

Research Note

Robustness Testing of the xMAP Food Allergen Detection Assay: A Multiplex Assay for the Simultaneous Detection of Food Allergens

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ABSTRACT

The xMAP food allergen detection assay (xMAP FADA) can simultaneously detect 15 analytes (14 food allergens plus gluten) in one analysis. The xMAP FADA typically employs two antibody bead sets per analyte, providing built-in confirmation that is not available with other antibody-based assays. Before an analytical method can be used, its reliability must be assessed when conditions of the assay procedure are altered. This study was conducted to determine the effects on assay performance associated with changes in incubation temperature, amounts of the antibody bead cocktail, and concentrations of detection antibody and β -mercaptoethanol in the reduced-denatured extraction buffer. The analysis of buffered-detergent extracts revealed lower responses at 22°C than at 37°C, but temperature had no effect on the analysis of reduced-denatured extracts. Changes in β -mercaptoethanol and detection antibody concentrations had an effect on the detection of only milk in the reduced-denatured extracts. A slight change in the measured bead count was observed when one-fourth of the bead cocktail was used, and a large decrease in the bead count was noted when one-eighth of the recommended amount was used, but this number (≥ 25) was still sufficient to provide reliable results. Overall, the xMAP FADA was very robust to changes in the assay procedure, which may inadvertently occur.

HIGHLIGHTS

- Temperature affected the FADA with buffered-detergent but not reduced-denatured extracts.
- Detection antibody concentration had no effect on reduced-denatured extracts other than milk.
- Reliable results were obtained even with a reduced amount of bead cocktail.
- The FADA was robust to deviations from the manufacturer's suggested protocol.

Key words: Bio-Plex; Food allergen; Robustness; xMAP food allergen detection assay

Food allergies are an important public health issue that affects about 8% of children younger than the age of 18 years and 4% of adults in the United States, and about one-third of these individuals are allergic to multiple food allergens (8, 9, 13, 14). Celiac disease affects approximately 1% of the U.S. population (1). Because no cure for food allergies currently exists, strict avoidance of the offending foods in the diet is the only option for allergic individuals. Therefore, high-throughput testing for multiple allergens has become increasingly important. The routinely used antibody-based methods are not capable of testing for multiple food allergens and cannot be used to distinguish between homologous cross-reactive foods. To address these

needs, the U.S. Food and Drug Administration (FDA) and Radix BioSolutions (Georgetown, TX) developed the xMAP food allergen detection assay (xMAP FADA), which can simultaneously detect 14 food allergens (including nine tree nuts) and gluten. The xMAP FADA kits used in the present study included 25 bead sets for analyzing food allergens extracted with a buffered detergent (phosphate-buffered saline plus Tween [PBST]) and 5 bead sets for the analysis of allergens extracted with a reduced-denatured solution (sodium dodecyl sulfate- β -mercaptoethanol) (10). Each kit also included the AssayChex process control panel beads (Radix BioSolutions), which were used to monitor the performance of the instrument and reagents in the assay.

A major concern with the use of any analytical method is the robustness of the assay to any changes in the suggested protocol conditions. To assess robustness, the effects on assay results of changes in incubation temperature, the concentration of beads in the bead cocktail, and the

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concentrations of the detection antibody and the β -mercaptoethanol in the reducing-denaturing extraction buffer were evaluated.

MATERIALS AND METHODS

The food allergen reference materials and reagents used in this study were described previously (4). The xMAP FADA kits were purchased from Radix BioSolutions and contained 25 bead sets for the analysis of buffered-detergent extracts, 5 bead sets for the analysis of reduced-denatured extracts, process control panel bead sets, and calibration standards containing a mixture of 15 analytes (Table 1).

