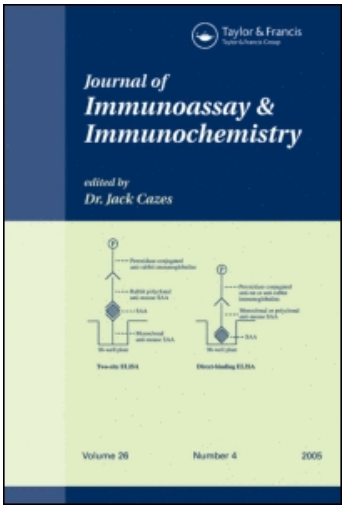

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Complexities of Clinical Assay Development and Optimization Prior to First-in-Man Immunization Trials - A Description of Immunogenicity Assay Development for the Testing of Samples from a Phase 1 Alzheimer's Vaccine Trial

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Complexities of Clinical Assay Development and Optimization Prior to First-in-Man Immunization Trials – A Description of Immunogenicity Assay Development for the Testing of Samples from a Phase 1 Alzheimer's Vaccine Trial

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Abstract: Immunogenicity is often a critical clinical endpoint in the assessment of vaccines prior to the submission of data to regulatory agencies. As a result, the assays used to measure immunogenicity must be highly characterized, well-controlled, and statistically supported. These goals are not easily attained, however, when the development of the assay must occur prior to the first-in-man studies. Two significant barriers exist in the development of these assays: (1) the lack of experience with the performance of a novel antigen in a clinical assay, and (2) the lack of available proper human clinical samples to create reference standards and assess sample matrices. To help to overcome these obstacles, we employed a screening experimental design to assess assay optimization.

Design of experiments (DOE) is a statistical tool that allows for the evaluation of all of the key assay parameters to determine the optimal conditions for the assay, as well as determine if there are any interactions of these parameters on the response of the assay. The multivariate approach that is integral to DOE helps to overcome the lack of experience with the assay reagents by facilitating an

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understanding of how the variables work together in the performance of the assay. Here, we outline the use of full and fractional factorial DOE in the optimization of a clinical assay on two platforms, Luminex® and ELISA, for the measurement of antibodies to the β-amyloid peptide (Aβ) for a novel first-in-man vaccine program. Both platforms are evaluated in an attempt to determine the assay best suited to the needs of the program. We also describe the specificity experiments performed to further characterize the utility of each assay platform.

Keywords: Alzheimer's, DOE, ELISA, IgG, Immunogenicity, Luminex®, Optimization

INTRODUCTION

Immunogenicity is often a critical clinical endpoint in the assessment of vaccines prior to submission to regulatory agencies. As a result, the assays used to measure immunogenicity must be highly characterized, well-controlled, and statistically supported. These goals are not easily attained, however, when the development of the assay must occur prior to the first-in-man (FIM) studies. The complexities related to immunoassay development and method validation have been reported previously by other scientists.[1]

One challenge to the development of a clinical immunogenicity assay prior to a FIM trial is the lack of experience with the novel antigen. While non-human pre-clinical data may exist, the species reactivity may not be representative of the human post-vaccination sample reactivity. Consequently, optimization of the assay reagents and parameters to the post-vaccination animal samples may not represent the optimal conditions for the human post-vaccination samples. Not all species may produce antibodies with comparable affinity, avidity, and class/subclass types, thus potentially affecting assay performance.[2] Additionally, as is the case presented here, the antigen of interest may be a naturally occurring non-infectious peptide to which varying levels of natural antibody may exist.[3–5] Accordingly, addressing the specificity of an immunogenicity assay for a novel antigen is challenging since reference methods or purified materials usually do not exist. Therefore, recovery methods are often used to address specificity of the assay.[6] As a result, the assay used to measure immunogenicity must be optimized to strike a balance between sensitivity (the ability to read a wide dynamic range of the analyte) and specificity (the ability of the assay to distinguish the analyte of interest in the sample matrix).

