**Statistical approaches to developing a multiplex immunoassay for
determining human exposure to environmental pathogens**

Swinburne A.J. Augustine^{a*}, Kaneatra J. Simmons^a, Tarsha N. Eason^b, Shannon M. Griffin^a,
Clarissa L. Curioso^c, Larry J. Wymer^a, G. Shay Fout^a, Ann C. Grimm^a, Kevin H. Oshima^a, Al
Dufour^a

^a National Exposure Research Laboratory, U.S. Environmental Protection Agency, 26 W. Martin
Luther King Drive, Cincinnati, OH 45268, USA

^b National Risk Management Research Laboratory, U.S. Environmental Protection Agency, 26
W. Martin Luther King Drive, Cincinnati, OH 45268, USA

^c Oak Ridge Institute for Science and Education, Oak Ridge, TN

* Corresponding author at: 26 W. Martin Luther King Drive, Cincinnati, OH 45268, USA.
Telephone: (513) 569-7132. E-mail address: augustine.swinburne@epa.gov (S. Augustine).

Abstract

There are numerous pathogens that can be transmitted through water. Identifying and understanding the routes and magnitude of exposure or infection to these microbial contaminants are critical to assessing and mitigating risk. Conventional approaches of studying immunological responses to exposure or infection such as Enzyme-Linked Immunosorbent Assays (ELISAs) and other monoplex antibody-based immunoassays can be very costly, laborious, and consume large quantities of patient sample. A major limitation of these approaches is that they can only be used to measure one analyte at a time. Multiplex immunoassays provide the ability to study multiple pathogens simultaneously in microliter volumes of samples. However, there are several challenges that must be addressed when developing these multiplex immunoassays such as selection of specific antigens and antibodies, cross-reactivity, calibration, protein-reagent interferences, and the need for rigorous optimization of protein concentrations. In this study, a Design of Experiments (DOE) approach was used to optimize reagent concentrations for coupling selected antigens to Luminex™ xMAP microspheres for use in an indirect capture, multiplex immunoassay to detect human exposure or infection from pathogens that are potentially transmitted through water. Results from *H. pylori*, *C. jejuni*, *E. coli* O157:H7, and *S. typhimurium* singleplexes were used to determine the mean concentrations that would be applied to the multiplex assay. Cut-offs to differentiate between exposed and non-exposed individuals were determined using finite mixed modeling (FMM). The statistical approaches developed facilitated the detection of Immunoglobulin G (IgG) antibodies to *H. pylori*, *C. jejuni*, *T. gondii*, hepatitis A virus, rotavirus and noroviruses (VA387 and Norwalk strains) in fifty-four diagnostically characterized plasma samples. Of the characterized samples, the detection rate was 87.5% for *H. pylori*, and 100% for *T. gondii* assays and 89% for HAV. Further, the optimized

48 multiplex assay revealed exposure/infection to several other environmental pathogens
49 previously uncharacterized in the samples.

50
51 **Keywords:** Multiplex immunoassay, assay optimization, Design of Experiments (DOE),
52 response surface methods (RSM), finite mixed modeling (FMM)

58 1. Introduction

59 Conventional approaches to studying human exposure or infection from pathogens
60 include epidemiological surveys that collect symptomology type data and ELISAs that detect the
61 presence of antibodies specific to pathogens. Determination of exposure or infection from
62 pathogens using survey-based epidemiological studies can be challenging because of difficulties
63 enrolling participants and high cost. These observational survey-based approaches may suffer
64 from selection bias and problems of recall (Lilienfeld 1983). Additionally, determination of the
65 etiologic agents may be challenging because symptoms and incubation periods may not be
66 unique to particular pathogens. Combining epidemiologic assessment with assays to measure

immunological status can be very effective in determining which pathogens present exposure risks to a given population.

Recent technological advances have provided the ability to study multiple analytes simultaneously with very small sample volumes (Ellington *et al.*, 2010; Fu *et al.*, 2010; Choi *et al.*, 2013). One such technology, the Luminex xMAP (Luminex Corp., Austin, TX) bead-based immunoassay is theoretically capable of multiplexing up to 500 unique assays in as little as 50 μ l of sample. This is in contrast to ELISA-type antibody-based immunoassays that can measure only one analyte at a time, are time-consuming, require larger volumes of patient sample for multiple tests, and are often costly. However, well-designed immunoassays and assay optimization tests are time consuming and costly due to the number of factors that must be managed. They require determining settings for multiple parameters and balancing sample availability with the use of expensive reagents and equipment. Hence, it is important that these assays are systematically developed to ensure they are precise and produce optimal results.

