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Application of multi-factorial design of experiments to successfully optimize immunoassays for robust measurements of therapeutic proteins

Chad A. Ray*, Vimal Patel, Judy Shih, Chris Macaraeg, Yuling Wu, Theingi Thway, Mark Ma, Jean W. Lee, Binodh DeSilva

Pharmacokinetics and Drug Metabolism, Amgen Inc., Thousand Oaks, CA, United States

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ABSTRACT

Developing a process that generates robust immunoassays that can be used to support studies with tight timelines is a common challenge for bioanalytical laboratories. Design of experiments (DOEs) is a tool that has been used by many industries for the purpose of optimizing processes. The approach is capable of identifying critical factors and their interactions with a minimal number of experiments. The challenge for implementing this tool in the bioanalytical laboratory is to develop a user-friendly approach that scientists can understand and apply. We have successfully addressed these challenges by eliminating the screening design, introducing automation, and applying a simple mathematical approach for the output parameter.

A modified central composite design (CCD) was applied to three ligand binding assays. The intra-plate factors selected were coating, detection antibody concentration, and streptavidin–HRP concentrations. The inter-plate factors included incubation times for each step. The objective was to maximize the log S/B (S/B) of the low standard to the blank. The maximum desirable conditions were determined using JMP 7.0. To verify the validity of the predictions, the log S/B prediction was compared against the observed log S/B during pre-study validation experiments.

The three assays were optimized using the multi-factorial DOE. The total error for all three methods was less than 20% which indicated method robustness. DOE identified interactions in one of the methods. The model predictions for log S/B were within 25% of the observed pre-study validation values for all methods tested. The comparison between the CCD and hybrid screening design yielded comparable parameter estimates.

The user-friendly design enables effective application of multi-factorial DOE to optimize ligand binding assays for therapeutic proteins. The approach allows for identification of interactions between factors, consistency in optimal parameter determination, and reduced method development time.

1. Introduction

In drug development, immunoassays are essential tools for measuring large molecule drugs, biomarkers, and determining immunogenicity against therapeutic proteins. Immunoassays offer flexibility, relatively high throughput, and simplicity for the operator. However, fully optimizing an immunoassay by evaluating all possible combinations of factors using single factor experiments is time prohibitive. As a result, assay developers often settle for methods that are "good enough" that may lack the robustness necessary to support sample analysis. Because there are several factors to evaluate and potential interactions exist between the factors, multi-factorial design of experiments (DOE) should be explored as an alternative to traditional single variable experiments. Multi-factorial DOE has been utilized throughout the pharmaceutical and biotechnology industries in the areas of manufacturing and process development for many years [1–3]. The technique is a cost-effective approach for testing the effects of many variables simultaneously [4]. Sittampalam et al. [5] described an approach for ELISA optimization using experimental design techniques. In this particular example, the authors conducted a Plackett–Burman screening design followed by a central composite design to optimize the immunoassay. They demonstrated that their DOE approach generated similar results to the traditional single variable optimization in a short timeframe, and more importantly they were able to define the interactions between factors. Lauwers et al. applied the Plackett–Burman screening design to optimize an RT-PCR ELISA for detection of enterovirus. The post-design conditions yielded a fourfold more sensitive assay [6]. Lamar and Petz applied DOE techniques for the optimization of an ELISA to detect intact beta-lactam antibiotics in food. They applied a Plackett–Burman screening

* Corresponding author. Current address: 1 Amgen Center Drive, Thousand Oaks, CA 91320-1799, United States. Tel.: +1 805 447 9414; fax: +1 805 480 3057.
E-mail address: caray@amgen.com (C.A. Ray).

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Table 1
Parameters tested using hybrid screening approach for each method.

	Method		
	A	B	C
Capture antibody concentration (μg/mL)	Fixed at 2	1–5	2.5
Detection antibody concentration (ng/mL)	1–10	500–2000	50–300
NA-HRP or sulfo-TAG (ng/mL)	NA	[a]Fixed with detection	100–400
Capture incubation (h)	1–2	1–2	1–3
Detection incubation (h)	0.5–2	1–2	1–3
Neutravidin or Tag incubation (min)	NA	30–60	Fixed 30
TMB substrate incubation (min)	10–20	NA	10–30

[a] A equal concentration of sulfo-TAG to detection antibody was used at each condition tested.

design followed by a Box–Behnken design to optimize the assay. From the response surface analysis, they were able to select conditions that maximized robustness and the process was more efficient than traditional approaches [7].

Our laboratory has implemented multi-factorial DOE for optimizing therapeutic protein immunoassays. Some modifications to the approach recommended by Sittampalam were employed, which included: elimination of the Plackett–Burman screening design, minimization of serum matrix effects prior to optimization, incorporation of automation, and finally implementing a different approach for analyzing the data. Due to the complexity of calculating the precision profile [8], we chose to optimize on S/B as an output variable instead of the precision profiles.

This manuscript illustrates the benefits of implementing multi-factorial DOE in the context of protein therapeutic bioanalysis. These benefits include consistent estimates for optimum parameters, identification of interactions between parameters, development of more robust immunoassays, and resource savings. We also provide details on the set-up, application, and qualification of the hybrid screening design.