Another barrier to the development of properly controlled immunogenicity assays is the lack of appropriate reference standards and positive control samples for the assay (those which mimic the matrix and

antibody profile of the samples to be tested). For vaccines targeted toward infectious diseases, it is sometimes possible to obtain human controls from naturally infected individuals. However, while preserving the matrix of the clinical samples to be analyzed, antibody profiles against infectious diseases may differ between patients that have suffered natural infection versus those that have been vaccinated.[7–9] Conversely, serum from pre-clinical studies, laboratory animal vaccinees, may exist which may or may not mimic the antibody profile, and in addition, will not be representative of the matrix of the samples to be tested. Because the assay may be developed with assay controls that do not mimic either the matrix or the antibody profile of the samples to be tested, the assay's true accuracy in detecting human antibodies to the antigen may not be reflected.[2]

Designed experiments can help reduce the time and resources typically invested in the development and optimization of a clinical assay facing an array of unpredictable variables. Design of experiment (DOE) is a method used to study experimental variables and to evaluate the impact these variables have on the response of an assay relative to changes in these variables.[10,11] Utilizing either full or fractional factorial testing of these assay variables provides information not only about the variables and about the interaction these variables may have on each other, but also through specific modeling, may provide information about levels of conditions not directly tested. Its use in the development and optimization of an assay saves time and resources. It also provides an extra level of confidence that the variability attributed to each parameter is appropriately determined. This manuscript describes the use of full and fractional factorial DOE in the development and optimization of an immunogenicity assay used for the detection of human IgG to the β-amyloid peptide (Aβ) used in a Phase 1 Alzheimer's disease vaccine program, on a Luminex®-based and an ELISA-based platform. Optimized assays on both platforms were evaluated in an attempt to determine the assay that is best suited to the needs of the program. Furthermore, the manuscript details the specificity assays used to characterize the fitness of each of the two platforms.

EXPERIMENTAL

General Reagents

Aβ Peptide

The Aβ1-40 peptide was synthesized with a biotin molecule covalently attached to the carboxyl terminal. The Aβ1-40 peptide was solubilized

in DMSO (data not shown). The Aβ1-40 peptide was chosen as the assay antigen because it resembles the physiological Aβ1-42 species but does not aggregate as significantly as the Aβ1-42 peptide.[12]

Reference Monkey Sera for Assay Standard and Controls

Assay positive controls and standards were derived from rhesus macaques vaccinated with a candidate peptide-based vaccine 9 times at monthly intervals, with bleeds collected 4 weeks following each injection.

Blocking Reagents

Five different blocking reagents were used across the optimization of the two assay platforms. The five reagents can be split into 3 categories: Animal protein-Normal goat serum (Sigma cat#G-6767) and globulin-poor goat serum (which consists of normal goat serum incubated with saturated ammonium sulfate to precipitate all Ig); Non-animal protein – Superblock (Pierce cat#37515), and Chemical Blockers – Polyvinyl alcohol (PVA, Sigma, cat#P18136)/Polyvinylpyrrlidone (PVD, Sigma cat#PVP-360) used per Ref. [13] and PBS with Tween-20 (see Tables 1 and 2).

General Protocol for Total IgG Aβ Luminex® Assay

Bead Coupling

LumAvidin microspheres (beads) were purchased from Radix Biosolutions, Ltd and custom coupled with avidin. The microspheres have been internally dyed with different intensities of red and infra-red fluorochromes to produce 100 distinct spectrally addressable microspheres, potentially allowing for antigen multiplexing.[14] The bead's spectral address was measured by a BioPlex array reader (BioRad Labs, cat#171-000010) and indicated by a specific bead number (1-100). The beads were incubated in a centrifuge tube, with coupling buffer (PBS, 0.05% Tween-20, 0.05% sodium azide) and biotinylated peptide for 30 minutes (±15 mins) on a rotator/shaker. The beads were then spun down using a micro-centrifuge at 13,200 rpm. The supernatant was aspirated off and the beads were re-suspended in 1 mL of coupling buffer by sonication and vortexing. The beads were washed and re-suspended an additional 2 times and finally resuspended in 1 mL per 100 μL of starting volume of coupling buffer. The beads were then counted using a Beckman Coulter Z series particle counter (Beckman Coulter, AB40040).

Table 1. Parameters tested in the anti-Aβ Luminex® DOE-Column labeled "Ranges/Conditions tested" shows the ranges of each of the parameters tested or the conditions of a factor to be analyzed; Conditions in the "optimal" column indicate the conditions of each parameter that optimize the anti-Aβ Luminex® assay

Experimental condition	Ranges/ conditions tested	Optimal
Antigen concentration	100–400 μM	200 μM
Secondary antibody concentration	0.75 μg/mL–2 μg/mL	2 μg/mL
Assay diluent	1. Globulin poor goat serum 2. Normal goat serum 3. 0.05% PBS-T	Normal goat serum
Bead coupling time	15–45 minutes	30 minutes
Sample/bead incubation time	45–75 minutes	75 mins (65 ± 10)
Bead/secondary incubation time	45–75 minutes	45 mins (35 ± 10)