Sample preparation. Extracted proteins for each of the 15 raw (unroasted and unsalted) analytes (Table 1) at final concentrations of 125, 250, and 2,500 ng/mL were prepared from 0.1-g samples of each allergenic food. The two lower concentrations fall within the dynamic range of the xMAP FADA; the buffered-detergent extraction entails a 20-fold dilution, and the reduced-denatured extraction entails a 200-fold dilution of the sample. In contrast, the 2,500 ng/mL samples generate saturating responses. The samples containing 2,500 ng/mL produced higher levels of cross-reactivity, as would be expected for low avidity and less specific binding. This cross-reactivity was easily rectified by using more stringent binding conditions (10, 11). Similar results were observed for the 125- and 250-ng/mL samples; therefore, only the data generated by the 250-ng/mL samples are presented here.

xMAP FADA. The xMAP FADA was conducted as specified by the manufacturer and previously described in detail (2, 3) with the following modifications. The reduced-denatured extracts were prepared using either the recommended concentration of β -mercaptoethanol (2%) or one-half of the recommended concentration. The bead cocktails were prepared using the recommended volume of beads (1 $\times$) or one-quarter (0.25 $\times$) or one-eighth (0.125 $\times$) of the recommended volume of beads. The incubation steps were performed with a shaker as recommended at 37°C or at 22°C (room temperature). All samples were analyzed in triplicate, and the mean $\pm$ standard deviation of the median fluorescence intensity (MFI) was calculated.

Instrumentation. Magnetic beads were washed using a magnetic-ring stand (AM10050, Applied Biosystems, Foster City, CA) and incubated in the dark in an orbital mixing Peltier dry bath (SC20, Torrey Pines Scientific, Carlsbad, CA). The multiplex assay system (Bio-Plex 200, BioRad Laboratories, Hercules, CA) settings were doublet discriminator gate at 5,000 to 25,000, photomultiplier tube at low, number of microspheres collected at 100 per region, and total number of microspheres collected at 3,500 total classified, with a time limit of 45 s.

Statistical analysis. As necessary, the results were analyzed with the PRISM 6 program for Windows (GraphPad, San Diego, CA). Analyses used were a one-way analysis of variance with repeated measures followed by post hoc comparisons with Tukey's multiple paired comparison test.

RESULTS AND DISCUSSION

Background MFI. Before assessing the effects of variations in assay conditions, the background (S0) responses under typical conditions must be established (Table 2). The

background responses associated with the analysis of buffered-detergent extracts at 37°C were typical for the individual analytes except cashew-18, hazelnut-29, and pistachio-43 when their bead concentrations were cut to 0.125 $\times$, resulting in MFIs of 119 ± 9 , 120 ± 6 , and 287 ± 26 , respectively. When the incubation temperature was reduced to 22°C, the background values slightly decreased except for a few instances when the bead concentrations were reduced to 0.25 $\times$ and 0.125 $\times$ for gluten-28 (234 ± 11 and 231 ± 36) and peanut-37 (236 ± 29 and 298 ± 44) or 0.125 $\times$ for almond-12 (147 ± 43). In contrast, the background responses associated with the analysis of reduced-denatured extracts were typically invariant with bead concentration changes but substantially decreased when the incubation temperature was reduced from 37 to 22°C (Table 2).

Calibration standards. Figure 1 depicts the average MFI responses of various allergen calibration standards when assayed using incubation temperatures of 37 and 22°C. Plots include the results for the dominant (more intense anchor antibody) of the complementary antibody pairs. For most analyses conducted according to the buffered-detergent extraction protocol with PBST (Fig. 1A), lower responses were obtained at 22°C except for the almond-12, gluten-28, and pine nut-39 bead sets, which exhibited similar responses at lower allergen concentrations at both temperatures but at higher concentrations had reduced responses at 22°C compared with 37°C. In contrast, no difference was observed in the responses measured for egg, milk, or peanut extracted under reducing-denaturing conditions (Fig. 1B). A slight increase in the MFI responses was found for gluten at 22°C compared with 37°C, possibly related to the complexity of gluten and its unique solubility properties.

Detection of allergenic foods. Figure 2 shows the effects of incubation temperature on the detection of buffered-detergent extracts (Fig. 2A) and reduced-denatured extracts (Fig. 2B) prepared from the raw (unprocessed) allergenic foods using the bead concentrations recommended by the manufacturer (1 $\times$) and at 0.25 $\times$ and 0.125 $\times$ concentrations. The results shown are for the dominant (more intense) of the complementary antibody pairs. For the buffered-detergent extracts, MFI responses for all bead sets were lower at 22°C than at 37°C, although pistachio-43 and walnut-47 had significantly lower responses ($P < 0.05$) at the lower temperature (Fig. 2A). This effect was mostly the same regardless of the bead concentration. For the reduced-denatured extracts, no differences in the response at 37 and 22°C were observed at all bead concentrations (Fig. 2B).

