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Taguchi Design-Based Optimization of Sandwich Immunoassay Microarrays for Detecting Breast Cancer Biomarkers

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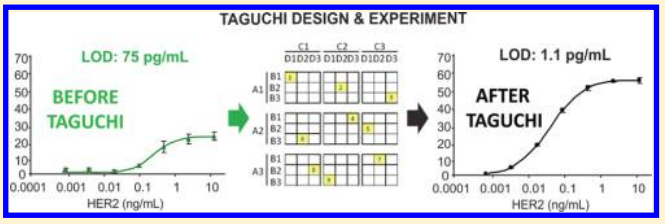
Supporting Information

ABSTRACT: Taguchi design, a statistics-based design of experiment method, is widely used for optimization of products and complex production processes in many different industries. However, its use for antibody microarray optimization has remained underappreciated. Here, we provide a brief explanation of Taguchi design and present its use for the optimization of antibody sandwich immunoassay microarray with five breast cancer biomarkers: CA15-3, CEA, HER2, MMP9, and uPA. Two successive optimization rounds with each 16 experimental trials were performed. We tested three factors (capture antibody, detection antibody, and analyte) at four different levels (concentrations) in the first round and seven factors (including buffer solution, streptavidin-Cy5 dye conjugate concentration, and incubation times for five assay steps) with two levels each in the second round; five two-factor interactions between selected pairs of factors were also tested. The optimal levels for each factor as measured by net assay signal increase were determined graphically, and the significance of each factor was analyzed statistically. The concentration of capture antibody, streptavidin-Cy5, and buffer composition were identified as the most significant factors for all assays; analyte incubation time and detection antibody concentration were significant only for MMP9 and CA15-3, respectively. Interactions between pairs of factors were identified, but were less influential compared with single factor effects. After Taguchi optimization, the assay sensitivity was improved between 7 and 68 times, depending on the analyte, reaching 640 fg/mL for uPA, and the maximal signal intensity increased between 1.8 and 3 times. These results suggest that Taguchi design is an efficient and useful approach for the rapid optimization of antibody microarrays.



Sandwich immunoassay-based antibody (Ab) microarrays are emerging as a high-throughput tool for the simultaneous quantification of multiple analytes. Capture Abs (cAbs) immobilized on a microspot bind target analytes. After washing, the chip is incubated with the detection Abs (dAbs) that will bind to the immobilized analytes, which will be sandwiched between a cAb and a dAb. The binding of analyte is quantified by incubating the chip with a secondary Ab conjugated to a signal-amplifying molecule that transduces binding into a readable signal. In conventional immunoassays such as in the enzyme-linked immunosorbent assay (ELISA), an enzyme is conjugated to the dAb and converts an invisible substrate into a light-adsorbing one that can be used to quantify binding. In microarrays, fluorescent labels are preferred because they remain localized while allowing highly sensitive imaging. An antibody microarray can achieve a sensitivity comparable to that achieved by the classical sandwich ELISA, and is thus especially attractive for detecting multiple low-abundance targets in complex biological samples, such as cancer biomarkers in blood.[1,2]

Although sandwich assays are routine, they are, in fact, complex, and a plethora of factors influence microarray assay performance,[3,4] including ones that are linked to microarray manufacture, such as the (a) surface chemistry of the chip,[5−7] (b) humidity during spotting,[8,9] (c) composition of the spotting buffer,[8,10] and (d) stability of immobilized cAbs.[11] The performance of the assay depends on many more critical factors, notably (e) concentration of cAbs, (f) incubation time and drying of spotting solution, (g) composition of blocking buffer, (h) time of blocking, (i) buffer used for sample dilution, (j) sample incubation time and (k) temperature, (l) level of agitation and mixing during incubation, (m) composition of rinsing buffer, (n) rinsing time, (o) agitation during rinsing, (p) composition of the detection buffer, (q) concentration of dAbs, (r) incubation time of the dAbs, (s) affinity and stability of dAbs, (t) concentration of detection binder (e.g., secondary Ab or fluorescently labeled streptavidin), (u) buffer composition, (v) incubation time, and (w) nature of detection label (fluorescent or enzyme), and so on. A major challenge to a multiplex sandwich assay development on a microarray is the optimization of the various assays and identifying the critical factors and the optimal level for

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each one of them. An additional challenge is the difficulty in assessing when the optimal levels have, in fact, been found. If one considers that factors may not be independent; that, thus, the optimal level for any one factor may depend on the levels of other factors that interact with one another; and that in an array, there may be different optimal levels for each analyte, finding the overall optimum constitutes a major challenge.