Assay Procedure

Diluted serum (standards, controls, and samples diluted in Run Buffer [PBS, 10% normal goat serum (NGS, Sigma, cat#G-6767) and 0.05% Tween-20]) were incubated with the peptide-coupled beads for 65 (±10) minutes in 96-well Multiscreen PVDF (polyvinylidene fluoride) plates (Millipore Co., cat#MABVN1250) on a plate rotator at 700 rpm. The plates were then washed 3 times with 200 μL/well of wash buffer (PBS/0.05% Tween-20) and vacuum aspirated using a Millipore vacuum manifold (Millipore corp, MAVM096OR). The beads were then incubated with 2 μg/mL of an anti-human IgG phycoerythrin-labeled secondary antibody (BioTrend Chemikalien GmbH, cat#I127) for

Table 2. Parameters tested in the anti-Aβ ELISA DOE – Conditions in the "optimal" column indicate the conditions of each parameter that optimize the anti-Aβ ELISA.

Parameter	Range/Conditions tested	Optimal
Antigen concentration	1–9 μg/mL	2 μg/mL
Blocking buffer	Normal goat serum Superblock PVA and PVD	Normal goat serum
Secondary antibody concentration	0.06 μg/mL–1.4 μg/mL	0.62 μg/mL
Substrate incubation times	1 hour–2 hours	1 hour, 45 minutes

35 (±10) minutes on a plate rotator at 700 rpm. The plates were washed again, 3 times, and finally the beads were re-suspended in 120 μL of assay wash buffer. The IgG anti-Aβ1-40 bound to the beads was detected using a Bioplex array reader.

Luminex® General Data Analysis

Sample titers were determined by interpolation of the median fluorescent intensity (MFI) response of the sample against a standard control (pool of immunized monkey) serum. The reference standard dilution series was modeled using a weighted four-parameter logistic function, and the test sample concentration was interpolated from the fitted standard curve. Data (in MFI units) were either processed using the Bio-Plex Manager 4.1.1 software (Bio-Rad, Hercules, CA) or a custom developed Excel spreadsheet.

General Protocol for Total IgG Aβ ELISA

Plate Coating

The antigen was diluted in assay coating buffer (PBS, 0.05% Tween-20) to its working concentration. One hundred microliters (μL) of diluted antigen was added to each well of NeutrAvidin coated ELISA plates (Pierce cat#15507). The coated plates were incubated for 12 to 24 hours at 2–8°C. The plates were washed 3 times and 200 μL blocking buffer (PBS, 10% NGS, 0.05% sodium azide, 0.05% Tween-20) was added to each well. The plates were covered and incubated for 90 minutes (±3 minutes) at 23°C.

Assay Procedure

Standard, control, and sample sera were 4-fold serially diluted in serum diluent (PBS, 10% NGS, 0.05% sodium azide, 0.05% Tween-20) and 100 μL of each was dispensed into antigen coated wells on the NeutrAvidin plates. The plates were incubated for 2 hours at 23°C. The plates were washed with wash buffer (PBS, 0.05% Tween-20) followed by the addition of 100 μL alkaline phosphatase labeled goat anti-human IgG conjugate diluted in conjugate diluent (PBS, 0.05% Tween-20) and incubated for 1 hour at 23°C. The plates were then washed and 100 μL of phenolphthalein monophosphate (PMP) substrate was added. The plates were incubated for up to 2 hours at 23°C, and then 200 μL of stop buffer (2% sodium phosphate in sterile distilled water) was added. The absorbance was measured at 550 nm by an ELISA plate reader.

ELISA General Data Analysis

Standard curves were fit with a four-parameter logistic function using weighted (squared mean weighting) nonlinear regression. Titers for the unknown samples were interpolated from each of the fitted standard curves in each run.

Design of Experiment (DOE)

DOE is a strategy in which multiple experimental variables are studied together and an evaluation is performed on how these factors collectively affect a measured response, as well as interact with each other. DOE was used in this study to determine the optimal conditions at which the dynamic range was maximized and within-assay variability was minimized. The dynamic range of the standard reference curve was optimized by maximizing the asymptote corresponding to high antibody concentration and by minimizing the slope. The within-assay precision was minimized to balance the impact of decreasing the slope (which increases the imputed uncertainty in the estimated titers of unknown samples).

