Adventures in Solid Phase Optimization – How I Learned to Stop Worrying and Love DOE

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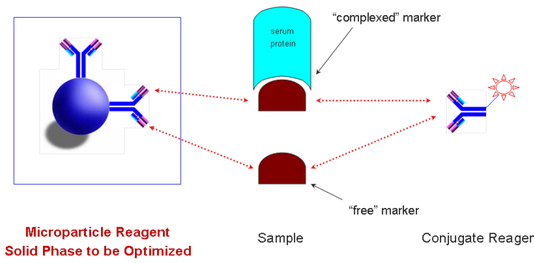
Introduction

Experimental design as used for immunoassay development in the *in vitro* diagnostics industry requires a thorough understanding of all aspects of a given product's composition and manufacturing. For immunoassays, a multiplicity of interactions between reagent components, sample composition, and process parameters make characterization of the process so complex and time consuming that the advantages of experimental design, or DOE, become immediately apparent. It is the strength of DOE to use statistics to define and/or optimize processes with fewer resources than standard experimental approaches.

Here we present a specific case that illustrates the use of DOE in a manufacturing problem that includes optimization of that process. The coating of a microparticle solid phase with antibodies for a specific analyte failed to yield the performance necessary as assessed by three different metrics (responses). Simultaneous optimization with only two DOE experiments corrected the problems, characterized and optimized the manufacturing process, and allowed the product to go to market.

The Problem - Objective



Preliminary protocols for making the reagents necessary for the immunoassay have been completed, and the initial performance evaluated. Three performance parameters were of particular importance:

1. Equimolarity = the ability to see complexed and free marker equivalently, operationally defined as a signal ratio of 1.0 ± 0.1 for complex/free
2. Microparticle stability, defined as 100 ± 10% of 2-8ºC signal after 3 days storage at 45ºC
3. Panel values = within 5% of target values across the dynamic range of the assay

The performance in the initial evaluation:

1. Equimolarity marginal at about 1.09
2. Microparticle stability < 90%
3. Panel values within target range (1-3% below actual target)

Thus, the experimental objective is to determine a manufacturing formula to meet all three of the above goals.

Additional information: the loss in stability was related to free antibody coming off of the solid phase during storage as a function of time, suggesting a lack of covalent coupling of the antibody to the microparticles. Dropping the antibody concentration for coating improved the stability, but then the panel values read lower than acceptable, and equimolarity, already marginal, departed further from the target.

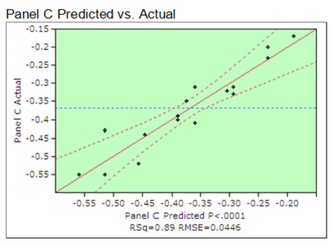
THE FACTORS

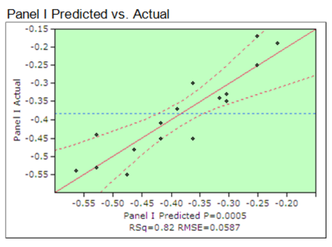
Four input factors to the microparticle manufacturing process were evaluated. The first three are the obvious "active ingredients" for the coupling process: the concentration of the coating antibody (in mg/mL), the concentration of the microparticles (in % solids), and the concentration of the coupling reagent, affectionately known as EDAC (a carbodiimide, for those chemically curious, in mg/mL). The fourth factor is the concentration of sodium chloride in mM. Ionic strength is known to impact the interaction of proteins with surfaces, and the concentration of NaCl is one easy way of manipulating this characteristic of the coupling reaction.

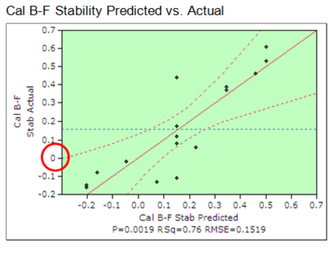
THE DESIGN

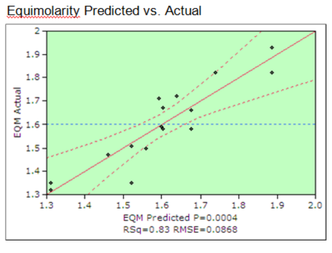
A screening design was chosen first to minimize the number of runs and determine the most important factors: 4 factors, 2 levels with midpoint: 11 unique preps including 5 duplicate preps for a total of 16.