Let us consider the example of a sandwich immunoassay with four factors to be optimized (i.e., cAb, analyte, dAb, and streptavidin-Cy5 dye conjugate (SA-Cy5)), each with three levels (concentrations). To find the optimal condition, one approach would be to test all possible combinations of each factor tested at every level, corresponding to a total of $3^4 = 81$. This could be achieved by carrying out 81 experimental trials (see Figure S1 in the Supporting Information (SI)). This approach, called full factorial design, is already laborious when considering as few as four parameters and three levels, and becomes unfeasible as the number of factors or levels increases. For example adding one extra factor and one extra level would require $4^5 = 1024$ experimental trials. Optimization of multifactorial processes, including ELISA, is often performed by varying a single factor while keeping the others constant and measuring the process output. This is called the one-factor-at-a-time (OFAT) method,[4] and all factors are optimized iteratively, which is slow, costly, and inefficient compared with rationally designed optimization methods described below. In our example, four successive runs with each three experimental trials with varying concentration for the factor being tested would be necessary. Moreover, the OFAT method is susceptible to the initial conditions because the optimal level for one factor may vary depending on the level of other factors when factors interact with one another (e.g., incubation time was found to depend on buffer composition in this study as is described below (see Figure S1 in the SI)).[12] The optimal conditions identified using OFAT may thus be wrong, and OFAT offers no means of determining when and whether the optimum has been reached.

Efficient methods for optimizing multiple factors simultaneously have been developed and are called design of experiments (DoE). With DoE approaches, only a fraction of the full factorial combinations (so-called fractional factorial design) of a process are tested following a particular strategy that maximizes the likelihood of finding the true optimum, which often involves statistical analysis.[13,14] The various DoE methods differ on the rationale and strategy used to select the subset of possible combinations that will be tested experimentally. Taguchi design is a DoE that has become the most widely used quality engineering technique today in industry for optimization of product and production process.[15] When applying the Taguchi method to a process (e.g., ELISA), the experimenter needs to choose an appropriate orthogonal array. An orthogonal array is a table that for a given combination of (i) number of factors (e.g., Ab concentration, incubation time, buffer, etc.) and (ii) number of levels (e.g., preselected values used in the experiment for concentration, incubation time, and buffer composition, respectively) defines the series of experiments and the levels for each parameter in each experiment according to the Taguchi design rules. Referring to our example of four factors with three levels ($3^4 = 81$ combinations) discussed above, it could be optimized using only 9 experimental trials with Taguchi design based on an orthogonal array named $L_9(3^4)$ that defines the levels of each factor for each trial (see explanations in the Supporting Information and Figure S2).

The result of a Taguchi experiment yields the optimal values for each factor, and its "weight" which is expressed as the percentage in overall signal variation to which this factor contributed by altering its level within the predefined range. These numerical results can subsequently be exploited to focus the process optimization and quality control on the most critical factors. The Taguchi method thus represents a highly efficient way to optimize and improve processes that are depending on large numbers of factors.

Despite the remarkable achievements of Taguchi design in the past several decades, application of this method in biological research is still sparse,[16] and its application to optimizing immunoassays remains underappreciated. The method has been shown to be useful for optimizing ELISA in one study[17] and, more recently, for protein microarrays in which it was used to optimize the fabrication process using electrodeposition so as to maximize the binding of proteins.[18] However, to the best of our knowledge, the Taguchi method has not been exploited for optimizing multiplex immunoassays or antibody microarrays, yet they are particularly difficult to optimize because many different parameters need to be taken into account simultaneously, and thus might, in fact, benefit the most from rational design of experiments methodologies. Here, we report on the use of the Taguchi design for the optimization of five sandwich immunoassays carried out using an Ab microarray against five breast cancer biomarkers, including cancer antigen 15-3 (CA15-3), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (HER2), matrix metallopeptidase 9 (MMP9), and urokinase plasminogen activator (uPA). These proteins are either currently used as breast cancer biomarkers in clinical practice[19] or have recently been identified as candidate biomarkers.[20−23] Our optimization strategy involved two rounds of Taguchi experiments evaluating two different sets of factors. Although the assay protocols and the levels for the various factors (e.g., concentration, incubation time, etc.) were selected on the basis of data from the literature and our own set of experiments and were considered to be optimized, rigorous Taguchi design-based optimization led to remarkable improvements of the limit of detection (LOD), the maximal signal intensity, and the dynamic range of the multiplexed assays.