Determining key or critical assay parameters typically involve evaluating output response given changes in parameter settings one-factor-at-a-time (OFAT). In addition to being time and resource intensive, OFAT provides no means of determining or exploring important interactions between factors. As an alternative, DOE offers an efficient means of investigating the impact of changes in system parameters, exploring factor combinations and identifying parameter settings for optimizing experimental results (Montgomery 1997; Khuri and Mukhopadhyay 2010). DOE has effectively been used in assay development to provide critical insight for optimizing experimental conditions for applications to include the detection of anti-drug neutralizing antibodies in human serum; optimization of an automated digital microfluidic platform immunoassay; ligand-binding assay sensitivity; optimizing ELISAs; and measuring neutralizing

antibodies against *Clostridium difficile* toxins (Chen *et al.*, 2012; Choi *et al.*, 2013; Joelsson *et al.*, 2008; Joyce and Leung 2013; Sitta Sittampalam *et al.*, 1996; Xie *et al.* 2013).

The goals of the present study are to optimize reagent concentrations for detecting human IgG antibodies in characterized human plasma samples and determine cut-off points for the antigens in the assay using FMM (Baughman *et al.*, 2004). Multiplex immunoassays are not as common as singleplex methods and have the additional requirement of needing multiple optimizations. To handle this complexity, we employ the use of DOE for designing and optimizing an assay for simultaneously detecting antibodies to multiple pathogens in commercial samples. The optimized settings from these tests will later be used to investigate bathing related exposures or infections from waterborne pathogens.

2. Materials and Methods

Designing a Luminex-based, indirect capture, multiplex immunoassay requires determining the optimal concentrations for antigen (Ag), primary antibody (1° Ab), biotinylated secondary antibody (2° Ab), and reporters, as well as, determining the optimal pH for coupling of antigens to the microspheres. A pH of 5.0 was previously determined to be best for the coupling reactions (Griffin *et al.*, 2011) and we found that 2° Ab concentration and streptavidin-R-phycoerythrin (SAPE) (Life Technologies, Carlsbad, CA, USA) worked best as recommended by Luminex (SAPE concentration = biotinylated 2° Ab concentration $\times$ 1.5). Preliminary experiments showed that the reagent concentrations necessary to achieve MFIs that approached the upper Limit of Detection (LoD) of the Luminex instrument (up to 30,000 MFI) tended to vary from antigen to antigen; accordingly, a range of concentrations was tested to optimize each assay (Table 1).

Multiple sets of xMAP carboxylated microspheres were obtained from Luminex Corp. (Austin, TX, USA) at a concentration of 12.5×10^6 beads/ ml each. Biotin-labeled affinity purified goat anti-human IgG (λ) and rabbit anti-goat IgG (H+L) were obtained from KPL, (Gaithersburg, MD, USA). Biotin-labeled donkey anti-rabbit IgG (H+L) and donkey anti-guinea pig IgG (H+L) were purchased from Jackson ImmunoResearch, (West Grove, PA, USA).

2.1. Two-step carbodiimide coupling of antigens to xMAP[®] carboxylated beads and confirmation of antigen coupling using animal-derived antibodies:

The Luminex microspheres were activated and coupled as described in Griffin *et al.*, (2011). Coupling confirmation was performed using serial dilutions of commercially available, animal-derived primary capture antibodies specific to each antigen to ensure that the beads were coupled properly and that the dynamic range of the assay could be achieved. Briefly, a working bead mixture was prepared by diluting the coupled bead stocks to a final concentration of 100 beads/ μ l of each unique bead set in PBS-1% bovine serum albumin (BSA). Two-fold serial dilutions of anti-species IgG primary antibody were prepared per the manufacturer's recommendations. A MultiScreen BV 96 well 1.2 μ m filter plate (Millipore, Billerica, MA, USA) was pre-wet with 100 μ l of PBS-1% BSA, excess buffer was removed with a vacuum manifold and 50 μ l of the working bead mixture was added to each well. The serially diluted species-specific antibody was added to the beads, the reactions were mixed gently with a multi-channel pipettor, covered, and allowed to incubate in the dark, at room temperature for 30 min at 500 rpm on a VWR[™] microplate shaker (Radnor, PA, USA).

The supernatant was removed by vacuum manifold, and the wells were washed twice with 100 μ l of PBS-1% BSA, 0.05% Tween 20 buffer and excess buffer removed by vacuum

manifold. The beads were gently resuspended in 50 μ l of PBS-1% BSA buffer with a multi-channel pipettor, and 50 μ l of a 16 μ g/ml of biotinylated anti-species IgG secondary detection antibody was added to each well. The filter plates were covered and allowed to incubate in the dark at room temperature for 30 min on a plate shaker. After incubation, the wells were washed twice with 100 μ l of PBS- 1% BSA, 0.05% Tween 20 buffer and excess buffer removed by vacuum manifold. Finally, 50 μ l of 24 μ g/ml SAPE was added to each well, mixed gently with a multi-channel pipettor and incubated as above for 30 minutes and washed twice as above. Excess buffer was removed by vacuum manifold, the beads were resuspended in 100 μ l of PBS-1% BSA, and the plate analyzed on a Luminex 100 analyzer.