DOE for Luminex® Optimization

A full factorial DOE was used to optimize the anti-Aβ Luminex® assay, an example of which is illustrated in Figure 1(a). Six major factors of variation were tested in this design, including: antigen concentration, secondary antibody concentration, assay diluent, bead coupling time, sample bead incubation time, and bead/secondary incubation time (Table 1). For this experiment, 450 standard curves and sets of control

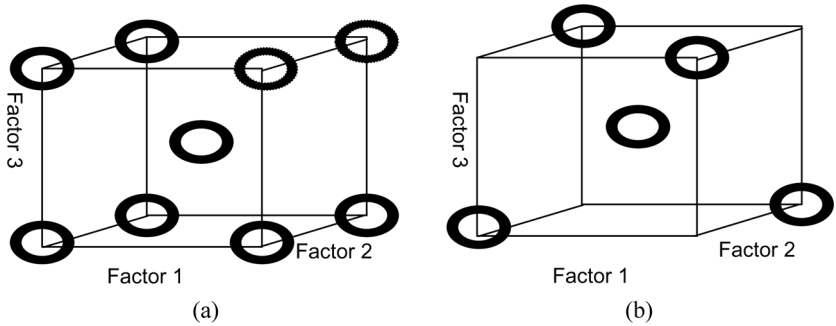


Figure 1. Example of DOE full factorial (a) and fractional factorial (b) designs. Black circles represent examples of parameter combinations that would have been tested in the Luminex® and ELISA platforms, respectively.

data were generated using each combination of the three parameters for each of the conditions tested.

DOE for ELISA Optimization

A fractional factorial DOE was used to optimize the anti-Aβ ELISA (an example of this design is illustrated in Figure 1(b)). Four major factors of variation were tested: antigen concentration, blocking buffer/sample diluent, secondary antibody dilution, and substrate incubation time (Table 2). Twenty-four standards and sets of control data were generated using either a combination of the extreme conditions or a combination of the center values of the parameters being evaluated. Additionally, 21 human samples with unknown status of Alzheimer's disease were tested at each of the experimental conditions.

DOE Analysis

The standard curves were fitted using a weighted (squared mean weighting) 4-parameter logistic nonlinear regression function. Since the objective of the experiment was to find the "optimal" conditions which maximized the dynamic range and minimized variability, the same regression function and weighting was used for all of the standard curves. A multivariate analysis of covariance (MANCOVA) was performed to assess the impact of the variables studied on the regression parameter estimates. Additionally, in the ELISA, optimal conditions were chosen based on the ability of the assay to have the maximum separation between the high reacting samples and the low reacting samples. To determine optimal assay parameters, a desirability function was used, where each response was assigned a value of 1 or zero, 1 representing a completely preferred result and 0 representing a completely non-preferred result.[15] For each set of conditions, the geometric mean of the desirability values for each parameter within the set of conditions is then combined and a desirability value for the set of conditions is established. The desirability values for each set of conditions are then compared. The set of conditions with the combined desirability values closest to 1 is considered the most optimal.[16]

Demonstration of Assay Specificity

Luminex®

Specificity in the Luminex® assay was demonstrated using a competitive inhibition of antibody binding approach. Sera from post-vaccination

Rhesus macaques, as well as sera from 10 non-vaccinated humans, were incubated with 1000 μM and 500 μM of free Aβ1-40 peptide, an irrelevant peptide (PKCε receptor, Biosource International, cat#77-121), and an equivalent volume of distilled water. The diluted samples were pre-incubated overnight at 4°C on a plate shaker and then assayed as described above.

ELISA

Since the solid phase antigen binding in an ELISA may differ from that on the beads, it was determined that the results of a liquid phase competition experiment may not truly represent the specificity of the binding demonstrated by the assay. As a result, spiking and recovery experiments were performed, where known quantities of post-vaccination monkey serum were spiked into 8 non-vaccinated human sera. The human sera were spiked with three different levels of monkey sera (60, 40, and 20% of total reactivity) and the curves were compared to the curve of the post-vaccination monkey standard control serum.

RESULTS

Optimization of Multiple Experimental Assay Conditions by using DOE

Luminex®

We first sought to determine the peptide concentration that maximizes sensitivity by extending the dynamic range of the assay. During the assay exploratory developmental period prior to the DOE, experiments were performed to determine the effect of a full range of antigen concentrations on the standard curves using a post-vaccination monkey serum (Figure 2). These results showed that a peptide concentration of 200 μM results in reproducible standard curves with good sensitivity and dynamic range. At concentrations greater than 200 μM, the standard curves' fluorescent values were suppressed.