Figure 3 shows the effects of changing β -mercaptoethanol and detection antibody concentrations (recommended [1 $\times$] and one-half of recommended [0.5 $\times$]) and incubation temperature (37 and 22°C). The MFI responses for the egg-65, peanut-72, and gluten-73 bead sets were relatively unchanged when β -mercaptoethanol and detection antibody concentrations were changed at both temperatures. However, the response for milk-67 was significantly lower ($P < 0.05$) when the detection antibody concentration was reduced to 0.5 $\times$ at both temperatures.

TABLE 1. *xMAP food allergen detection assay design*

Allergen	Bead set no. ^a		Calibration standards (ng/mL protein) ^b						
	Buffered-detergent extraction protocol	Reduced-denatured extraction protocol	S1	S2	S3	S4	S5	S6	S7
Almond	12	13	2.9	5.2	9.4	17	30	55	99
Brazil nut	14	15	2.9	5.2	9.4	17	30	55	99
Cashew	18	19	0.5	0.8	1.5	2.7	4.8	8.7	16
Coconut	20	21	0.6	1.1	2.1	3.7	6.7	12	22
Crustacean	22		15	28	50	90	162	292	525
Egg ^c		65	5.8	10	19	34	61	110	198
Gluten	27	28	3.7	6.6	12	21	39	69	125
Hazelnut	29	30	1.2	2.1	3.8	7	12	22	40
Macadamia nut	33	34	4.8	8.7	16	28	51	91	165
Milk ^c		66	1.5	2.6	4.7	8	15	27	49
Peanut	37	38	1.5	2.8	5	9	16.2	29.2	52.5
Pine nut	39	42	8.1	15	26	47	85	152	274
Pistachio nut	43	44	2.9	5.2	9.4	17	30	55	99
Soy	45	46	9.2	17	30	54	96	174	313
Walnut	47	48	9.2	17	30	54	96	174	313

^a Bead sets associated with the buffered-detergent (25 sets) and reduced-denatured (5 sets) extraction protocols were individually numbered.

^b Calibration standards were reported as soluble protein prepared as described by the manufacturer.

^c Lots produced subsequent to those used in this study for the buffered-detergent extraction protocol included bead sets 25, 26, 35, and 36 for the detection of egg and milk.

Ratio analysis using complementary antibody pairs.

Complementary antibody ratios offer a strong confirmatory test for food allergen identification and for distinguishing cross-reactive analytes (2, 5, 7, 12). Table 3 shows the

complementary antibody response ratios as a function of incubation temperature and bead concentration. These ratios were mostly consistent and reproducible at 37°C for the individual analytes with both protocols at all three amounts