2.2. DOE optimization of assay parameters

Design Expert 8.0.7.1 (StatEase, Inc., Minneapolis, MN, USA) was used to design singleplex experiments using antigens to *H. pylori*, *C. jejuni*, *E. coli* O157:H7, and *S. typhimurium*. The concentration of Ag coupled to the microspheres, 1 $^{\circ}$ Ab, 2 $^{\circ}$ Ab and SAPE were used as factors. Since 2 $^{\circ}$ Ab concentration and SAPE vary together, the SAPE was used as an experimental factor but eliminated as a design factor. A full factorial design was initially used to assess the impact of factor changes on MFI response for all of the pathogens, after which, response surface methods (RSM) were employed in accordance with the optimization goal of this work. RSM uses statistical and mathematical approaches to explore the functional relationship between controllable factors and output response. They are used for modeling and analyzing problems with multiple parameters, determining factor significance, developing mathematical models and optimizing system response (Montgomery 1997). There are a number of RSM approaches to include D-optimal, central composite designs (CCD) and Box-Behnken

designs (Khuri and Mukhopadhyay 2010, Montgomery 1997). Each can be employed in a variety of circumstances; however, we used a D-Optimal design for our studies. A D-Optimal design is a RSM developed to select design points in a way that minimizes the variance associated with the estimates of the specified model coefficients (Khuri and Mukhopadhyay 2010).

2.3. Developing the complete multiplex based on DOE results

Singleplex immunoassays were performed for the following organisms: *H. pylori*, *C. jejuni*, *E. coli* O157:H7, and *S. typhimurium* to determine the optimal parameter settings for each antigen/antibody pair. After using DOE to numerically optimize the Ag, 1° and 2° Ab concentrations for each organism, the results for each reagent was pooled and a mean was computed from these results. The mean concentration value for each reagent was then applied to hepatitis A virus, rotavirus, and *T. gondii*. The noroviruses had been previously optimized in Griffin *et al.* (2011). Antigen coupling and confirmation were conducted as described in Section 2.1 above. The complete multiplex immunoassay was performed on 96 well plates and analyzed for assay sensitivity and specificity.

Assay specificity was evaluated by performing single detection cross-reactivity using the concentration of 1° Ab at the highest point of the linear range of the assay. Individual 1° Abs were tested in the presence of a multiplex of antigen-coupled beads and the appropriate biotinylated 2° Ab and SAPE. The degree of cross-reactivity (defined as the percentage of MFI detected for a given antigen analyte relative to the specific MFI for a target antigen analyte) was calculated for all antigens. An assay was excluded from the multiplex if the maximum reporter

signal observed in coupling confirmation tests was less than 18,000 MFI and/or cross-reactivity to any non-target antigen was greater than 10%.

2.4. Assay validation of the optimized DOE parameters using commercially obtained diagnostically characterized human plasma samples

Serial dilutions (ranging from 1:20 to 1:6400) of characterized human plasma samples were tested using the beads that had been confirmed to be properly coupled with animal-derived anti-species antibodies to determine the dynamic ranges of those samples. A total of 54 commercially obtained plasma samples were analyzed in this study (44 positives and 10 negatives). 33 plasma samples were purchased from Access Biologicals (Vista, CA, USA) and 21 samples were obtained from SeraCare Life Sciences (Milford, MA, USA). These plasma samples were from patients diagnostically characterized with *H. pylori* and *T. gondii* ((n = 16 (5 positives and 3 negatives each)), HAV ((n=17 (14 positives and 3 negatives))), *L. pneumophila* (n=10) and *S. typhimurium* ((n=11 (10 positives and 1 negative))) infections. The *L. pneumophila* plasma was donated by SeraCare but the vendor was not confident about positivity. With the exception of the *L. pneumophila* samples, the characterization of the remaining 44 plasma samples was confirmed by the suppliers: Access Biologicals, using the ADVIA Centaur kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) and SeraCare Life Sciences using Calbiotech EIA (Calbiotech, Spring Valley, CA, USA) for the target organisms. The plasma samples were first tested by the Luminex platform using the optimized DOE reagent concentrations to confirm that they were either positive or negative. Once the results were validated in singleplex by Luminex, the characterized samples were exposed to a multiplex of the antigen-coupled bead sets.