MATERIALS AND METHODS

Antibodies and Analytes. CA15-3 and CEA cAbs, dAbs, and analytes (i.e., antigens) were obtained from United States Biological (Swampscott, MA); HER2, MMP9, and uPA cAbs, dAbs, and analytes were from R&D Systems (Minneapolis, MN). dAbs were obtained in the biotin-conjugated form, except for CA15-3 and CEA, which were biotinylated in our laboratory using the ProtoArray Mini-Biotinylation kit (Invitrogen Carlsbad, CA). Bradford methodology (Pierce, Rockford, IL) was used for assessment of the concentrations of the Abs used.

Antibody Microarray Fabrication. The cAbs were individually prepared to the concentrations defined in Table 1 in 50 mM carbonate−bicarbonate buffer, pH 9.6 (Sigma-Aldrich) containing 5% (w/v) of trehalose. Sixteen replicate arrays of cAbs were arrayed on epoxy slides[5,6,24] (Nexterion Slide E, Schott, Elmsford, NY) using an inkjet microarrayer (Nanoplotter NP2, GeSiM, Germany) with a 500 μm pitch.

Antibody Sandwich Microarrays Assays. Incubation times for various steps were 2 h unless indicated otherwise. Printed slides were incubated overnight at 4 °C in a sealed box, then blocked at room temperature with 1% bovine serum albumin

Table 1. List of the Five Analyte-Ab Reagents and the Three Factors (analyte, dAb, cAb) with Four Levels That Were Investigated Using Taguchi Orthogonal Array $L_{16}(4^5)$ Shown in Supporting Information Table S1

		factors		
assay	levels	analyte (ng/mL)	dAb (μg/mL)	cAb (μg/mL)
CA15-3[a]	1	0.04	1	100
	2	0.4	0.2	200
	3	4	0.04	400
	4	40	0.008	800
CEA	1	0.05	0.2	50
	2	0.5	1	100
	3	5	2	200
	4	50	4	400
HER2	1	0.05	0.0625	100
	2	0.5	0.25	200
	3	5	1	400
	4	50	2	600
MMP9	1	0.01	0.0625	100
	2	0.1	0.25	200
	3	1	1	400
	4	10	2	600
uPA	1	0.01	0.0625	100
	2	0.1	0.25	200
	3	1	1	400
	4	10	2	600

[a] CA15-3 analyte concentration is U/mL.

(BSA, Jackson ImmunoResearch, West Grove, PA) prepared in either 50 mM phosphate buffered saline (PBS) or 50 mM Tris−HCl buffered saline (TBS). Analytes were diluted in PBS-0.05% Tween 20 (PBST) containing 0.1% BSA, and a 60-μL aliquot was applied to each replicated array with cover lid and incubated on a microplate shaker at 25 °C. In the first optimization round, analytes were applied individually, and in the second round, as a mixture. The sequence with which test samples were added to each array was randomized to avoid bias. A single biotinylated dAb (60 μL) at a concentration defined by the experiment was applied to each array. Tyramine signal amplification[25] (TSA, PerkinElmer, Waltham, MA) was performed by incubating biotinylated tyramine with horseradish peroxidase (HRP) for 7 min. SA-Cy5 (Sigma) was applied at 1 μg/mL for 30 min initially or as defined by the experiment. Washing steps were performed between incubations on a Fluido 2 microplate washer (Biochrom Anthos, UK) with PBST, and each slide was washed five times. In the end, the slides were copiously rinsed with PBST and distilled water and dried under a stream of nitrogen. For technical details regarding signal acquisition and data analysis, refer to the SI.

Experimental Design. *Taguchi Optimization Round 1.* The effects of three factors, cAb, dAb, and analyte, were evaluated for the five biomarkers. Each factor was tested at four concentrations (levels), which were selected for each reagent set on the basis of published data on microarrays and on our own experience[26,27] (see Table 1). The range of analyte concentrations was from low picograms per milliliter to low nanograms per milliliter (or from mU (millunits) per milliliter to U per milliliter for CA15-3). The Taguchi orthogonal array $L_{16}(4^5)$ with 16 trials was selected because it could be carried out on a single slide featuring 16 replicate Ab microarrays, each enclosed within a single well of a 16-well slide module (Grace Bio-Laboratories, Bend, OR), with each well representing one experimental trial. Three factors and one two-factor interaction between cAb and analyte (which we thought to be the most likely to occur) were assigned to separate columns of the orthogonal array, as shown in Table 1. All five analytes were optimized independently.