2. Experimental

2.1. Materials and equipment

The following equipments were used: Meso Scale Discovery (MSD) standard MA6000 96 plates (MSD, Gaithersburg, MD), Sector Imager 6000 (MSD) and Spectra Max 340PC (Molecular Devices, Sunnyvale CA) plate readers, ELX-405 plate washers (Biotek, Winooski VT), Titermix 100 plate shakers (Brinkmann, Westbury NY), model 2005 incubators (VWR, West Chester PA), and Freedom EVO liquid handlers (Tecan, CH-8708, Mannedorf Switzerland).

2.2. ELISA reagents

All therapeutic protein standards and immunoassay reagents were produced and prepared by Amgen Inc. (Thousand Oaks, CA). Sera from cynomolgus monkey and human were obtained from Bioreclamation (Oceanside, CA). All solutions were stored at 2–8 °C. MSD Read Buffer T with Surfactant 4× (catalog number R92TC-1) was purchased from MSD. 20× KPL wash buffer catalog number 50-63-00 was purchased from KPL, Inc. (Gaithersburg, MD). Dulbecco's phosphate buffer saline (without $CaCl_2$ and $MgCl_2$) was purchased from Invitrogen (Carlsbad, CA). Neutravidin was from R&D Systems (Minneapolis, MN) and the TMB substrate solution from BioFX (Owings Mills, MD).

2.3. ELISA procedures

2.3.1. Method A

Method A was developed at Amgen (Thousand Oaks, CA) for the quantification of a therapeutic protein in serum. The 96-well standard plates were coated at a concentration of 2 μg/mL with an anti-idiotypic mouse monoclonal antibody to the therapeutic protein. The plates were incubated with 1% BSA blocking buffer to block any unbound surfaces. After a wash step, 100% serum standards (STD), quality controls (QC), and blank (BL) were diluted 1:100 with an assay buffer and loaded into the wells. The therapeutic protein present in the STD and QC bound to the immobilized capture reagent. After a wash step, a HRP-conjugated monoclonal anti-human Fc antibody was added to the wells as a detection antibody. The detection antibody concentration was optimized using multi-factorial DOE. The concentrations tested varied between 1 and 10 ng/mL. The detection antibody bound to the therapeutic protein captured during the first step. After incubation, the plate was washed and a TMB substrate solution was added. The plates were read after incubation with a Spectramax plate reader at 450–650 nm. The optical density (O.D.) changes were proportional to the amount of the therapeutic protein bound by the capture reagent. The conversion of the O.D. signal of the samples to concentration was achieved through computer software mediated comparison to a standard curve assayed on the same plate, which is regressed according to a 4 or 5 parameter (Auto-Estimate) regression model with a weighting factor of $1/Y$. Table 1 summarizes the parameters that were tested using multi-factorial DOE. The detection antibody concentration was tested at four different concentrations within a plate. All four antibody concentrations had a seven point standard curve with blank assayed in triplicate (intra-plate factor) (Fig. 1b). The same plate design was applied to the inter-plate factors: capture, detection, and TMB incubation times (example of an inter-plate design Table 2). The objective of the optimization was to improve the sensitivity and performance of the ELISA. In order to accomplish improved sensitivity, the log S/B at the lower limit of quantification (LLOQ) was maximized. In addition, the ULOQ absorbance value was set to match an O.D. between 1.8 and 2.2.

2.3.2. Method B

Method B was similar to method A with some modifications. The most notable difference was the detection platform was electrochemiluminescence. The coating reagent was a recombinant protein receptor. The concentration of the recombinant protein

Table 2
Example of inter-plate schedule.

Plate	STD incubation (h)	Detection antibody incubation (h)	Sulfo-TAG (h)
1	1	2	1
2	1	1	0.5
3	2	2	0.5
4	2	1	0.5
5	1	1	1
6	1	2	0.5
7	2	2	1
8	2	1	1
9	1.5	1.5	0.75

(a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	LL-1	LL-2	LL-3	LL-4	LL-5	LL-BL	LL-1	LL-2	LL-3	LL-4	LL-5	LL-BL
B	LL-1	LL-2	LL-3	LL-4	LL-5	LL-BL	LH-1	LH-2	LH-3	LH-4	LH-5	LH-BL
C	LH-1	LH-2	LH-3	LH-4	LH-5	LH-BL	LH-1	LH-2	LH-3	LH-4	LH-5	LH-BL
D	MM-1	MM-2	MM-3	MM-4	MM-5	MM-BL	MM-1	MM-2	MM-3	MM-4	MM-5	MM-BL
E	MM-1	MM-2	MM-3	MM-4	MM-5	MM-BL	HL-1	HL-2	HL-3	HL-4	HL-5	HL-BL
F	HL-1	HL-2	HL-3	HL-4	HL-5	HL-BL	HL-1	HL-2	HL-3	HL-4	HL-5	HL-BL
G	HH-1	HH-2	HH-3	HH-4	HH-5	HH-BL	HH-1	HH-2	HH-3	HH-4	HH-5	HH-BL
H	HH-1	HH-2	HH-3	HH-4	HH-5	HH-BL						

(b)