With a preliminary concentration of antigen targeted, we next-evaluated the effect of the most critical assay incubation times and reagents on assay sensitivity (the ability to detect the analyte of interest at high and low levels), dynamic range, and precision by using a DOE approach. Specifically, the assay conditions depicted in Table 1 were evaluated, and included the use of normal goat

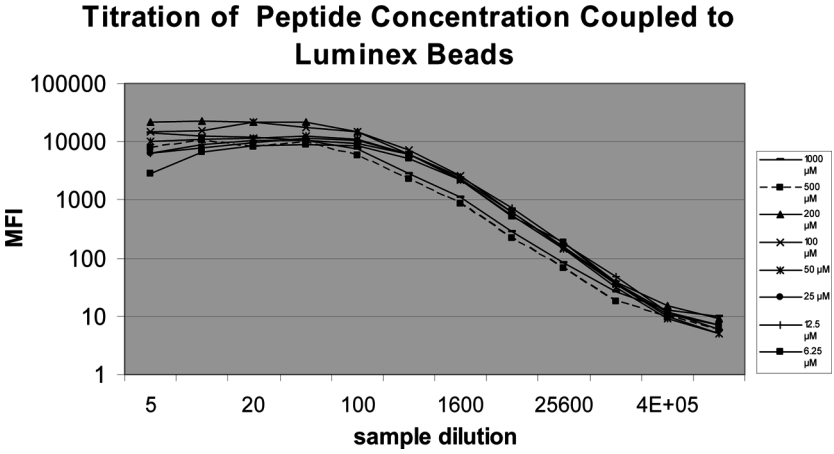


Figure 2. Peptide Titration in Luminex® – Several concentrations of biotinylated-Aβ peptide were coupled to LumAvidin microspheres and tested in the Luminex® platform with post-vaccination monkey serum in order to determine the most optimal coupling concentration. Based on the curves, 200 µM was chosen as it shows the widest dynamic range with little to no prozone effect. (All concentrations listed are micromolar).

serum in the assay diluent, an antigen concentration of 200 µM, and a secondary antibody concentration dilution of 1:50 (2 µg/mL). The desirability function values at different incubation times at these conditions are shown in the black square in Figure 3. Generally, longer sample incubation and shorter secondary incubation times yielded higher desirability values.

ELISA

We evaluated extreme and midpoint combinations of the most critical assay parameters using the assay standard control serum, as well as serum from 21 non-vaccinated human donors. The determination of optimal assay parameters was based on the combination of tested parameters that allowed for the widest dynamic range, as well as the largest separation between high responding and low responding human samples previously screened in the Luminex® platform. The results can be seen in Table 2. Generally, the parameters that best optimized the standard control serum did not demonstrate the best separation between samples; the converse was also true. As a result, the parameters that were selected indicate a compromise between the experimental objectives.

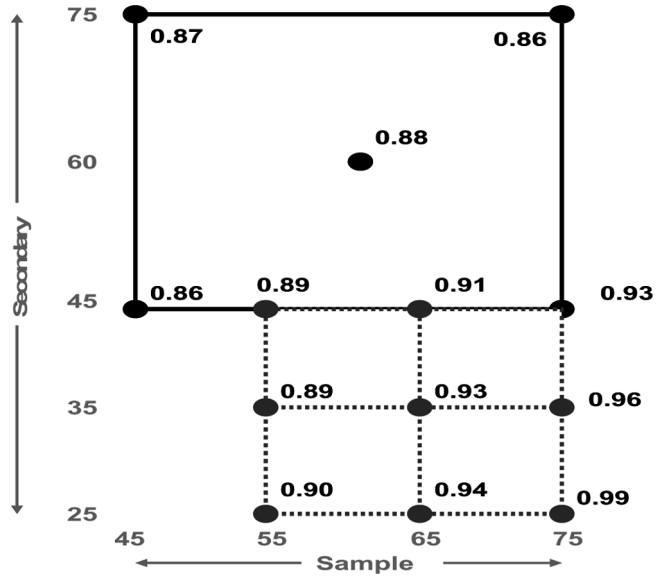


Figure 3. Desirability function values for different incubation times (in minutes). A combination of incubation times is represented by each dot; the values indicate the desirability value, where 0 is a non-preferred result and 1 is the optimal, preferred result. Dots with values closest to 1 represent combinations of incubation times that help achieve the desired results. Incubation times tested in the DOE are indicated in solid black, while extrapolated desirability values for incubation times that were not directly tested are indicated in dotted grey.

Demonstration of Assay Specificity

Luminex®

Specificity studies using free Aβ1–40 peptide as an inhibitor were performed in post-vaccination monkey serum and serum from non-vaccinated humans. Both the 1,000 and 500 μM concentrations of the free Aβ1-40 peptide were able to inhibit as much as 85% of the reactivity in the post-vaccination monkey serum. Competition with an irrelevant peptide (PKCϵ receptor) or water did not significantly reduce the reactivity. The results are illustrated in Figure 4(a). The human samples, however, seem to show a subject specific response to competition, with some samples showing no reduction in reactivity and others showing a significant reduction after competition with free Aβ1-40 peptide, Figure 4(b).