Taguchi Optimization Round 2. The optimization for seven additional factors was carried out and included buffer solution, SA-Cy5 concentration, and incubation times for five assay steps: blocking, analyte incubation, dAb incubation, TSA amplification, and signal development with SA-Cy5. The experiments were run according the Taguchi orthogonal array $L_{16}(2^{15})$ (see Table 3). This orthogonal array accommodated the above seven factors and five two-factor interactions, as shown in Table S4 in the Supporting Information. The three unassigned columns were used for calculating the experimental error (see paragraph below for explanations). Each factor was tested at two levels: the first one adopted from the initial protocol and the second one either higher; lower; or in case of buffers, with a different composition. The cocktail of analytes for this experiment contained 1 U/mL of CA15-3 and 1 ng/mL of the other analytes HER2, MMP9 and uPA. The Taguchi experiment was performed on 8 slides spotted with 16 replicate arrays to test each of the reagent sets in duplicate. The optimal levels identified for each of the four assays were then used to identify a consensus level that would be optimal for all assays performed on this microarray.

When assigning the factors to be tested onto an orthogonal array, some columns were left empty in each experiment (Tables 1 and 2 and Supporting Information Table S4). Each column represents a specific factor whose effects on the system performance (i.e., assay signal) was assessed, whereas unassigned columns represent "virtual" levels because they do not correspond to any change of experimental factors. The variation of signal obtained for "virtual" levels thus corresponds to experimental error, or "noise". The signal variation obtained for the real factors can thus be assessed for statistical significance in consideration of the noise by statistical analysis of variance (see also Supporting Information).[17]

Binding Curves. Binding curves before and after optimization were established using negative control to set the zero value. To eliminate cross-reactivity, each of the four dAbs was incubated separately. The curve was fitted by the four-parameter logistic model and the regression R^2 calculated using SigmaPlot 11 (Systat Software, Chicago, IL). The LOD was defined as corresponding to the lowest concentration (on a fitting curve) that exceeds the zero analyte concentration signal plus three times the standard deviation.

RESULTS AND DISCUSSION

The net signal was chosen as the parameter for optimization in the Taguchi experiments. Background noise due to nonspecific binding and interferences is widely recognized to limit the performance of immunoassays in practice.[28] For ELISAs, which are carried out in microplate wells, only an average background signal can be measured. For the Ab microarrays in this study, the fluorescence signal in the areas surrounding each spot was also recorded and subtracted from the spot signal.[6] The assays were optimized for net fluorescence intensity, which was obtained by subtracting the surrounding background signal from each spot, and by subtracting the signal from negative control

Table 2. The Factors Tested in the Second Experimental Round with the Values for Levels 1 and 2 for Each of Them[a]

	Factors						
levels	analyte time (h)	dAb time (min)	TSA time (min)	SA-Cy5 time (min)	SA-Cy5 (μg/mL)	buffer	blocking time (h)
1	1	45	10	15	1	PBS	2
2	2	120	5	30	0.5	TBS	1

[a] The full Taguchi array is shown as Supporting Information Table S4.