	1	2	3	4	5	6	7	8	9	10	11	12
A	S-7-1	S-7-1	S-7-1	S-7-2	S-7-2	S-7-2	S-7-3	S-7-3	S-7-3	S-7-4	S-7-4	S-7-4
B	S-6-1	S-6-1	S-6-1	S-6-2	S-6-2	S-6-2	S-6-3	S-6-3	S-6-3	S-6-4	S-6-4	S-6-4
C	S-5-1	S-5-1	S-5-1	S-5-2	S-5-2	S-5-2	S-5-3	S-5-3	S-5-3	S-5-4	S-5-4	S-5-4
D	S-4-1	S-4-1	S-4-1	S-4-2	S-4-2	S-4-2	S-4-3	S-4-3	S-4-3	S-4-4	S-4-4	S-4-4
E	S-3-1	S-3-1	S-3-1	S-3-2	S-3-2	S-3-2	S-3-3	S-3-3	S-3-3	S-3-4	S-3-4	S-3-4
F	S-2-1	S-2-1	S-2-1	S-2-2	S-2-2	S-2-2	S-2-3	S-2-3	S-2-3	S-2-4	S-2-4	S-2-4
G	S-1-1	S-1-1	S-1-1	S-1-2	S-1-2	S-1-2	S-1-3	S-1-3	S-1-3	S-1-4	S-1-4	S-1-4
H	BL-1-1	BL-1-1	BL-1-1	BL-1-2	BL-1-2	BL-1-2	BL-1-3	BL-1-3	BL-1-3	BL-1-4	BL-1-4	BL-1-4

Fig. 1. Plate layout of intra-plate variables. For each design a combination of two variables were tested simultaneously within a plate. (a) Plate maps for methods B and C. The two letters in each well represent the relative concentrations of both reagents tested. For example, LL is low coating and low detection concentrations. The numbers represent the standard concentrations from low to high (1 being the low (LLOQ) and 5 being the high (ULOQ)). A total of 5 combinations of factors were tested on the plate and each standard point was assayed in triplicate per condition. (b) Plate map for method A. One intra-plate factor was tested, so the arrangement was different. The naming convention was S (standard)-1 (standard number)-1 (condition). Only four detection antibody concentrations were tested where 1 represents the lowest antibody concentration and 4, the highest antibody concentrations. A seven point standard curve was assayed in triplicate where 1 represents the lowest standard concentration and 7 the highest. In addition a blank (BL) was run.

receptor was optimized using multi-factorial DOE. The concentrations tested ranged from 1 to 5 μg/mL. Like method A plates were blocked, incubated, and washed. The minimum required dilution was 1:20. The detection antibody was a biotin-conjugated monoclonal anti-idiotypic therapeutic protein antibody. The concentration of this antibody was also optimized using multi-factorial DOE. The concentrations tested ranged from 500 to 2000 ng/mL. Streptavidin–sulfo-TAG was added to the plate at an equivalent concentration to the detection antibody. A 1:4 diluted tripropylamine read buffer (4× MSD read buffer) was added, incubated and read on the Sector Imager 6000. Electrochemiluminescent signals (ECL counts) were proportional to the amount of the therapeutic protein bound by the capture reagent. The recombinant receptor and the detection antibody concentrations were varied in five different combinations described in Fig. 1a. The capture, detection, and SA–sulfo-TAG incubation times were also tested using multi-factorial DOE (Table 2). In this case only the log S/B at the lower limit of quantification (LLOQ) was maximized.

2.3.3. Method C

Method C was also a standard sandwich capture ELISA. An anti-idiotypic mouse monoclonal antibody to the therapeutic protein was added at a concentration of 2.5 μg/mL. The samples had a minimum required dilution of 1:4. The biotin-conjugated anti-idiotypic mouse monoclonal antibody concentration was optimized using multi-factorial DOE. The concentrations tested varied from 50 to 300 ng/mL. The neutravidin–HRP concentration was also varied from 100 to 400 ng/mL. The detection antibody concentration and the neutravidin–HRP concentrations were tested at five different combinations within a plate. All five combinations had a five point standard curve with blank assayed in triplicate (Fig. 1a). Like the other methods, the signal to noise ratio was maximized.

2.4. Process overview

The most difficult aspect of implementing multi-factorial DOE for optimization of ligand binding assays is the upfront planning and development of tools necessary to execute the experiment. Proof of concept experiments were done by manual pipetting. This proved to be a laborious and time consuming process which yielded inconsistent results. As a result, several scripts were developed using a Tecan Evo automated pipetting instrument. The scripts used a 96-well pipetting head to deliver reagents to the plates. A 96-deep-well block is configured by adding the appropriate reagent to each well of the block according to the plate maps (Fig. 1a and b). Once the block is prepared, it is placed on the Tecan EVO deck with assay plates that need reagent addition. The reagents are added one plate at a time using the 96-channel head. Proper planning and organization of the DOE is also essential for a successful run. Necessary volumes of standards, capture and detection antibodies, and other reagents are scaled up, calculated, and verified to reduce any systematic error that could bias the data set. Any other variables such as temperature and equipment that can influence data reproducibility are controlled. Incubation time is tracked via a spreadsheet that lists the DOE conditions. The analyst documents the start time of an incubation and the spreadsheet calculates the time when the plate(s) are ready for the next step in the assay. A Microsoft Office Excel 2003 (Microsoft Corporation, Seattle, WA) macro was developed to calculate the DOE response of S/B ratio. These data were then compiled and added into the JMP (SAS, Cary, NC) DOE file for analysis.