TABLE 2. *Average Blank MFI responses of xMAP FADA bead sets*

Allergen	MFI response (mean $\pm$ SD) ^a					
	37°C			22°C		
	1 $\times$ beads	0.25 $\times$ beads	0.125 $\times$ beads	1 $\times$ beads	0.25 $\times$ beads	0.125 $\times$ beads
Buffered-detergent protocol						
Almond-12	44 $\pm$ 3	39 $\pm$ 1	65 $\pm$ 1	15 $\pm$ 0	78 $\pm$ 16	147 $\pm$ 43
Brazil nut-14	35 $\pm$ 3	38 $\pm$ 1	69 $\pm$ 11	22 $\pm$ 1	36 $\pm$ 13	37 $\pm$ 14
Cashew-18	58 $\pm$ 2	65 $\pm$ 4	119 $\pm$ 9	45 $\pm$ 2	56 $\pm$ 22	56 $\pm$ 18
Coconut-20	38 $\pm$ 4	38 $\pm$ 1	32 $\pm$ 2	21 $\pm$ 1	28 $\pm$ 2	32 $\pm$ 8
Crustacean-22	30 $\pm$ 3	32 $\pm$ 1	34 $\pm$ 8	16 $\pm$ 1	20 $\pm$ 1	31 $\pm$ 19
Gluten-28	133 $\pm$ 6	120 $\pm$ 3	141 $\pm$ 9	112 $\pm$ 2	234 $\pm$ 11	231 $\pm$ 36
Hazelnut-29	48 $\pm$ 3	50 $\pm$ 1	120 $\pm$ 6	30 $\pm$ 1	44 $\pm$ 11	45 $\pm$ 12
Macadamia-33	40 $\pm$ 4	41 $\pm$ 2	44 $\pm$ 4	28 $\pm$ 2	44 $\pm$ 5	43 $\pm$ 8
Peanut-37	55 $\pm$ 5	66 $\pm$ 6	97 $\pm$ 6	38 $\pm$ 1	236 $\pm$ 29	298 $\pm$ 44
Pine nut-39	94 $\pm$ 5	99 $\pm$ 3	90 $\pm$ 6	82 $\pm$ 4	62 $\pm$ 8	75 $\pm$ 30
Pistachio-43	47 $\pm$ 4	61 $\pm$ 1	287 $\pm$ 26	36 $\pm$ 4	35 $\pm$ 13	37 $\pm$ 12
Soy-46	27 $\pm$ 4	28 $\pm$ 2	43 $\pm$ 5	12 $\pm$ 0	43 $\pm$ 33	34 $\pm$ 11
Walnut-47	106 $\pm$ 8	113 $\pm$ 3	126 $\pm$ 8	88 $\pm$ 2	112 $\pm$ 33	103 $\pm$ 25
Reduced-denatured protocol						
Egg-65	166 $\pm$ 37	134 $\pm$ 10	127 $\pm$ 8	74 $\pm$ 2	66 $\pm$ 2	68 $\pm$ 2
Gluten-73	1,240 $\pm$ 37	1580 $\pm$ 26	1580 $\pm$ 39	300 $\pm$ 5	377 $\pm$ 13	351 $\pm$ 15
Milk-67	495 $\pm$ 30	460 $\pm$ 9	429 $\pm$ 28	393 $\pm$ 8	376 $\pm$ 14	394 $\pm$ 5
Peanut-72	110 $\pm$ 21	100 $\pm$ 3	102 $\pm$ 4	73 $\pm$ 2	64 $\pm$ 2	72 $\pm$ 5

^a Only the higher (anchor) antibody bead set responses are shown. The 1 $\times$ bead treatment was designated as the background (S0) response.