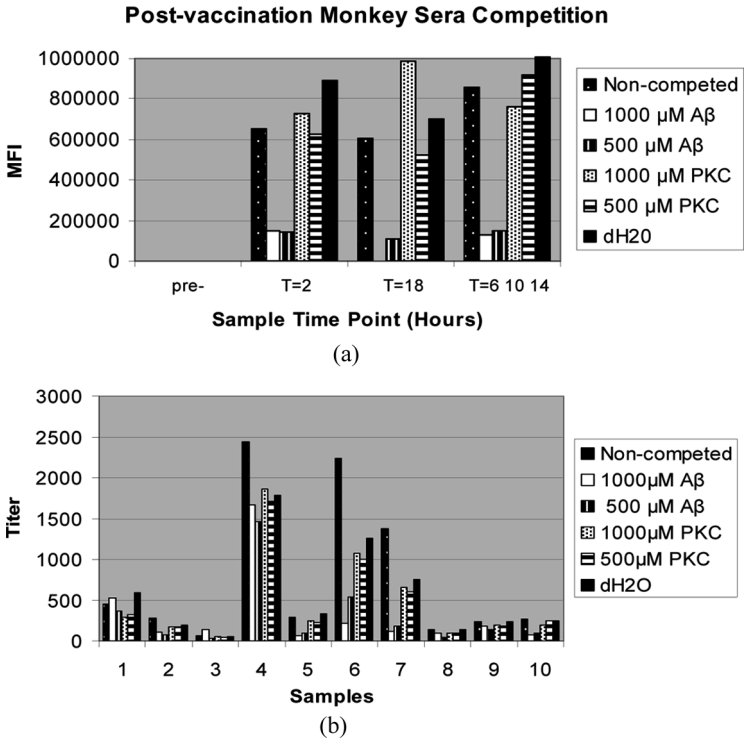
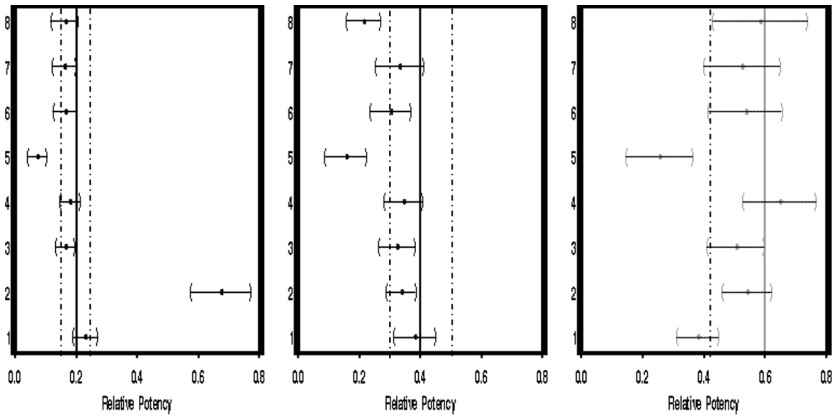


Figure 4. Sample specific results from anti-Aβ Luminex® Specificity Competition experiments – Each bar represents a different inhibitor or condition used for competition. (All concentrations listed are micromolar (µM). (a) Median Fluorescent intensity results from post-vaccination monkey sera competition. Each sample is comprised of a mixture of the serum from 3 post-vaccination monkeys. The x-axis values represent the time in weeks post-vaccination, where "T = 6 10 14" is the combination of 3 monkey sera at each of three time points. (b) Results from non-vaccinated human serum competition. Each sample is comprised of 1 individual serum and titers are interpolated from the monkey serum standard run with the samples.

ELISA

Specificity studies based on the recovery of Aβ-specific antibodies from human matrix were performed. In each of the eight samples tested, and at each of the three spiking concentrations, anti-Aβ antibodies from the post-vaccination monkey sera were recovered. Approximately 87.5% of the samples tested fell within 30% of the expected value for at least 2 of the 3 concentrations tested (Figure 5).



(a) (b) (c)

Figure 5. Results from the anti-Aβ ELISA serum spiking experiments. The graphs indicate the recovery of Aβ1-40 antibodies from post-vaccination monkey serum from each of 8 non-vaccinated human samples relative to the expected value of recovery. The solid line in each graph represents the expected value if 100% of the expected activity is recovered and the dotted lines in each graph represent 30% RSD from each expected value. Graphs (a), (b), and (c) show the individual sample recoveries (with 95% CI) when the samples are spiked with 20%, 40%, and 60% of the post-vaccination monkey serum, respectively.