2.5. Analytical validation: accuracy and precision

Analytical performance of the ELISA was defined in a comprehensive pre-study validation. The guidelines described by

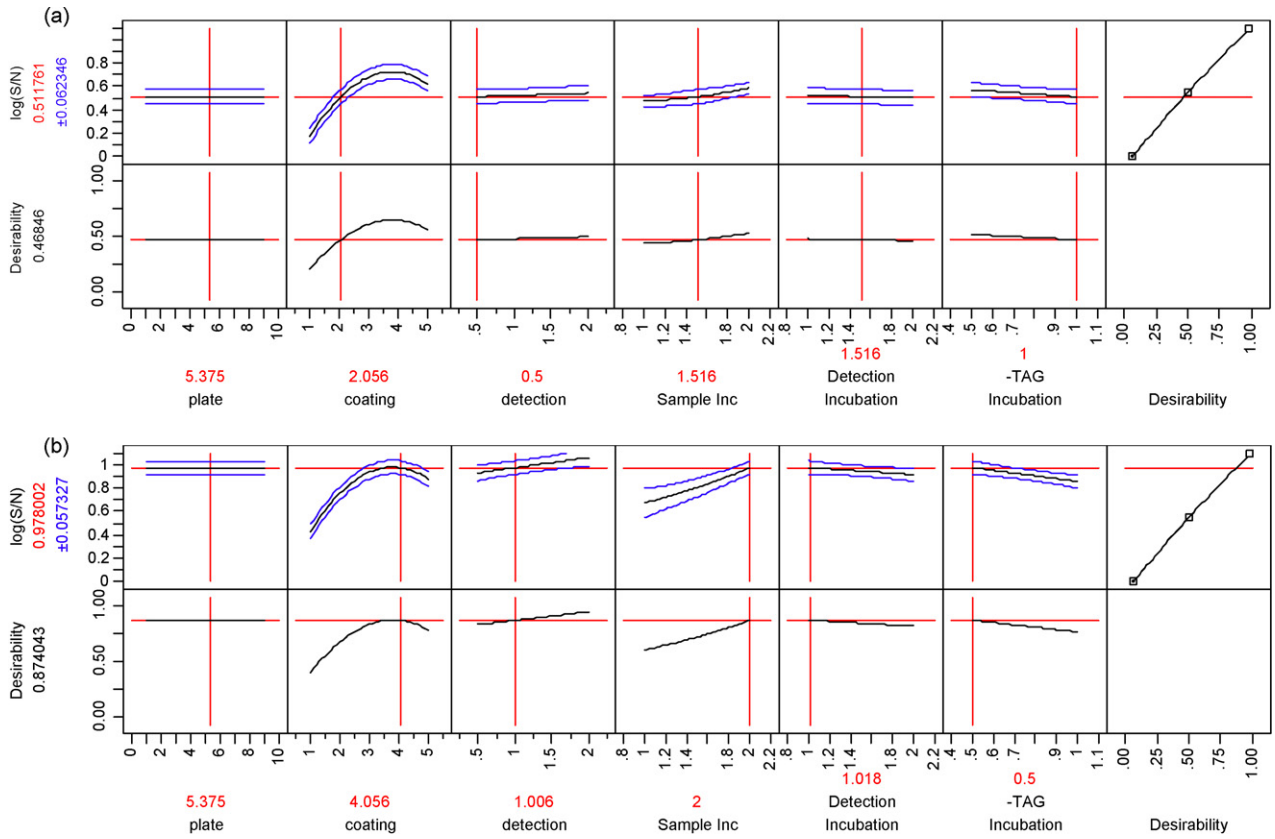

Fig. 2. Prediction tracings for log signal to blank. After multi-factorial DOE a prediction profile was generated, and the two figures represent the original conditions and the most desirable based on DOE. (a) A prediction trace applying the best single factor design conditions for method B. The $\log_{10}$ mean ratio and 95% confidence interval of S/B at LLOQ was 0.512 ± 0.062. (b) The maximum desirable prediction for the log mean ratio and 95% confidence interval S/B at LLOQ ratio was 0.978 ± 0.057.

DeSilva et al. [9] were applied for pre-study validation. Validation samples were prepared by adding the antigen to the serum at five concentrations. The validation samples were assayed a total of 30 times over the course of 3 days, and by 3 operators. The *a priori* criteria required that the bias and inter-assay precision described as total error must be <25% [9]. Method robustness was reflected by the pre-study validation performance data.

2.6. Model confirmation experiments

To test the model predictions, pre-study validation log S/B values were computed for all accuracy and precision runs and compared against the JMP predicted maximum desirable log S/B. During accuracy and precision runs, the mean O.D. at the LLOQ and the mean O.D. of the blank for each run was computed. The mean signal was divided by the mean blank value and then the S/B was log transformed. All six runs were combined and the mean log S/B was computed. That value was compared against the JMP predicted log S/B. The predicted log S/B and 95% confidence intervals were based on the actual data collected during the DOE optimization experiments.

2.7. Comparison of the hybrid screening design and CCD

In order to verify that the hybrid screening design was generating comparable results to the CCD, an experiment was conducted where the same ELISA method was optimized with both approaches. The within plate conditions were the same for both the hybrid and CCD, and the inter-plate parameters were randomly generated by JMP software for each approach. The conditions tested were coating concentration, detection concentration, capture incubation time, detection incubation time, and TMB time. The CCD had 16 inter-plate conditions and the hybrid screening design had 9 inter-plate conditions. The output variables were to maximize the S/B at the LLOQ and the ratio of the 1500 ng/mL anchor standard curve point to the 1000 ng/mL ULOQ point. The goal of including the latter output variable was to increase or maintain the dynamic

Table 3
Conditions: before and after DOE optimization.