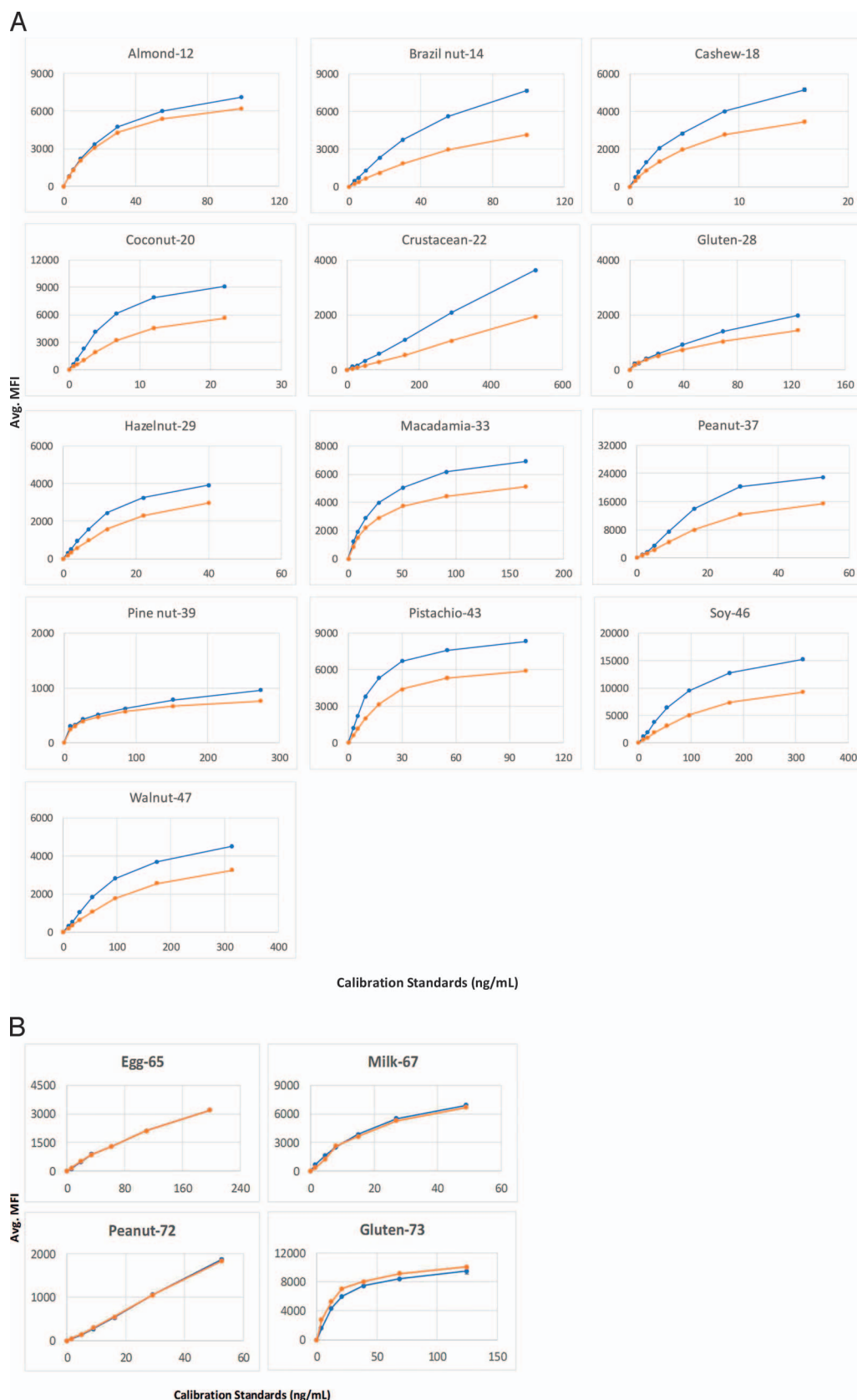
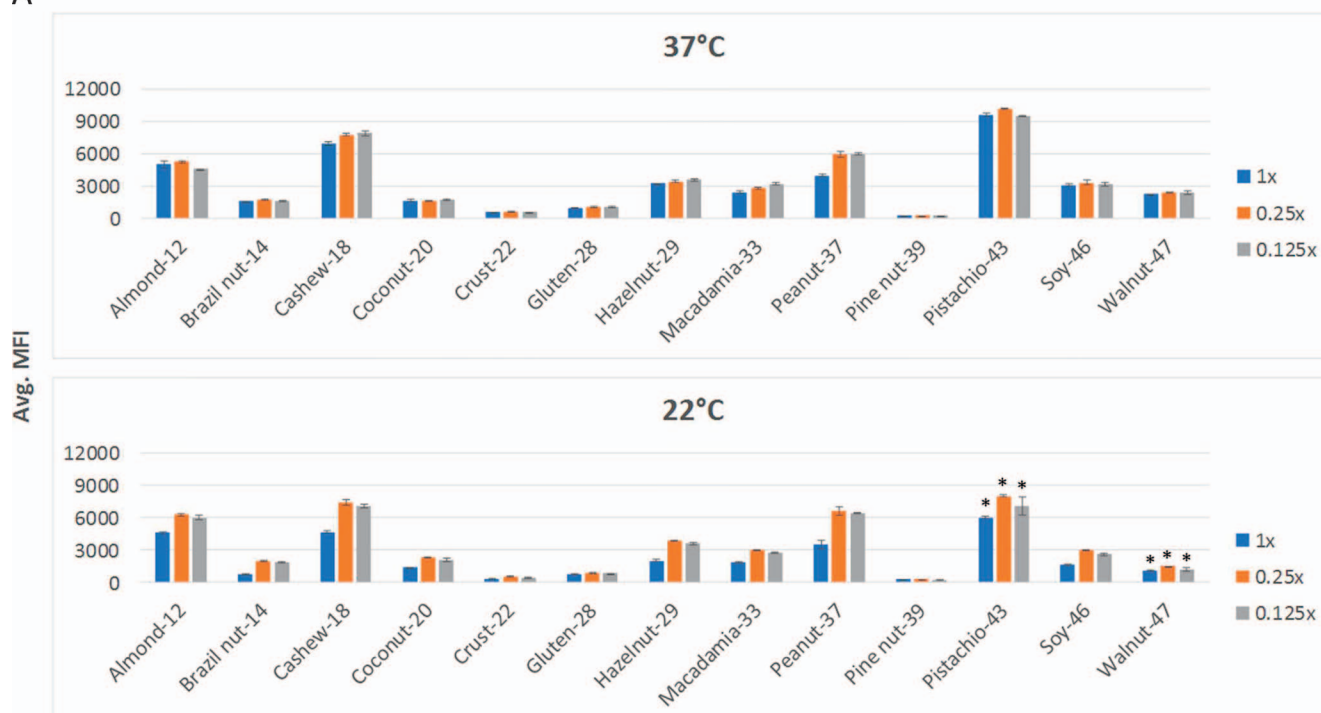