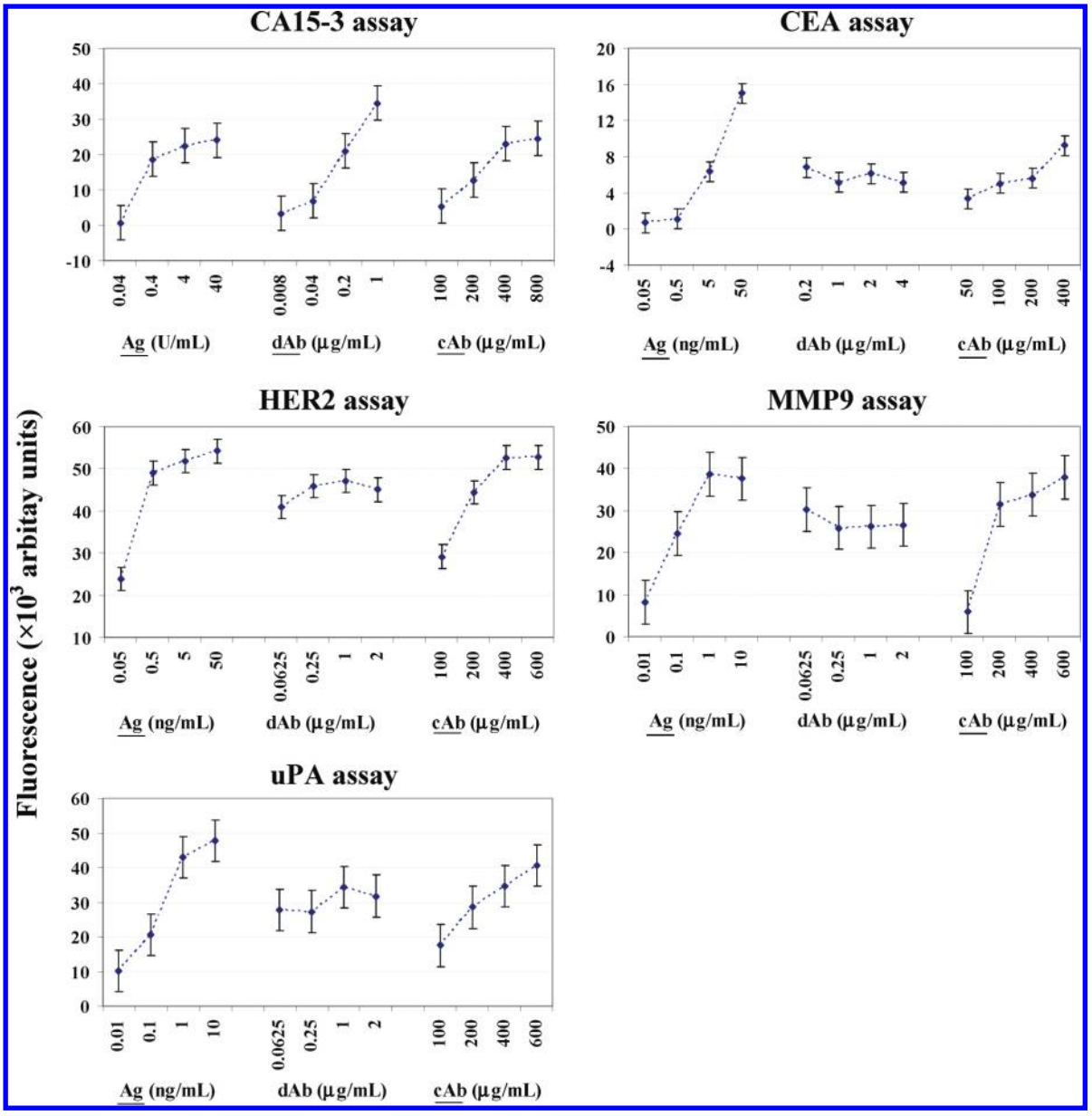

Figure 1. Response graphs for CA15-3, CEA, HER2, MMP9, and uPA sandwich immunoassay on microarray tested with the $L_{16}(4^5)$ array. Data are presented as the mean fluorescence signal of the four trials at indicated concentrations, and the error bars represent the least significant difference (LSD) ($\alpha = 0.05$) based on the Taguchi experimental results (shown in Tables S2 and S3 in the Supporting Information). Significant factors are underlined. Analyte and cAb concentrations strongly influenced the signal for all reagent sets, whereas for the dAb, no statistically significant change was observed except for CA15-3. The CEA analyte responses were flat for the lower concentrations, indicating a poor sensitivity compared with other assays.

spots from the spot being measured. Using this approach, the background signal is intrinsically minimized, which will help improve the LOD, which is strongly dependent on the background noise.

Optimization and Selection of Reagent Sets (Round 1). Using the Taguchi $L_{16}(4^5)$ orthogonal array, three factors (cAb, dAb, and analyte), each with four different levels (concentrations) were investigated simultaneously along with a two-factor interaction

between cAb and analyte in a 16-trial experiment (Table 1). The concentration range for each factor was selected on the basis of prior knowledge about workable assay conditions while taking into account cost, which led us to select different concentration ranges and, for example, a low concentration for CA15-3, since it yielded strong assay signals. The averaged spot signal intensity and standard deviations obtained for various experimental conditions for the five assays are shown in Table S2 in the Supporting Information, and assay signals are shown in Figure 1.