Parameter	A		B		C	
	Before	After	Before	After	Before	After
Capture antibody concentration (ng/mL)	Fixed at 2000		2000	4000	Fixed at 2500	
Detection antibody concentration (ng/mL)	10	6	250	500	150	200
NA-HRP or sulfo-TAG (ng/mL)	NA		Fixed with detection		200	200
Capture incubation (h)	2	1.83	2	2	2	1
Detection incubation (h)	2	1.25	2	1	2	1
Neutravidin or Tag incubation (min)	NA		60	30	Fixed at 30	
TMB substrate incubation (min)	10	13	NA		30	20

range without losing the sensitivity. The prediction profiles were compared for both DOE approaches.

3. Results

3.1. Hybrid screening design

Using JMP 7.0 a hybrid screening design was developed. The inter-plate factors had two levels which are characteristic of a screening design and the intra-plate factors had three levels tested to determine curvature similar to a response surface or a central composite design. The hybrid approach was introduced to make the workload more manageable from 16 plates to 9 plates. A total of 9 plates (8 factors and 1 center point) were tested varying two intra-plate factors with high, mid, and low levels for each condition (Fig. 1) and three inter-plate factors high and low with one center point (Table 2). Following immunoassay analysis, the log S/B (signal at LLOQ to blank ratio) was computed for each of the conditions tested for a total of 45 results. A representative JMP prediction trace was shown in Figs. 2 and 3. A prediction trace is the predicted

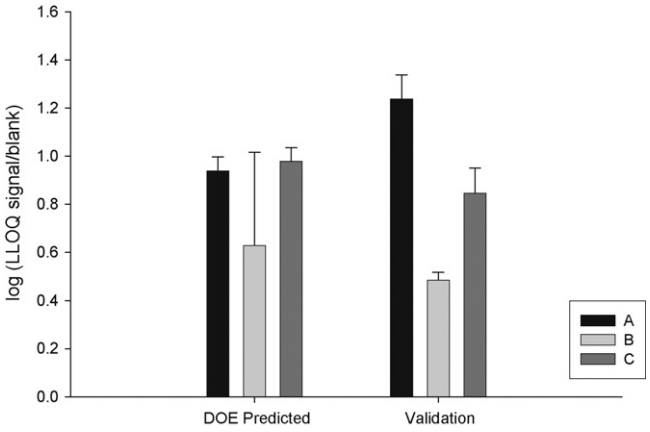

Fig. 3. Confirmation of the DOE predicted conditions. Three methods were optimized using multi-factorial DOE. To confirm the validity of the predictions, pre-study validation S/B at LLOQ data was compared to the DOE predictions for all 3 methods. The bars represent the mean $\log_{10}$ S/B and the error bars represent the 95% confidence of the mean.

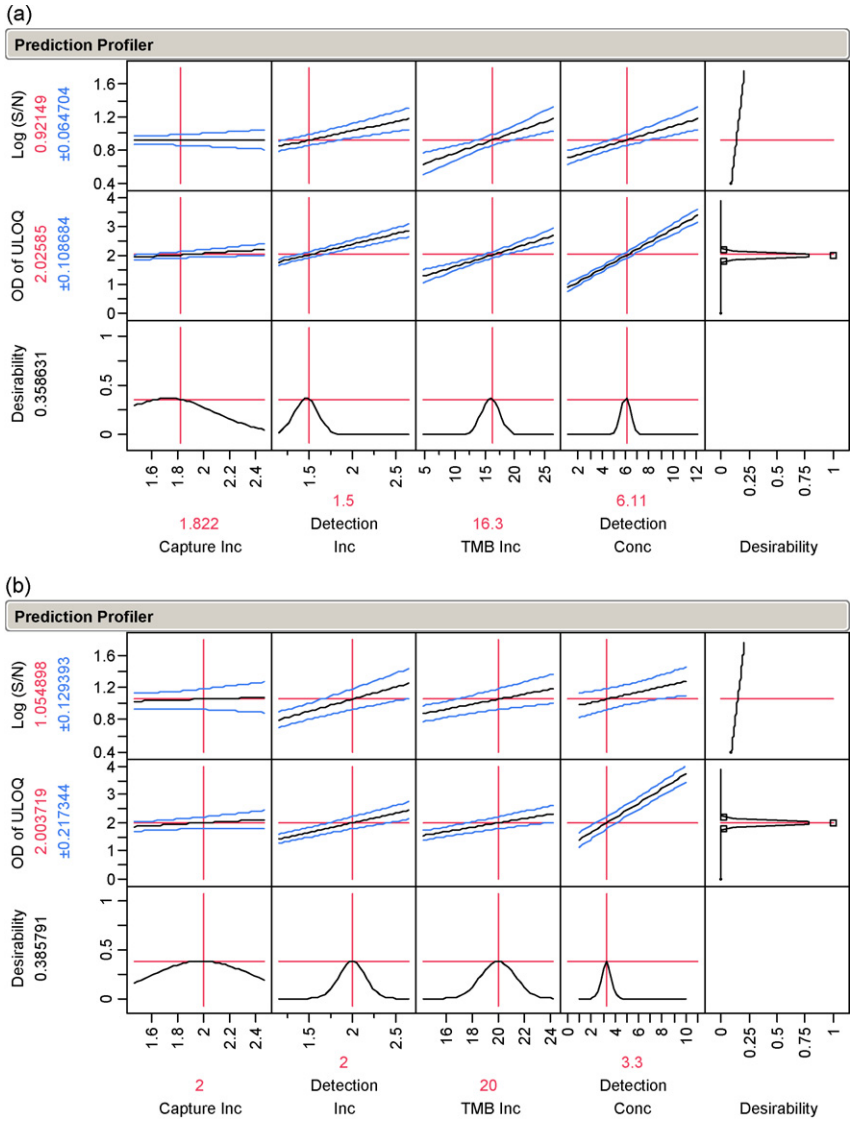

Fig. 4. Identification of interactions between assay conditions in Method A. (a) The maximum desirable conditions were detection concentration of roughly 6 ng/mL and the corresponding detection incubation and TMB time were optimal at 1.5 h and 16 min, respectively. (b) To illustrate the interactions between factors another prediction profiler tracing was included with a detection concentration set at 3 ng/mL and the corresponding detection incubation optimum became 2 h and the TMB optimal time shifted to 20 min.