FIGURE 1. Effects of temperature on calibration standards analyzed according to the protocol for the buffered-detergent extracts (A) and the protocol for the reducing-denaturing extracts (B). The mean ($n = 3$) MFI (minus background) for the more intense (anchor) of the complementary antibody pairs is plotted versus the concentration of extractable soluble protein in the calibration standards. Incubation was at either the recommended temperature of 37°C (blue lines) or 22°C (orange lines); all other aspects of the assay were as recommended by the manufacturer.

A



B

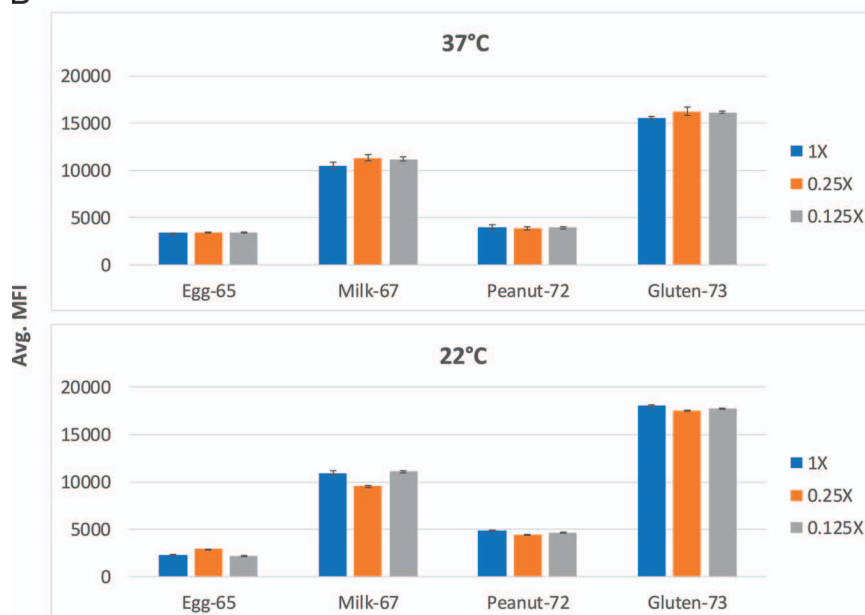


FIGURE 2. Effects of bead count and temperature on the detection of allergenic foods. Raw allergenic foods were analyzed using the buffered-detergent (A) or reduced-denatured (B) extraction protocols as described by the manufacturer with the bead cocktail prepared using beads at the concentrations recommended by the manufacturer (1X) or at one-quarter (0.25X) or one-eighth (0.125X) of the recommended concentration. Incubation steps were performed at both 37 and 22°C. The mean ($\pm$ SD, $n = 3$) MFI is plotted for the more intense (anchor) of complementary antibody pairs. Crust, crustacean. Asterisks indicate significant differences at $P < 0.05$.

of bead cocktail (1X, 0.25X, and 0.125X). The same level of consistency and reproducibility was also observed at 22°C except when the bead concentrations were decreased to 0.25X and 0.125X for coconut and gluten, which had relatively lower ratios, and hazelnut, which had relatively higher ratios. When comparing the results obtained at 37°C with those at 22°C, the gluten, walnut, and pine nut ratios differences were 101, 55, and 29%, respectively. For all

other analytes, differences in their ratios between 22 and 37°C were $\leq 20\%$. These results indicate that the complementary antibody ratios can provide a consistent secondary confirmatory endpoint.