2.5. Assay controls, cross-reactivity and signal to noise ratio (S/N)

Two assay controls were utilized to ensure proper performance of the multiplex immunoassay. First, one set of beads was used as a control for nonspecific binding of antibodies to the beads. These beads were treated exactly like the actual bead sets used in the analysis except that they were not coupled to any antigen before being exposed to the antibodies. The second control consisted of antigen-coupled beads but the antibodies were replaced with 1% PBS-BSA. The MFI values from the background controls (no antigen microspheres and antigen-coupled beads with no primary antibody) were used to determine non-specific binding and the MFI values were subtracted from each sample result.

Several cross-reactivity assays were performed with the animal-derived antibodies including monoplex (one antigen with its specific antibody), duplex (one antigen and its specific antibody) and multiplex (multiple antigens, one antibody and controls). Since it was not possible to obtain human-derived positive and negative controls for each antigen, a determination was made to remove antigen/antibody pairs that demonstrated levels of cross-reactivity >10% using the animal-derived primary antibodies. As a surrogate to measure assay sensitivity, a signal to noise ratio (S/N) was calculated by dividing the specific antigen signals by the MFI of the uncoupled control beads.

2.6. Establishing cutoffs to distinguish exposure status: exposed vs. unexposed

Given the lack of information regarding the true status of exposure/infection for the uncharacterized antigens in the multiplex, we used finite mixed models (FMM) (Titterton *et al.*, 1985; McLachlan and Peel, 2000) to predict exposure sub-group distributions based on

MFIs. Parameters for respective sub-group membership distributions were estimated via maximum likelihood for a family of models using the expectation-maximization technique due to the incomplete nature of the observed data (i.e. lack of information regarding individual exposure/infection status). For each pathogen, the number of sub-group distributions hypothesized to exist ranged from 1 to 3, and these distributions compared by the Akaike Information Criterion (AIC). The AIC adjusts the log likelihood by the number of parameters involved in the model. Probabilities of correct classification or misclassification for a future sample were developed for each final model. Fujii *et al.* (2014) applied the FMM approach to identify underlying distributions of exposed and infected populations to develop surveillance of tropical diseases. In addition, several underlying (mixed) distributions were considered. These include the Gaussian, log normal and their mixtures, and the truncated Gaussian. Fifty-four plasma samples were assayed by an independent laboratory and their resulting immune status as (presumably) negative/positive compared to predictions from the respective mixed models. Analysis was performed using SAS (Cary NC, version 9.3) and Microsoft Excel 13.

3. Results

3.1. Experimental design

Our analysis involved an examination of a range of concentrations for each immunoassay factor (Table 1). Experiments were designed using commercial animal-derived IgG antibody concentrations to confirm that the antigens were properly coupled to microspheres and provided a rough estimate of how the beads would react in the presence of antibodies in a human sample. Table 2 provides a sample of the DOE design matrix with MFI response. A preliminary DOE attempted to model the behavior of all of the antigens in one design but resulted in lack of fit due

to the differences between the antigens in the assay. Hence, separate D-Optimal RSM were developed and run to assess the response profile of each individual antigen. Each of the models met the underlying conditions of normality, independence and non-constant variance. Thus, they were sufficient for use in characterizing the impact of parameter changes and optimizing reagent concentrations for each Ag. As expected, analysis of variance (ANOVA) results reflected that the concentration of 1° Ab is the dominant factor impacting the MFI response, followed by the 2° Ab concentration and the BC (1° Ab and 2° Ab) and AC (Ag and 2° Ab) interactions (Fig. 1). Multiple two factor interactions (2FI) were identified in the ANOVA for *E. coli* O157:H7. *H. pylori* and *C. jejuni* also had a number of statistically significant higher order terms. Such observations provide insight into the Ag specific variations observed in the MFI response as a function of changes in reagent concentrations. These differences have a direct impact on the development of the multiplex immunoassay, and by understanding and accounting for them, we were able to determine the settings that worked best in the complete multiplex immunoassay.

Since the main goal of the DOE was to minimize the reagent concentrations necessary to elicit maximum MFI response from the coupled beads, the numerical optimization tool in Design Expert was employed to optimize each of the Ag specific models with the goal of maximizing MFI while imposing equally weighted minimization constraints on Ag, 1° Ab, 2° Ab concentrations and standard error in MFI. The optimization results for the Ag specific studies are summarized in Figure 2 (upper panel: RSM plots, lower panel: optimized concentrations). Note, there is quite a distinctive profile for each Ag. The response surface plots developed from the models reflect significant curvature in the *C. jejuni* and *H. pylori* response surfaces resulting from multiple interaction and higher order effects. Such a finding indicates there are complex factor effects for these organisms and the DOE for these antigens may be augmented with

additional runs and center points to more accurately capture the response characteristics. However, the designs used in this work provide enough information to compare and contrast key factors for the multiplex immunoassay.