response as one variable is changed while the others are held constant. As an example, method B data demonstrated a maximum desired log S/B prediction of 0.978 ± 0.057 as depicted in Fig. 4. In order to achieve the maximum desirable log S/B, the parameters were adjusted to the optimal value. The original procedure had a predicted log S/B of 0.512 ± 0.062 shown in Fig. 2. The maximum desirable conditions for each method were compiled in Table 3. In addition, the original conditions have been included for comparison. There were considerable changes in assay conditions on all methods for optimization indicating that the original methods were not adequately optimized for performance sensitivity and desirable robustness.

3.2. Confirmation of the DOE results

In order to test the validity of the optimized DOE predictions, S/B values were compiled from pre-study validation. During pre-study validation the DOE recommended optimized conditions were tested. The log S/B prediction at the LLOQ results demonstrated mean and 95% confidence limits of 0.846 ± 0.104 for pre-study validation for method B. The predictions were 11% different for the pre-study validation data. Method A had a mean and 95% confidence limits for log S/B prediction at the LLOQ of 0.939 ± 0.058 compared to the observed mean and 95% confidence limits for the log S/B at the LLOQ of 1.238 ± 0.100 for pre-study validation experiments. The observed values were within 25% of the predicted value. Another optimization parameter that was tested for method A was to match a target for optical density (O.D.) at the ULOQ between 1.8 and 2.2. The observed mean O.D. and 95% confidence values for pre-study validation was 2.442 ± 0.196. The observed mean O.D. values were within 20% of the mean target value. These data verified the predictive potential of the hybrid screening approach for optimizing immunoassays (Fig. 4).

3.3. Interactions between factors

The single factor experiments cannot determine if factors interact with each other. Using multi-factorial DOE, it is possible to determine interactions and the results show that interactions did occur between different factors in the 3 cases of immunoassays. Method A demonstrated interactions between the detection antibody concentration, detection incubation time, and TMB time. Fig. 5a and b illustrates the impact of changing the detection antibody concentration on the other factors. The prediction profiler tracing depicted optima of detection concentration at 6 ng/mL, 16 min for TMB and 1.5 h for detection incubation time. If the concentration of the detection antibody was decreased to 3 ng/mL the optimal detection incubation time would be increased to 2 h and the TMB time to 20 min. From the slope of the lines on the prediction tracing, it was clear that the highest impact factor on both log S/B and the OD of the ULOQ was the detection antibody concentration. A factor of minimal effect on both output parameters is capture incubation time as illustrated by the relatively flat line in Fig. 6a and b.

3.4. Accuracy and precision

Accuracy and precision of the methods were tested by measuring validation samples of known concentrations over multiple days by multiple operators. This design was to include tests on multiple sources of variation for robustness; assays that demonstrate total error (bias + imprecision) values less than 20% were considered robust. Each of the methods optimized using multi-factorial DOE demonstrated total error values of 20% or less at all levels of validation samples. Method A was an interesting case because a previous pre-study validation was conducted prior to optimization

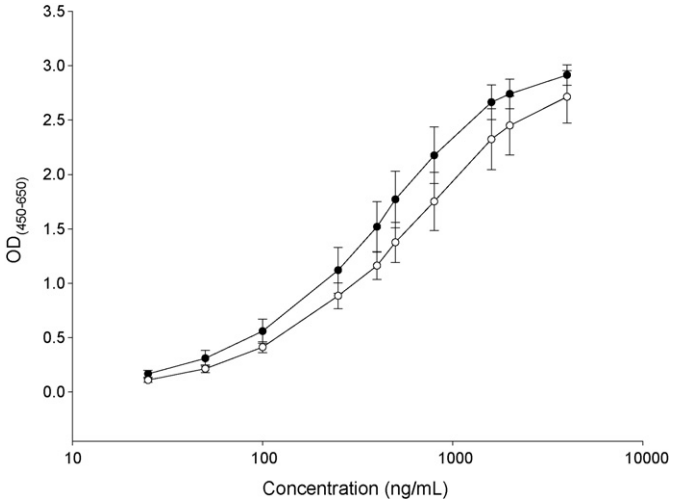

Fig. 5. Standard curves during pre-study validation for method A. The solid circles represent standard curve before DOE optimization. The open circles represent the standard curves after DOE modifications. The standard curves represent the mean and standard error of the mean for $n = 9$ runs.

using DOE. The non-DOE optimized method demonstrated total error values that exceeded the *a priori* total error acceptance criteria of 25%. Following DOE, the conditions were modified and the pre-study validation exercise was repeated. All of the total error values ranged from 8 to 20%. The original conditions did not accommodate the interactions between the detection antibody concentration and the detection and TMB incubation times. As a result, the assay demonstrated saturation at the upper region of the standard curve (Fig. 6). To avoid the saturation, reduction in O.D. at the ULOQ was achieved by selecting conditions resulting within the target range of 1.8–2.2 O.D. units. Fig. 6 also illustrates the difference in the slope between the 1500 and the 1000 standard curve point in the pre-DOE and the post-DOE design.