Bead count. The bead count in the sample portion monitored with the Bio-Plex system is a useful indicator for assessing the precision of the data. In previous xMAP

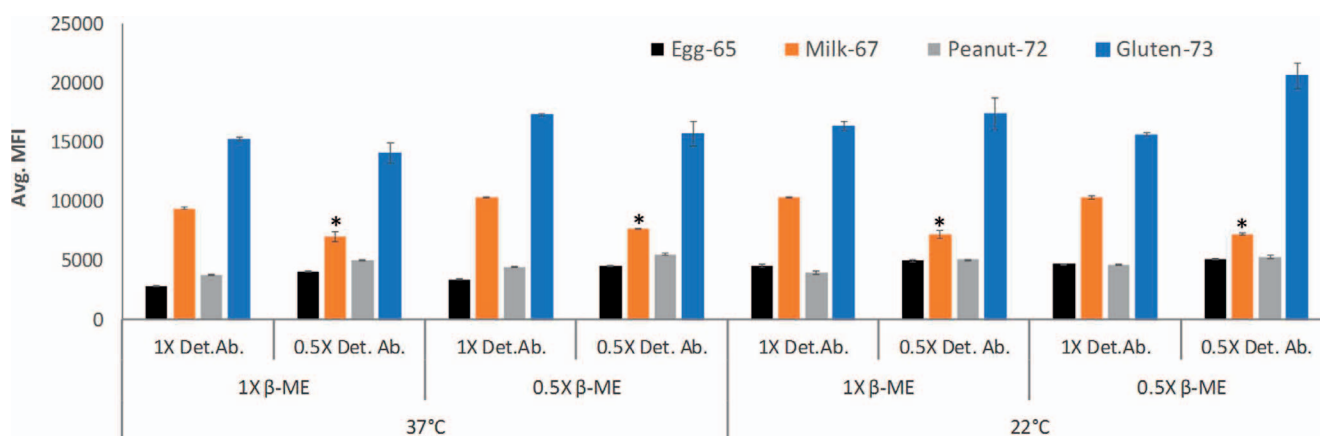


FIGURE 3. Effects of β -mercaptoethanol (β -ME) concentration, incubation temperature, and detector antibody (Det. Ab.) concentration on the analysis of allergenic foods with the reduced-denatured extraction protocol. Analyses were conducted as stipulated by the manufacturer except for the concentration of β -mercaptoethanol was as recommended (1X, 2%) or one-half the recommended concentration (0.5X, 1%). Reactions were incubated at either 37 or 22°C. The mean ($\pm$ SD, $n = 3$) MFI is plotted. Asterisks indicate significant differences at $P < 0.05$.

FADA experiments, variances increased with low bead counts, and bead counts of ≥ 25 were considered desirable. In the xMAP FADA kits, the concentration of beads recommended for use in the cocktail was deliberately designed allow compensation for possible loss during washing or other manipulations. Thus, although changes in bead count were observed when using 0.25X and 0.125X the recommended concentration of beads, the average bead count still was ≥ 25 and the data had the typical levels of variance. At 37°C, the average bead counts with the protocol for the analysis of buffered-detergent extracts were 113 ± 16 (range, 75 to 134), 86 ± 13 (range, 49 to 107), and 58 ± 11 (range, 23 to 67) for 1X, 0.25X, and 0.125X bead concentrations, respectively. With the protocol for the analysis of reduced-denatured extracts, the bead counts

were 448 ± 53 (range, 386 to 521), 110 ± 12 (range, 90 to 118), and 63 ± 8 (range, 49 to 68) for 1X, 0.25X, and 0.125X bead concentrations, respectively. Decreasing the incubation temperature to 22°C did not have any effect on the bead counts.