Results With Checkpoints



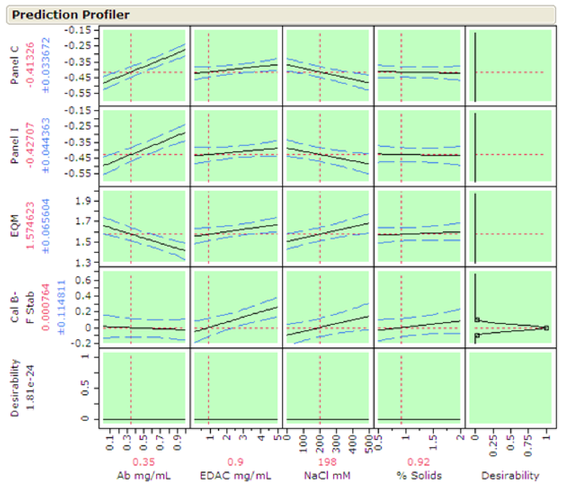






These plots show a reasonable ability to predict these responses of interest, with most R Square values above 0.80. Note that the only target actually met in the data collected for this design is for stability (zero difference from control; red circle). Thus, it was decided to attempt optimization with the Prediction Profiler using these results first for stability. Of particular relevance here is the flat line for the Desirability function for the panels and the equimolarity responses. This is because the target is not on the y-axes for these responses, indicating that the input factors cannot be adjusted to any setting within these ranges that will yield the desired response.

Prediction Profiler optimized for stability



Making a microparticle preparation using the optimized settings predicted by the Prediction Profiler, it was found that, consistent with predictions (i.e., the model was indeed good enough to act as a response surface even though the design was a simpler screening design), the stability was 100%. Unfortunately, panel values were also as predicted: ~ 40% below target (-0.4) and equimolarity was 1.6 (target is 0.9 – 1.1).

These observations were further enhanced with a second checkpoint using the model to predict responses based on the current process values for the input. The model created by the analysis of the data of this first DOE predicts the following responses:

- Stability = 85% (NOT acceptable)
- Panel values = 1-3% below target (acceptable; within range)
- Equimolarity = 1.09 (*barely* acceptable)

This was the current performance of the assay.

CONCLUSION, PART 1

The conclusion of this first DOE analysis provides an important lesson on the utility of DOE. The four inputs studied here do not provide a means to simultaneously optimize stability, equimolarity, and panel values. To do so, you must *either* a.) accept a tradeoff in response outputs, i.e., change the design goals (which is not a good idea if those design goals have been properly formulated from customer requirements), *or* b.) entertain a new perturbation/parameter in the assay system, i.e., look at something new, a different parameter not yet evaluated, or something radically new to the entire system.

A CRITICAL ADVANTAGE OF USING DOE

"Lack of Success" is not the same as "Failure." *One of the greatest benefits of DOE is the ability to terminate unfruitful lines of investigation using the objective evidence of the validated models generated to scientifically justify this decision.* This occurs when the model predictions have been verified (validating the model) and when those predictions show the impossibility of meeting all necessary goals simultaneously.

> "Eliminate all other factors, and the one which remains must be the truth."
> ·Sherlock Holmes, *The Sign of the Four*

WHAT TO DO?

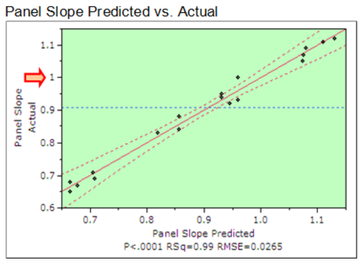
At this point, a preliminary experiment done months earlier surfaced again, suggesting a new direction to evaluate in earnest. Initially, the data were set aside as "interesting results" having no practical utility. It had been observed that if you added the monoclonal Ab (MAb) against the free marker to the solid phase (microparticle) diluent to create a pseudo-complexed marker from the free marker, all forms of the marker appeared to look alike in the "total" assay. This addresses the panel values and the equimolarity issue. The new hypothesis in the form of a question, therefore, was, can we optimize the solid phase coating for stability and then adjust the panel values and equimolarity results with this MAb in the diluent?

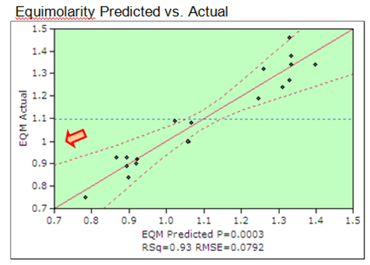
THE FACTORS & DESIGN, PART 2

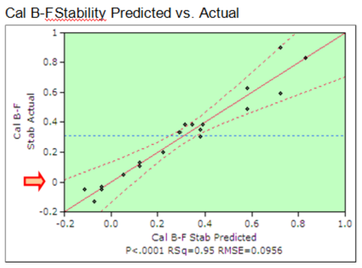
Based on the first DOE the % solids and NaCl concentrations were fixed and dropped from this study, leaving only three factors. With fewer factors to evaluate, and a stronger need to create a model that would be predictive, a *D*-optimal Response Surface Method (RSM) with 3 factors, 3 levels, and 5 duplicate reps was designed and executed.

THE RESULTS, PART 2

In this instance, in order to capture more than just two panels, the metric used to determine the panel performance was the slope of the plot of observed values versus the target values, making the goal a slope of one. Equimolarity and stability were measured as before. Considerably better R square values for this data were observed and target values (arrows) are on the y axis:







Another JMP output that is informative is the sorted parameter estimates. When sorted, the factor having the greatest influence on the response is on the top. The results for the Panel Slope are in agreement with our hypothesis that the most important factor is the concentration of the MAb in the microparticle diluent. Likewise, the same input factor is the primary driver of the equimolarity response. Stability shows a different story, but one expected based on the theory of our process. The concentration of the coupling reagent plays the most important role in the generation of that response.