In all assays, the factor analyte exhibited significant effects and produced a strong signal variation. (see also Table S3 in the Supporting Information). The onus to validate the Abs for a particular application, such as antibody microarrays, is on the end user, and the selection of suitable Ab pairs is the critical step of the optimization process. The inclusion of the analyte as an optimization factor for ELISA was first proposed by Jeney et al. because the experimental results allow predicting the binding range and serve to validate the reagents.[17] By using this approach, the CEA reagents were found to perform poorly because they showed no signal increase between the two lowest concentrations (Figure 1). A full binding curve established subsequently confirmed this finding (Figure S3 in the Supporting Information). The disease-free cutoff (threshold) values for CEA in breast cancer are reported to be around 2−5 ng/mL in serum samples,[29−31] and the performance was therefore deemed insufficient considering that blood-based assays require sample dilution and are generally less sensitive because of the sample matrix effects.[27,32] CEA was thus not considered for the second optimization round. The binding results for the other reagent sets did meet clinical cutoffs for breast cancer as reported in the literature, which is 20−30 U/mL for CA15-3,[30,31] 15 ng/mL for HER2,[33,34] and 264 ng/mL for MMP9.[23]

The ANOVA method was used to evaluate the significance of individual factors (and of the two-factor interaction that was tested) on the assay signal (Table S3 in the Supporting Information). Analyte and cAb concentrations were found to be highly significant in all five biomarker assays ($P < 0.01$ or $P < 0.001$), whereas dAb concentration was significant only for CA15-3 ($P < 0.001$). None of the five assays was markedly affected by the two-factor interaction between analyte and cAb concentration, indicating that these factors are independent within the concentration ranges tested. The experimental error varied from assay to assay, but remained much lower than the effects of the main factors.

Optimization of General Assay Factors (Round 2). For CA15-3, HER-2, MMP9, and uPA, seven factors, including the incubation time for five major incubation steps, the concentration of SA-Cy5, the blocking buffer composition (BSA solution prepared with PBS or TBS), and five two-factor interactions were tested in a 16-trial experiment defined by the Taguchi array $L_{16}(2^{15})$ (Table 3 and SI Table S4). Many of these factors were selected because of their potential influence on the background signal; notably, blocking (e.g., buffer and blocking time) and reagent concentrations (e.g., SA-Cy5 and dAb). The assay results and the results of ANOVA are shown in Tables S5 and S6 in the Supporting Information, and all significant parameters are represented graphically in Figure 2. The SA-Cy5 concentration was the most important factor and contributed to more than 50% of signal variation for each assay ($P < 0.001$). Replacing PBS with TBS as the blocking solution improved the fluorescent signal between 8.6% and 15.2% for MMP9 and HER2 assays, respectively, presumably due to quenching of the unreacted epoxy groups of the slide by the primary amine of TBS. Interestingly, the optimal concentration for the dAbs was found to be 0.2 μg/mL for many pairs, which was lower than the original concentration of 1 μg/mL that was used, and may be a consequence of reducing the background signal both in the vicinity of the spot and on the spot itself. Increasing the analyte incubation time from 1 to 2 h significantly increased the signal for the MMP9 assay only. However, in this experiment, 1 ng/mL of analytes was used, whereas in clinical samples, their concentration may be significantly lower, and thus, mass transport may become a limiting factor, which led us to choose the longer incubation time of 2 h. Two hours of incubation with BSA−TBS gave better results than with BSA−PBS and was most significant for MMP9 (Figure 2), whereas for some assays, a slight loss in signal was seen, but which did not reach statistical significance, 2 h was selected.