While the recommended 2° Ab level is similar for the Ags, the optimal Ag and 1° Ab for *E.coli* O157:H7 and *S. typhimurium* tended to be much higher, yet elicited a lower MFI than for *H. pylori* and *C. jejuni*. As a means of demonstrating the impact of optimizing the immunoassay parameters with the goal of minimizing reagent use and maximizing MFI response, the Ag, 1° Ab and 2° Ab concentrations from the Ag specific studies were compiled (Fig. 2: lower panel) and compared to the mean of the reagent concentration range for the original assay design (Table 1). Results illustrate that while the mean amount of the 1° Ab concentration is nearly identical, the concentrations of Ag and 2° Ab are dramatically different (Fig. 2) between the mean of DOE optimized results (Table 1) and original assay. Hence, applying DOE to optimize the immunoassay resulted in a recommendation of significant reductions in Ag, 1° Ab and 2° Ab concentrations (62.68%, 6.81% and 41.87%, respectively).

3.2. Cross-reactivity

Cross-reactivity was used as an indicator of assay specificity and is reported in Table 3. Antigens highlighted in gray were not included in the final multiplex panel because the maximum MFI detected during coupling confirmation tests was less than 18,000 and/or cross-reactivity between a primary detection antibody and non-target antigen was greater than 10% by MFI. Among those antigens included in the assay, two exceptions were made. Cross-reactivity between the two noroviruses was 84.9% when using the guinea pig anti-norovirus polyclonal antibody. This was expected because the primary detection antibody was developed by

immunizing guinea pigs with multiple genotypes of GI and GII noroviruses. However, when rabbit anti-GII.4 primary detection antibody was employed, cross-reactivity with the GI Norwalk virus antigen was reduced to 0.4%. Also, 20.6% cross-reactivity was detected between the hepatitis A virus antigen and anti-*T. gondii* antibody. This cross-reactivity was not seen with further tests using human derived positive and negative control samples for HAV or *T. gondii*. Thus, both antigens were included in the multiplex panel. All other antigens tested achieved maximum reporter signals during coupling confirmation and demonstrated minimal cross-reactivity.

3.3. Plasma sample analysis

Of the diagnostically characterized plasma samples for *H. pylori*, *T. gondii*, and HAV (n = 33) using the optimized DOE settings, the accuracy of the Luminex assay was 87.5%, 100% and 89% respectively. One *H. pylori* characterized negative sample had an MFI value above our cut-off while two HAV samples that had been characterized as positive by the vendor were negative in our assay. However, because the specific antibody concentrations in the plasma samples were not determined for these samples, it was not possible to conclude whether there was a sensitivity issue between the testing methods employed by the vendors and the Luminex assay. The *S. typhimurium* and *L. pneumophila* antigens did not detect the presence of IgG antibodies in their corresponding characterized samples (n = 21) but antibodies to other antigens were detected in those samples. The range of MFI minus the background values of three antigens (*H. pylori*, *T. gondii* and hepatitis A virus) where characterized positive and negative plasma samples were available are shown in Figure 3 (upper panel). The S/N ratio of the characterized positive versus the characterized negative samples was computed by dividing the

MFI reading for each antigen in the assay by the MFI reading of the uncoupled control beads for each well. The mean S/N of the positively characterized samples (48.13) was 4.4 times that of negative samples (10.94). All 54 characterized plasma samples were also tested for the presence of antibodies against the antigens for which they had not been previously tested. Many of the samples were positive for other antigens on the panel as indicated by the MFI (Fig. 3, lower panel).

Figure 4 graphically shows results from the finite mixed modeling of the observed mixed distribution of the respective targets in all 54 characterized plasma samples by MFI. Parameters of the underlying populations and cut-off points are shown in Table 4. The left three columns give the $\log_{10}$ mean and standard deviation of the distribution of $\log_{10}$ MFI among presumed unexposed persons, along with the $\log_{10}$ MFI at the 95th percentile for these distributions. This results in a cut-off value for classifying an individual as unexposed (or “minimally exposed”) based on the 95th percentile of their distribution, beyond which such individuals’ exposure statuses would be improperly declared as positive (*i.e.*, “false positives”). Correspondingly, the second set of three columns give $\log_{10}$ mean and standard deviation among presumed “positive” persons along with their probability of false identification as negative samples (“false negatives”). In the cases of Norwalk virus and rotavirus, where three compartment mixed models were developed to better characterize the populations, these comparisons are made among all three possible combinations of components, giving the cut-off values for false positives among presumed unexposed and unexposed populations and corresponding false negative rates among individuals in the next higher exposure category based on these cut-offs.