Seeing such results that agree with the known the chemistry of the process enhances the confidence that the models created are describing reality.

Panel Slope

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
[MAb] ug/mL in diluent	0.1764243	0.008012	22.02		<.0001
(EDAC mg/mL-6)*([MAb] ug/mL in diluent-1)	0.0118634	0.002058	5.76		0.0003
Ab mg/mL	0.0876801	0.016448	5.33		0.0005
(Ab mg/mL-0.5)*(EDAC mg/mL-6)	-0.021259	0.004288	-4.96		0.0008
(Ab mg/mL-0.5)*([MAb] ug/mL in diluent-1)	-0.057951	0.019001	-3.05		0.0138
([MAb] ug/mL in diluent-1)*([MAb] ug/mL in diluent-1)	-0.050856	0.017105	-2.97		0.0156
EDAC mg/mL	-0.004822	0.001845	-2.61		0.0281
(EDAC mg/mL-6)*(EDAC mg/mL-6)	-0.000842	0.000616	-1.37		0.2048
(Ab mg/mL-0.5)*(Ab mg/mL-0.5)	0.0592606	0.067526	0.88		0.4030

Equimolarity

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
[MAb] ug/mL in diluent	-0.250386	0.023948	-10.46		<.0001
([MAb] ug/mL in diluent-1)*([MAb] ug/mL in diluent-1)	0.1138585	0.051129	2.23		0.0530
Ab mg/mL	0.1009066	0.049166	2.05		0.0703
(EDAC mg/mL-6)*([MAb] ug/mL in diluent-1)	-0.011816	0.006152	-1.92		0.0870
(EDAC mg/mL-6)*(EDAC mg/mL-6)	0.0022738	0.00184	1.24		0.2479
(Ab mg/mL-0.5)*(Ab mg/mL-0.5)	-0.071951	0.201843	-0.36		0.7297
EDAC mg/mL	0.0019068	0.005514	0.35		0.7374
(Ab mg/mL-0.5)*(EDAC mg/mL-6)	0.0026686	0.012818	0.21		0.8397
(Ab mg/mL-0.5)*([MAb] ug/mL in diluent-1)	0.0070362	0.056798	0.12		0.9041

Cal B-F Stability

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
EDAC mg/mL	0.0634739	0.006657	9.54		<.0001
Ab mg/mL	-0.236815	0.05936	-3.99		0.0032
(Ab mg/mL-0.5)*(EDAC mg/mL-6)	-0.03898	0.015475	-2.52		0.0328
(Ab mg/mL-0.5)*(Ab mg/mL-0.5)	0.5580778	0.243692	2.29		0.0478
[MAb] ug/mL in diluent	0.0488119	0.028913	1.69		0.1256
([MAb] ug/mL in diluent-1)*([MAb] ug/mL in diluent-1)	0.0978185	0.06173	1.58		0.1475
(EDAC mg/mL-6)*([MAb] ug/mL in diluent-1)	0.0112314	0.007428	1.51		0.1648
(Ab mg/mL-0.5)*([MAb] ug/mL in diluent-1)	0.1006162	0.068574	1.47		0.1764
(EDAC mg/mL-6)*(EDAC mg/mL-6)	0.0004016	0.002222	0.18		0.8606

Turning then to the simultaneous optimization of all three responses:

Optimum conditions *predicted* from the model analysis:

[Ab] = 0.75 mg/mL
[EDAC] = 2.5 mg/mL
[MAb in diluent] = 2.0 µg/mL

Observed responses in confirmation runs at these levels (checkpoints):

Stability = 94-96% (acceptable)
Panels = within 3% of target (acceptable)
Equimolarity = 0.94 – 0.97 (acceptable)

Conclusion: dancing in the halls might commence!

UNEXPECTED CHECKPOINT ACCURACY

Remember that I mentioned preliminary experiments that suggested the viability of this strategy as the basis for trying the approach. The scary part is how accurately the RSM model generated months later predicts the results of that preliminary experiment. In this case, panel values were monitored by the slope of the regression line between observed panel values versus known panel values (target therefore is one, with a permissible range of 0.95-1.05).

Output	*[MAb in diluent] mg/mL*	PREDICTIONS FROM THIS MODEL ***Predicted Target***	*Predicted 95% CI*	***OBSERVED Previously***
Panel Slope	0.10	**0.71**	0.64 - 0.79	**0.71**
	0.50	**0.80**	0.72 - 0.87	**0.78**
	1.00	**0.88**	0.81 - 0.96	**0.93**
Equimolarity	0.10	**1.25**	1.04 - 1.45	**1.23**
	0.50	**1.10**	0.89 - 1.31	**1.08**
	1.00	**0.97**	0.76 - 1.18	**0.99**

Acknowledgement

I would like to acknowledge John Wass, statistician extraordinaire, former co-worker, and good friend, for motivation, helpful hints, and dutiful review of the content of this poster.