3.5. Direct comparison between hybrid screening design and CCD

A direct comparison between the traditional response surface model (CCD) and the hybrid screening design was conducted to test the agreement between the predictions of both approaches. The parameter estimates determined by the JMP software prediction profiler were very similar (Fig. 6a and b). The log S/B and the ratio at the 1500–1000 estimates, shape of curve, and the direction were similar; however, the standard error values were greater for the hybrid screening design. This could be related to a technical error, because a plate was dropped during processing and was excluded from the hybrid screening model. It is also possible that the increased number of runs and conditions tested provided better estimates. Overall, the results confirmed that the estimates generated by the hybrid screening design were comparable to the traditional CCD.

4. Discussion

Multi-factorial DOE is not a new concept in the arena of process development and manufacturing. It has been used extensively [10–12]; however, it has only been used sparingly as an approach for optimizing immunoassays. Recently, our laboratory has adopted the technique for defining optimal parameters for immunoassays for therapeutic proteins. Besides being a consistent and rational approach, multi-factorial DOE provides two additional benefits: identification of interacting parameters that are not identifiable by single factor experiments and improvement of assay robustness.

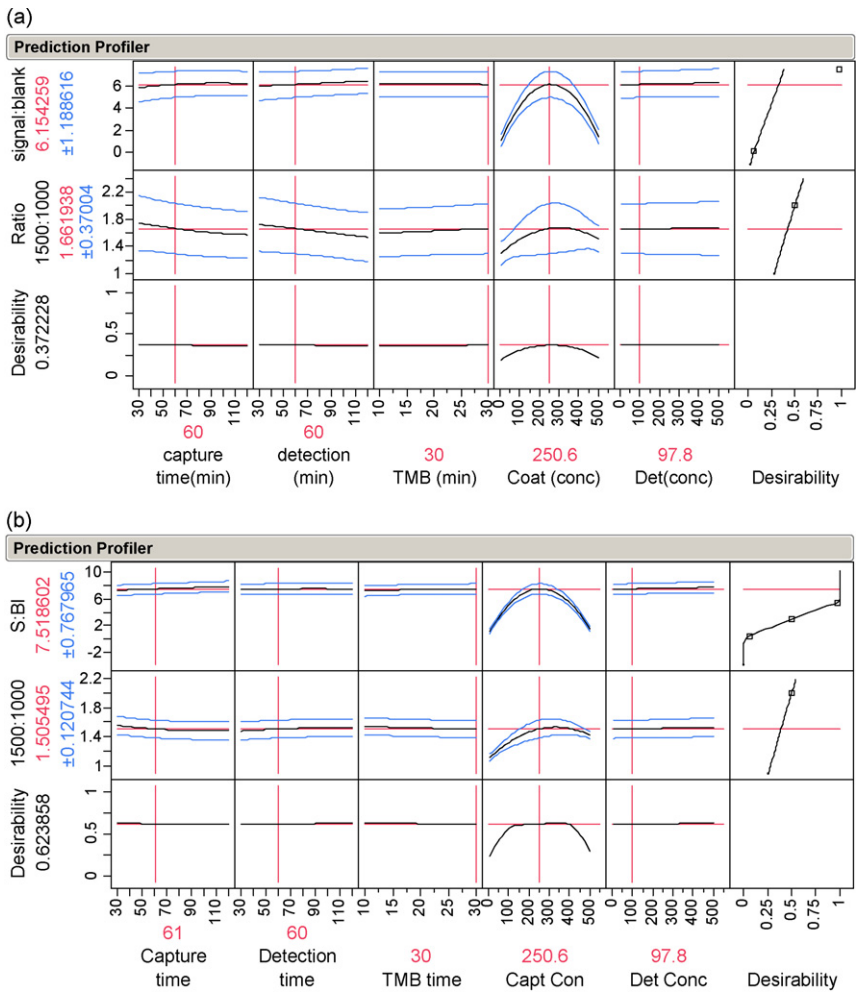

Fig. 6. Comparison of hybrid screening design and the CCD. After multi-factorial DOE a prediction profile was generated, and the two figures represent defined conditions for the hybrid screening design and traditional CCD. (a) A prediction trace of a 9 plate hybrid screening design. The mean ratio and 95% confidence interval of S/B at LLOQ was 6.15 ± 1.1 and the 1500:1000 ratio was 1.66 ± 0.37. (b) The 16 plate CCD maximum desirable prediction for the mean ratio and 95% confidence interval S/B at LLOQ ratio was 7.51 ± 0.76 and the 1500:1000 ratio and 95% confidence interval was 1.50 ± 0.12. The relative slopes and curve shapes were similar for all of the parameters tested.

These observed benefits are consistent with previously published immunoassay methods that utilized multi-factorial DOE [5,6]. We consider that the lack of publications was not because of a lack of success; instead it was an inability to overcome the resistance and skepticism by the end users. During the implementation process, it was clear that analysts were resistant for a few reasons. The most common complaint was the number of assays and the amount of time required to obtain results. Other reasons could be the lack of statistical and automation tools, expert help in statistical design, and difficulty in data interpretation.