In the case of *C. jejuni*, observed distribution of $\log_{10}$ MFI is described well by a single lognormal distribution. $\log_{10}$ MFI distribution of *C. jejuni* is unimodal with a comparative high

mean (7.8) and standard deviation (1.1). All others indicate generally excellent discrimination between exposed and unexposed subpopulations. Based on the estimates from the mixed models, Table 4 gives the implied false negative rates based on a pre-determined fixed false positive rate of 0.05. For Norwalk virus and rotavirus, three compartment mixed models were optimal. In the case of Norwalk virus, separation between unexposed and exposed populations were clear. However the middle “minimally exposed,” population was ambiguous on the basis of its overlap with the two extreme populations (0.522 and 0.340 false negative rates). Rotavirus infectivity populations lack this ambiguity, with clear separations and small probabilities of false negatives regardless of true exposure status.

4. Discussion and Conclusions

The design of experiments (DOE) method was successfully implemented to optimize coupling confirmation reagent concentrations in the multiplex immunoassay. This approach enabled us to determine which of the antigens could be utilized in the multiplex with little to no cross-reactivity, and which ones to exclude pending further studies. Moreover, we were able to observe the differences in behavior that antigens display when used in either a monoplex or a multiplex immunoassay. Antigens from *G. lamblia*, *Vibrio* species and *Shigella* species were removed from the multiplex assay because they cross-reacted with antibodies against other antigens in the multiplex assay. More specific antigen-antibody pairs will have to be tested to address the cross-reactivity issues. Although coupling confirmation and cross-reactivity tests for *E. coli* O157:H7, *L. pneumophila* and *S. typhimurium* were adequate, these assays would need to be further validated with either positive control samples or observed positivity in human saliva serum samples to ensure identification of samples that are positive in a population. The

characterized samples for *L. pneumophila* and *S. typhimurium* did not produce the results expected for their corresponding antigens, therefore these antigens were removed from the multiplex immunoassay pending further studies. A key benefit of the DOE approach is the ability to clearly identify important interactions between test parameters that would have been lost using the traditional OFAT approach. Previous studies have also made this observation (Altekar *et al.*, 2007, Joelsson *et al.*, 2008, Ray *et al.*, 2009).

DOE is an iterative process that affords the ability to derive valuable knowledge about a system with a minimum number of experiments (Chen *et al.*, 2012). This was certainly the case in our study. It would have been very expensive, labor intensive, and time consuming to attempt to determine optimal concentrations for all of the antigens in our multiplex immunoassay using OFAT. While OFAT is useful for studying main effects, it involves assessing each factor separately and provides little information on how factors interact. For example, it would appear that maximizing the Ag or 2° Ab concentrations would result in greater antibody binding, however we discovered that the interaction between the 1° Ab and 2° Ab concentration (BC) and Ag and 2° Ab concentrations (AC) were more critical than increasing the Ag concentration alone. In fact, we observed that increasing the Ag concentration tended to have an inverse impact on MFI response. By simply performing the random experiments as suggested by DOE which represent the range of values for each parameter, the optimal concentrations for the antigens in the assay were determined.

The mean reagent concentrations computed from the optimization results of the four monoplexes (performed separately for *H. pylori*, *C. jejuni*, *E. coli* O157:H7, and *S. typhimurium*) was used to set the optimal antigen, 1° antibody, and 2° antibody concentrations for the antigens in our multiplex immunoassay, with the exception of the noroviruses which had been previously

optimized (Griffin *et al.*, 2011). As demonstrated in the results of the positive and negative control human plasma sample for specific antigens and a S/N ratio of 4.4, the results showed considerable sensitivity and specificity when antibodies against them were present. Although the *L. pneumophila* and *S. typhimurium* assays did not perform as expected, we were able to test those characterized samples for exposure to other pathogens.

Immunoassay data can be difficult to interpret because the signals range from very high to very low, indicating a range of IgG reactivity that includes individuals that were recently infected, previously infected, previously vaccinated or have never been previously exposed. This makes it difficult to determine a cut-off between positive and negative individuals in a population, especially for acute infections. Finite mixed modeling offers an approach that exploits differences in the immune status of individuals in a population (Vyse *et al.*, 2006, Fujii *et al.*, 2014). Rota *et al.*, (2008), used this approach to optimize cut-off points from measles serological survey data from an exposed population. Because the exposure or infection status of subjects in the study is not known, we estimate the underlying distributions of unexposed, newly exposed and formerly exposed populations that is suggested by the overall distribution of the reactivity from the data using FMM. An additional limitation is the small sample size used in this assay to set the cut-off points. The approach used here is a demonstration of how to set a cut-off point for antibody response but it is currently being used in this laboratory to analyze large data sets from human plasma and saliva collected from epidemiology studies.