Overview. The potential for unintended deviations from the recommended conditions when performing an assay always exists. Therefore, the performance of this xMAP FADA was evaluated under conditions designed as worse-case scenarios. The focus was not on variables that might affect the target analytes but on assay performance. Thus, throughout the study, the samples used were the same as routinely used to standardize the performance of the xMAP FADA, that is, the same calibration standards and

TABLE 3. Effects of temperature and bead concentration on complementary antibody ratios

Allergen ^a	MFI ratio ^b						% difference (mean $\pm$ SD) ^c
	37°C			22°C			
	1× beads	0.25× beads	0.125× beads	1× beads	0.25× beads	0.125× beads	
Almond 13/12	0.31	0.31	0.29	0.35	0.36	0.36	18 $\pm$ 5
Brazil nut 15/14	0.4	0.41	0.4	0.46	0.46	0.47	15 $\pm$ 2
Cashew 19/18	0.87	0.88	0.91	0.83	0.82	0.83	−6 $\pm$ 2
Coconut 21/20	0.04	0.04	0.03	0.04	0.04	0.03	12 $\pm$ 7
Gluten 27/28	0.18	0.19	0.19	0.43	0.36	0.32	101 $\pm$ 33
Hazelnut 30/29	0.27	0.27	0.27	0.23	0.3	0.3	1 $\pm$ 13
Macadamia 34/33	0.09	0.09	0.09	0.09	0.1	0.1	8 $\pm$ 8
Peanut 38/37	0.82	0.82	0.84	0.7	0.65	0.67	−18 $\pm$ 3
Pine nut 42/39	0.11	0.12	0.11	0.13	0.15	0.15	29 $\pm$ 11
Pistachio 44/43	0.18	0.19	0.17	0.15	0.17	0.17	−6 $\pm$ 9
Soy 45/46	0.27	0.28	0.29	0.29	0.27	0.27	−2 $\pm$ 6
Walnut 48/47	0.1	0.12	0.12	0.15	0.17	0.20	55 $\pm$ 12
Milk 66/67	0.69	0.71	0.71	0.55	0.56	0.58	−20 $\pm$ 2

^a Crustacean has only one bead set, thus it is not included. In the reduced-denatured protocol, only milk had two bead sets.

^b Lower MFI divided by higher MFI (anchor) responses of complimentary antibodies with the recommended amount of beads (1X) or one-quarter (0.25X) or one-eighth (0.125X) of that amount.

^c Change in the mean calculated ratios between 37 and 22°C across bead concentrations.

raw unprocessed allergenic foods that were used to develop the calibration standards and for analyses in various other publications (2–6, 11, 12). For example, the effects of incubation temperature were designed to represent possible scenarios when a laboratory did not have an incubator or incubated the samples at room temperature (22°C) instead of 37°C.

Differently processed foods might have different levels of sensitivity to changes in assay conditions. However, the evaluation of all possible scenarios is beyond the scope of this study, which was focused on the primary measures used in assessing assay performance. Aside from possible changes to extraction efficiency, changes in assay performance should be minimal to inconsequential and comparable effects should be expected for the food samples and calibration standards being analyzed simultaneously. Increased variances are not expected to be a concern, as indicated by the excellent reproducibility found in this and previous studies and the inherently large signal:noise ratio associated with the calibration standards, typically >3 at the limit of quantification (equivalent to the S1). Concerns about extraction efficiency and the redox potential of the reduced-denatured extraction buffer are not minor and may be related to the reduced detection of milk when the concentrations of β -mercaptoethanol and detection antibody were decreased. Despite these issues, the xMAP FADA was highly robust and suitable for routine use.

In conclusion, the results of this study established that the xMAP FADA (with protocols for the analysis of buffered-detergent and reduced-denatured extracts) provides a robust platform for sensitive, reliable, and reproducible multiplex analysis of 15 analytes (14 food allergens plus gluten) concurrently in a single reaction. Variations in the input amount of bead cocktail (1 $\times$, 0.25 $\times$, and 0.125 $\times$ the recommended amount of beads) and the incubation temperature (37 and 22°C) in both buffered-detergent and reduced-denatured protocols did not affect the performance of the xMAP FADA. Variation in the β -mercaptoethanol and detection antibody concentrations (1 $\times$ and 0.5 $\times$ the recommended) in the reduced-denatured protocol had no significant effect on the assay performance, except for the milk-67 bead set.

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