Table 3. The Major Assay Factors Used in the Protocols before and after Taguchi-Design-Based Optimization

factors	initial protocol	optimized protocol
cAb concentration (μg/mL)	200	400
analyte incubation time (h)	2	2
dAb concentration (μg/mL)	1	0.2 (HER2, MMP9, uPA) 1 (CA15-3)
dAb incubation time (h)	2	1
TSA time (min)	7	5
SA-Cy5 (μg/mL)	1	1
SA-Cy5 time (min)	30	15
blocking buffer composition (1% BSA)	PBS	TBS
blocking time (h)	2	2

Interactions between different factors could potentially confound the identification of the optimal levels, including in sandwich immunoassays. The commonly used OFAT optimization method cannot identify such interactions, and hence, little is known on whether interactions might play a significant role in sandwich immunoassays. Recent studies using different DoE methods indicate that interactions seem to be common in ELISA.[35,36] This led us to choose a Taguchi orthogonal array adapted to the evaluating interactions,[15] and to quantify them, and we found several interactions that contributed to almost 10% of the observed signal variation (Figure 2 right column). TSA time and SA-Cy5 concentration interacted for CA15-3, HER2, and MMP9 assays, and buffer composition with blocking time of MMP9 and uPA assays (Table S6 in the Supporting Information), but their contribution was small compared to that of the most significant factor taken alone. For example, for MMP9, a high concentration of SA-Cy5 gave the strongest signal despite negative interactions with TSA time. Please note that interactions between the various Ab pairs, which are known to lead to cross-reactivity, were not optimized in this study, but would need to be assessed for real life applications.[26,27] The physical basis for the interactions observed here remains unknown, and further in-depth investigations are needed to uncover them.

Binding Curves before and after Optimization. On the basis of the Taguchi experiments and following consideration of the results for each individual assay, the consensus levels that were chosen are summarized in Table 3, along with the original