This work explored the impact of key multiplex parameters on the output response (MFI) with the goal of determining optimal conditions for obtaining maximum MFI while minimizing reagent concentrations for various commercially available antigens. Multiple DOE analyses were performed, optimal settings were found and important interactions were studied. RSM was

412 particularly beneficial in studying the full design space based on the parameter settings,
413 developing regression models to explore characteristics and optimizing reagent concentrations.
414 This approach proved effective at determining and distinguishing parameter importance as a
415 function of differences in antigen properties. DOE is a very promising tool that can be applied in
416 a variety of cases to provide insight on the impact of parameter changes, examining factor
417 interactions and optimizing experimental settings that result in time, resource and cost savings
418 even when dealing with quite complex problems. The establishment of cut-off points using
419 FMM allowed us to discriminate between individuals who were unexposed, exposed and
420 formerly exposed even with a limited sample size.

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Figure Legends

Figure 1. Summary of the ANOVA results from the Ag specific DOE studies: The figure displays the frequency of identifying certain statistically significant main and two factor interaction (2FI) effects. The 1° Ab concentration was identified as an important factor in each of the models, followed by the 2° Ab concentration, BC and AC interactions. Given the multiple statistically significant main and 2FI identified, *E. coli* appears to exhibit the greatest amount of complexity. Note: Factors are A: Antigen concentration, B: 1° Ab concentration and C: 2° Ab concentration.

Figure 2. Summary of DOE optimization results from Ag specific studies. The surface plots present a profile of the 1° and 2° Ab. interaction and corresponding MFI response. The reagent concentrations provided were determined by optimizing the response surface model given the goal of minimizing reagent use while maximizing MFI response.

Figure 3. Range of Median Fluorescence Intensity minus background values for antigens in the multiplex assay exposed to the 54 characterized plasma samples. Top panel: Observed MFI's for the specific antigens to the characterized samples. Bottom panel: Observed MFI's to non-characterized antigens in the characterized samples.

Figure 4. Finite mixed models for pathogens based on observed distributions. The dotted line (sometimes obscured by the component distributions, which are differentiated by solid and

dashed lines) is the sum of the component distributions, equal to 1. Relative areas under the component curves give the probability of false positive to false negative (from left to right). For comparison, histograms of the observed distribution are shown by bars and percent of the sample falling with each range are indicated by the values on the y-axis. Cutoff values, defining the 95th percentile of the distribution of log₁₀ MFI among the presumed “unexposed” individuals is shown as a vertical line.

Table 1. Factor and levels for the designed experiments. These settings were developed from preliminary experiments which indicated that reagent concentrations varied from organism to organism; hence we explored a range of concentrations.

Table 2. Sample of the DOE design matrix with corresponding MFI response.

Table 3. Evaluation of multiplex assay specificity. Maximum signal observed between target antigen and detection antibody as well as percent cross-reactivity of signal obtained between non target antigen and detection antibody are presented. Shaded (gray) area indicates antigens not included in final assay.

Table 4. Cut-offs and implied false negative rates based on calculated means and standard deviation of underlying unexposed and exposed populations.

Table 1

Factor		Levels								Mean
A	Ag									106.25
	concentration (µg/ml)	25	50	100	250					
B	1 ⁰ Ab									24.80
	concentration (µg/ml)	0	1.5625	3.125	6.25	12.5	25	50	100	
C	2 ⁰ Ab									9.5
	concentration (µg/ml)	4	8	10	16					

Run	Ag name	A	B	C	MFI
		Ag	1 ⁰ Ab	2 ⁰ Ab	
		concentration (μg/ml)	concentration (μg/ml)	concentration (μg/ml)	
1	<i>H. pylori</i>	25	100	8	23722
2	<i>H. pylori</i>	250	50	4	18933
3	<i>C. jejuni</i>	250	3.125	4	18558.5
4	<i>E. coli 0157:H7</i>	50	50	10	3500
5	<i>S. typhimurium</i>	25	3.125	16	977.5
6	<i>H. pylori</i>	250	6.25	10	17971.5
7	<i>E. coli 0157:H7</i>	50	3.125	4	2094
8	<i>E. coli 0157:H7</i>	250	50	10	5362