In order to overcome the resistance, we implemented approaches that reduced the workload and applied a more user-friendly data analysis approach for simplicity and manageability with limited resources. The workload reduction was a two part process, the first part required that the manual pipetting be reduced and the second part was to reduce the number of plates. In order to reduce manual pipetting, a Tecan EVO script was written using the 96 tip head to pipette reagents rapidly. In order to reduce the number of plates, we instituted the 9 plate hybrid screening design.

The 9 plate screening design was developed with lessons learned from a previous DOE optimization of a sandwich ELISA using the Plackett–Burman screening design followed by a CCD measuring the precision profile as an output. In that example [13], the assay demonstrated characteristics of a robust assay as measured by accuracy and precision. However, the selectivity test indicated that some of the samples were demonstrating serum matrix effects. Based on this experience, we incorporated matrix interference resolution prior to optimization. Method C was an example of an assay that had serum matrix effects. The matrix interference was eliminated by increasing the minimum required dilution and supplementing the assay buffer with salt, Tween 20, and BSA [14]. Once the modifications were made, the buffer and serum standard curves behaved similarly. If the matrix interference was not resolved, the optimization results would have been confounded by the different dilutions. Other lessons learned from the first exercise using multi-factorial DOE were the need to reduce the design to manageable number of plates and that assessing the precision profile was difficult to implement. Precision profiles required considerable statistical support in SAS programming, calculation and data interpretation. In addition, if the standard curve was unable to converge then the estimate of working range was unattainable, so from our perspective the S/B was simpler and only a slightly inferior output measurement.

We developed a basic screening design with 3 inter-plate variables at 2 levels and 2 intra-plate variables at 3 levels to test for curvature. The screening design yielded a total of 8 plates with one center point for a total of 9 plates, as demonstrated in methods B and C. The reduction in workload enticed more analysts to adopt DOE. The hybrid screening approach has proven reliable for both predicting the log S/B (Fig. 4) and for selecting optimal conditions for robust assay performance. The proven prediction of the

log S/B and the comparability of the hybrid screening to that of the classical DOE indicates the mathematical model is producing valid results. From a practical perspective, the total error in all of the methods has been less than 20%, so the approach is working for our intended purpose. More importantly, achieving this level of robust performance is critical for successful method validation and sample analysis under tight timelines.

An interesting illustration of multi-factorial experimental design was the unusual case of method A. This method was developed, optimized with a conventional non-DOE approach, and tested in a pre-study validation exercise. The results were outside of the *a priori* acceptance criteria. In order to identify the source of failure, multi-factorial DOE was applied. The results from the pre-study validation exercise indicated that the assay might approach saturation at concentrations around the ULOQ region (Fig. 6). In order to properly optimize the assay, a combination of log S/B and maximize the ratio of high anchor to the ULOQ (1500:1000) was incorporated. The prediction profiler trace 5a and b illustrated the major interactions existed between the detection antibody concentration, the detection antibody incubation time, and TMB time. Specifically the detection antibody concentration was inversely related to detection antibody incubation and TMB incubation time (Fig. 5a and b). After DOE optimization, the assay did not show saturation at the high end of the standard curve (Fig. 6). The post-DOE optimized method demonstrated acceptable accuracy and precision in pre-study validation experiments. This example illustrated two important points: (1) interactions between factors are easily observed using multi-factorial DOE; (2) applying the approach will improve the likelihood of meeting the assays intended purpose during pre-study validation experiments [15]. The multi-factorial interactions and non-interactions would not be determined by single factor experiments. This method also illustrates the time savings that multi-factorial DOE can provide. In this situation, more than 2 weeks of single factor experiments yielded an assay that failed to meet *a priori* acceptance criteria; however, 2 days of analysis generated parameter estimates that successfully met validation criteria of total error values less than 20%.

Our primary objective over the last 18 months was to define the utility of multi-factorial DOE in a ligand binding assay development laboratory. We have successfully demonstrated the value of this technique and we plan to expand the capabilities of DOE in this setting by improving the tools and extending the types of assays that use DOE for optimization. A fully automated DOE approach is currently not possible using Tecan EVO; however, using worklist functions of the Tecan Evo instrument combined with scheduling software, we believe that it will be possible to fully automate DOE. Fully automated assays will further increase the quality and utility of this approach. To expand the types of assays, we plan to test different immunoassay formats. One immunoassay format used extensively in immunogenicity and fairly common in pharmacokinetic analysis is the bridging immunoassay [16]. This format uses an identical capture and detection reagent for a bivalent antigen that forms a "bridge" between the two reagents. The assay development approach and configuration is different from non-bridging sandwich methods, posing unique challenges to optimization [17]. We believe that multi-factorial DOE will be an excellent tool for optimizing bridging assays in both a therapeutic drug measurement and in the immunogenicity environment, because of its ability to probe interactions between the capture and detection reagents. We plan to investigate the utility of multi-factorial DOE in the setting of immunoassays supporting immunogenicity. Another application for multi-factorial DOE is in the multiplex immunoassay optimization. The objective for multiplex immunoassays is to find a set of conditions that provides the best performance for all assays combined [18]. In that setting, a set of conditions must be selected where all of the assays are performed simultaneously, so interactions between the reagents could be occurring.

5. Conclusion

Multi-factorial DOE has proven to be a reliable and accepted approach for optimizing immunoassays for therapeutic proteins. In order to maximize the efficiency of the technique certain logistical and scientific considerations were made such as simplified calculations, reduction in serum matrix effects, incorporation of automation, and introduction of the hybrid screening design. The major benefits have been identification of interactions between factors, reduction in time to develop optimal parameter estimates, and improved robustness

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