DISCUSSION AND CONCLUSIONS

There is a wide range of technical and scientific challenges when developing, optimizing, and characterizing an immunogenicity assay prior to FIM trials. Because of little experience with the Aβ1-40 peptide, and the unavoidable use of serum from a different species with a different matrix to generate the reference standard, two platforms were evaluated and tested for potential use as a clinical immunogenicity assay. The Luminex® platform was chosen for evaluation since it can provide the flexibility to measure additional responses to other antigens in a single sample (multiplex) if needed, and provides a wide dynamic range indicating excellent sensitivity.[14,17] Unlike ELISAs, antigen-antibody interactions in the Luminex® platform occur on the surface of latex microspheres in solution, allowing for a three-dimensional antigen-antibody interaction. The ELISA platform was evaluated because, in general, it is a more economical platform and lends itself to bridging with current, high-throughput automation platforms.

Assay optimization is frequently carried out by changing one variable at a time, while other variables are held fixed. While seemingly easy to implement, this process is often quite resource intensive. More

importantly, this approach may fail to identify potentially important interactions between certain assay variables, resulting in an analytically suboptimal assay that could negatively influence the clinical findings. Therefore, the use of experimental design methodology, that allows degrees of multiple variables to be tested and optimized, simultaneously, has been highly recommended.[2] In this report, two platforms were optimized using the DOE strategy: the Luminex® using the low-risk full factorial approach, and the ELISA with the higher risk fractional factorial approach, where it may not be possible to distinguish some interactions from main effects. Both DOE approaches allowed for the quick determination of parameters that maximized the dynamic range and allowed for maximum sensitivity of each of the assays. Additionally, the DOE approach allowed for the elucidation of the parameter interactions. This was particularly important for the optimization of the ELISA assay where compromises needed to be made in the selection of the assay parameters. In short, the DOE approach to assay optimization allowed for a small amount of experimentation that led to highly characterized results.

Specificity is the ability to measure the analyte, in this case anti-Aβ antibodies, "unequivocally in the presence of other components, either exogenous or endogenous",[18] and in the absence of proper assay controls can be an important determinant of assay utility. Because of the differences in the platforms (i.e., fixed solid phase ELISA vs. floating solid phase Luminex®), two different approaches to assess specificity were used, i.e., competition for the Luminex® and recovery for the ELISA. The Luminex® results indicated that, in serum from post-vaccination monkeys (the only available vaccinated sample), the reactivity could be reduced up to 85% when the serum is pre-absorbed with homologous peptide. The human donors tested exhibited a sample-specific response, with some non-vaccinated patients showing reactivity that could be competed out, potentially indicating the presence of natural antibodies (a conclusion supported by Refs. [3–5], while the reactivity of other samples tested could not be reduced by competition, which may indicate some matrix interference in the assay. As remarked by Findlay et al. (2000), "some factors that may nonspecifically interfere with the antigen-antibody binding reaction include, hyperlipidemia, hemolysis, ionic strength, sample viscosity, serum proteins (e.g., complement and rheumatoid factor), anticoagulants, proteases, autoantibodies, binding proteins and heterophilic anti-IgG antibodies".

Recovery testing in immunogenicity assays is the determination of whether components in the serum matrix being investigated can inhibit specific antibodies from binding to the antigen under assay conditions, thereby affecting assay response.[2] The recovery experiments performed in the ELISA were evaluated at three different levels of spiked antibody from each of the samples tested. The results indicate that, in general, the human

matrix does not interfere with the measurement of Aβ 1-40 antibodies, since recovery of response was achieved in most samples tested.

We describe the use of DOE for assay optimization, and its utility in reducing the assay development time and allowing for controlled and optimized results. Subsequent pre-validation experiments for both platforms were performed and indicated that the parameters determined in the DOE allowed for the development of precise (within 30% total relative standard deviation for both platforms) and sensitive (2.5-fold and 3.5-fold dynamic range for the ELISA and Luminex® immunological assays, respectively) (data not shown). Because of the lack of proper human controls for the assays, it was important that specificity be demonstrated on both platforms. Specificity experiments performed for each platform indicate that, within the constraints of each platform, the assays are specific for anti-Aβ in post-vaccination monkey serum for the Luminex® and post-vaccination monkey serum and most human serum samples tested for the ELISA. Because of the subject-specific nature of the reactivity seen in the Luminex®, additional experiments and assay controls would be necessary to use the assay to test samples from a clinical trial. However, for the ELISA, the incorporation of the use of DOE and extensive specificity experiments has led to the development of an immunological assay acceptable for use in testing samples from a clinical trial.