Target analyte (catalog no.)	Maximum signal for target antigen (MFI)	Goat anti- <i>C. jejuni</i> (G5V04-500)	Goat anti- <i>E. coli</i> O157:H7 (01-95-90)	Goat anti- <i>Salmonella</i> CSA-1 (01-91-99)	Goat anti- <i>Shigella</i> spp. (01-90-01)	Goat anti- <i>G. lamblia</i> (B65640G)	Goat anti- <i>T. gondii</i> (ab23507)	Goat anti-HAV (B65808G)	Goat anti-Rotavirus (B65110G)	Rabbit anti- <i>Vibrio</i> spp. (01-90-02)	Goat anti- <i>H. pylori</i> (B92510G)	Rabbit anti-GII.4 Norovirus	Guinea pig anti-GI and GII norovirus
<i>C. jejuni</i> intact cells (50-92-93) ^a	25820		2.5	7.8	18.2	109.7	3.5	6.7	0.3	22.8	3.8	3.3	0.2
<i>E. coli</i> O157:H7 intact cells (50-74-01) ^a	20550	1.6		27.9	9.8	58.6	3	1.5	0.3	7.9	0.3	1	0.2
<i>S. typhi</i> intact cells (50-74-01) ^a	20688	1.4	4.2		18.7	86.1	2.1	4	0.4	13.5	0.4	2.6	0.3
<i>Shigella</i> spp.intact cells (50-90-01) ^a	17258	3.9	12.3	37.6		119.1	2.3	10.4	0.9	94.2	1.4	3.6	0.8
<i>G. lamblia</i> lysates ^b	5545	0.7	1.4	2	1.3		3.2	0.8	0.1	0.9	0.1	0.1	0.02
<i>T. gondii</i> p30 (R86300) ^c	26199	0.9	2	2.1	3.7	138.4		0.6	0.1	2.6	0.1	1.4	0.03
HAV from cell culture lysates (8505) ^c	18207	4.4	16.1	17	2	179.4	20.6		2.7	1.5	0.3	4.6	0.1
Rotavirus VP6 ^d	26114	1.1	2.6	3	2.3	107.1	5.1	4		1.2	0.3	1	0.1
<i>Vibrio</i> spp. intact cells (50-90-02) ^a	19565	3.7	12.9	33.1	26.2	170.2	6.3	6.5	1.5		1.6	5.3	1.3
<i>H. pylori</i> lysate (R14101) ^c	19197	1.7	1.6	2.1	1.5	60.9	4.8	2.1	0.5	1		5.1	0.4
Norovirus VA387 P particle ^e	25912	1.6	3	4.7	4.3	96.3	2.1	1.2	0.2	3.9	0.3		84.9
Norwalk virus P particle ^e	28605	2	3.6	8.3	5.6	134.8	6.1	2.4	0.3	5.1	0.6	0.4	
BSA control	ND	0.8	2	1.5	1.8	88.6	1.5	0.5	0.1	0.9	0.1	0.1	0.03

Table 4

	"Unexposed/minimally exposed" samples			"Positive" samples		
	Mean	SD	Cutoff ¹	Mean	SD	P(F-) ²
<i>Campylobacter</i>	3.370	0.4934	-	(1 Component Model) ³		
<i>Toxoplasma</i>	2.381	0.3061	2.884	3.614	0.2242	0.001
<i>Helicobacter</i>	2.546	0.1425	2.781	3.735	0.4896	0.026
Hepatitis A	2.615	0.4031	3.279	3.872	0.3004	0.017
Norovirus VA387	3.007	0.2120	3.356	4.028	0.4309	0.059
Norwalk						
- Component 1 vs. 2	3.373	0.4482	4.111	4.104	0.1910	0.522
- Component 1 vs. 3	"	"	"	2.324	0.0091	< 0.001
- Component 2 vs. 3	4.104	0.1910	4.419	"	"	0.340
Rotavirus						
- Component 1 vs. 2	3.549	0.1824	3.849	4.126	0.1334	0.019
- Component 1 vs. 3	"	"	"	4.415	0.0382	< 0.001
- Component 2 vs. 3	4.126	0.1334	4.346	"	"	< 0.001

¹ Cutoff is determined as that reactivity (log₁₀MFI) at the 95th percentile of the distribution of log₁₀MFI values among unexposed/minimally exposed subjects, equivalent to a 5% false positive rate. ² P(F-) is calculated from the distribution of positive (i.e. exposed) subjects as the proportion falsely categorized unexposed/minimally exposed based on the cutoff value. ³ Observed log₁₀MFI among *Campylobacter* antibody samples are best modeled by just a single distribution. This describes a wide range of exposures, as evidenced by the large log standard deviation.

Figure 1

p-Values from Separate RSM ANOVAs: Main and 2FI

Antigen	A	B	C	AB	BC	AC
<i>H. pylori</i>	0.0114	< 0.0001	0.0064		0.0416	
<i>C. jejuni</i>		< 0.0001				0.0246
<i>E. coli</i> 0157:H7		< 0.0001	0.0002	0.0029	0.0173	0.0088
<i>S. typhimurium</i>		0.0019				

Figure 2

	Optimized Concentration (µg/ml)			
Antigen	Antigen	1 ^o Ab	2 ^o Ab	MFI
<i>H. pylori</i>	29.89	13.45	5.70	23071.90
<i>C. jejuni</i>	36.20	12.80	4.42	23437.50
<i>E. coli</i> 0157:H7	48.45	28.48	6.43	9654.91
<i>S. typhimurium</i>	44.08	37.73	5.54	6668.49
Mean	39.66	23.12	5.52	

Figure 3

Figure 4