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REFERENCES

1. Colburn, W.A. Biomarkers in drug discovery and development: from target identification through drug marketing. J. Clin. Pharm. **2003**, *43*, 329–341
2. Mire-Sluis, A.R.; Barret, Y.C.; Devanayaran, V.; Koren, E.; Liu, H.; Maia, M.; Parish, T.; Scott, G.; Shankar, G.; Shores, E.; Swanson, S.J.; Tanigushi, G.; Wierda, D.; Zuckerman L.S. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. J. Immunol. Meth. **2004**, *289*, 1–16.
3. Du, Y.; Dodel, R.; Hampel, H.; Buerger, K.; Lin, S.; Eastwood, B.; Bales, K.; Gao, F.; Moeller, H.J.; Oertel, W.; Farlow, M.; Paul, S. Reduced levels of amyloid β-peptide antibody in Alzheimer's disease. Neurology **2001**, *57*, 801–805.
4. Weksler, M.; Relkin, N.; Turkenich, R.; LaRusse, S.; Zhou, L.; Szabo, P. Patients with Alzheimer's disease have lower levels of serum anti-amyloid peptide antibodies than healthy elderly individuals. Exp. Gerontol. **2002**, *37*, 943–948.

5. Jianping, L.; Zhibing, Y.; Wei, Q.; Zhikai, C.; Jie, Jinbiao L. Low Avidity and level of Serum Anti-Aβ Antibodies in Alzheimer's Disease. Alzheimer Dis. Assoc. Disord. **2006**, *20*, 127–132.
6. Rifai, N.; Gillette, M.A.; Carr, S.A. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat. Biotech. **2006**, *24*, 971–983.
7. Bogger–Goren, S.; Baba, K.; Hurley, P.; Yabuuchi, H.; Takahashi, M.; Ogra, P.L. Antibody response to varicella-zoster virus after natural or vaccine-induced infection. J. Infect. Dis. **1982**, *146*, 260–265.
8. Asano, Y.; Hiroishi, Y.; Itakura, N.; Hirose, S.; Kajitan, Y.; Nagai, T.; Yazaki, T.; Takahashi, M. Immunoglobulin subclass antibodies to varicella-zoster virus. Pediatrics **1987**, *80*, 933–936.
9. Isa, M.B.; Martinez, L.; Giordano, M.; Passeggi, C.; de Wolff, M.C.; Nates, S. Comparison of immunoglobulin G Subclass profiles induced by measles virus in vaccinated and naturally infected individuals. Clin. Diagn. Lab. Immunol. **2002**, *9*, 693–697.
10. Sittampalam, G.S.; Smith, W.C.; Miyakawa, T.W.; Smith, D.R.; McMorris, C. Application of experimental design techniques to optimize a Competitive ELISA. J. Immunol. Meth. **1996**, *190*, 151–161.
11. Haaland, P.D. *Experimental Design in Biotechnology*; Marcel Dekker: New York, 1989; 37–59.
12. Monsonego, A.; Zota, V.; Karni, A.; Kreiger, J.; Bar-Or, A.; Bitan, G.; Budson, A.; Sperling, R.; Selkoe, D.; Weiner, H. Increased T cell reactivity to amyloid β protein in older humans and patients with Alzheimer's disease. J. Clin. Invest. **2003**, *112*, 415–422.
13. Waterboer, T.; Sehr, P.; Pawlita, M. Suppression of non-specific binding in serological Luminex® assays. J. Immunol. Meth. **2006**, *309*, 200–204.
14. Dunbar, S.A.; Vander Zee, C.A.; Oliver, K.G.; Karem, K.L.; Jacobson , J.W. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex® Lab-MAP™ system. J. Microbiol. Meth. **2003**, *53*, 245.
15. Derringer, G.; Suich, R. Simultaneous optimization of several response variables. J. Qual. Tech. **1980**, *12* (4), 214–219.
16. *NIST/SEMATECH e-Handbook of Statistical Methods*; http://www.itl.nist.gov/div898/handbook/, 11Oct 2007.
17. Pickering, J.W.; Martins, T.B.; Schroder, M.C.; Hill, H.R. Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for quantitation of antibodies to tetanus, diphtheria, and haemophilus influenzae Type b. Clin. Diagn. Lab. Immunol. **2002**, *9*, 872–876.
18. Findley, J.W.A.; Smith, W.C.; Lee, J.W.; Nordblom, G.D.; Das, I.; DeSilva, B.S.; Khan, M.N., Bowsher, R.R. Validation of Immunoassays for bioanalysis: A pharmaceutical industry perspective. J. Pharm. Biomed. Anal. **2000**, *21*, 1249–1273.

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