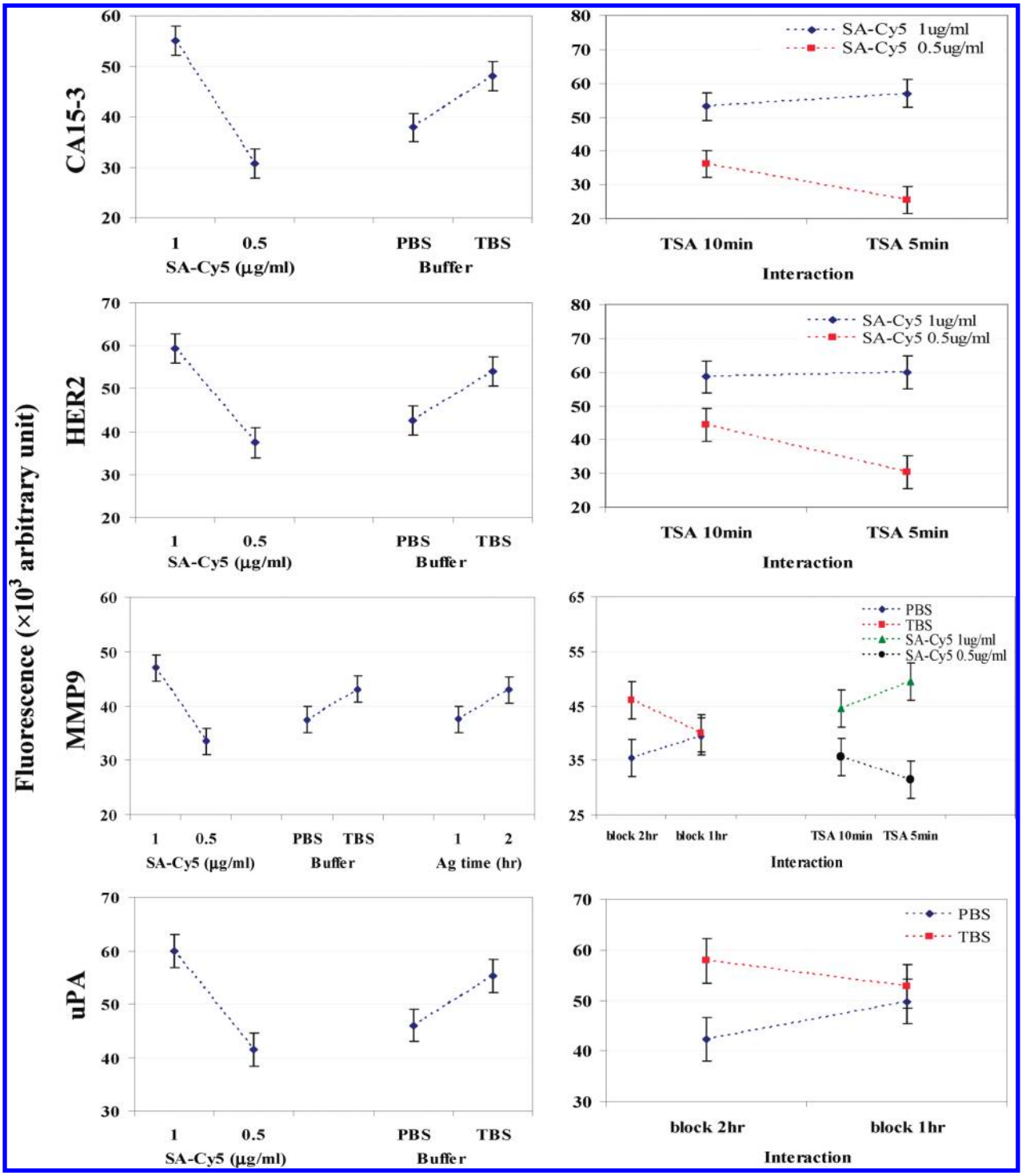

Figure 2. Response graphs for the effects of significant factors and interactions on signal outputs as tested in sandwich immunoassay using the Taguchi L_{16} (2^{15}) array. Data are presented as the mean fluorescence, signal and the error bars are the LSD based on the Taguchi experiment results shown in Tables S5 and S6 in the Supporting Information.

levels. Binding curves were generated for CA15-3, HER2, MMP9, and uPA for the full assay range using the protocol before and after optimization (Figure 3). Both protocols gave rise to sigmoidal binding curves that could be fitted with high correlation factors $0.9784 \leq R^2 \leq 0.9999$. Signal intensity, dynamic range, background signal, and sensitivity were all greatly improved after Taguchi optimization. Notably for MMP9, high background levels in the surrounding areas led to negative values for low analyte concentration before optimization. The LOD of the assays was decreased between 6.5 and 68 times, and the signal intensity at saturation was increased between 1.8 and 3 times. The results illustrate how the sum of seemingly minor changes outlined in Table 3 can lead to major improvements. In addition, the method allowed quantitative rationalization of

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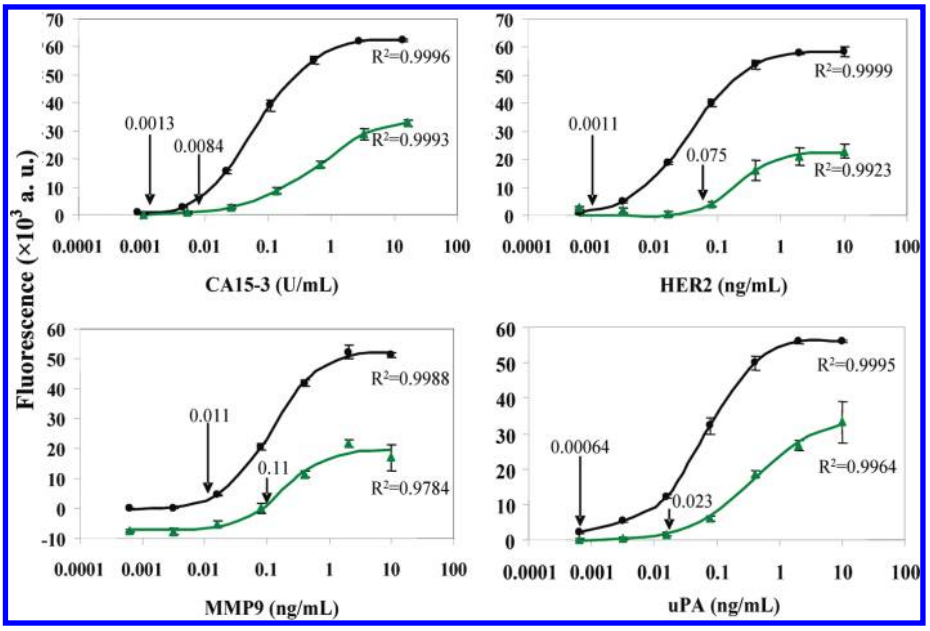

Figure 3. Standard curves obtained using a sandwich immunoassay microarray for four analytes before (green line with up-pointing triangles) and after (black line with circles) Taguchi design-based optimization. The regression R^2 value and the LOD for each curve are indicated. After optimization, the LOD, the maximal intensity, and the dynamic range were all markedly improved, and for Her2, the LOD improved by a factor of 68.

the choice of the consensus assay conditions for all four reagent sets on the basis of each individual response. These assay parameters were established under serum-free conditions, but preliminary tests confirm that they are also suitable for use with serum, while they may be further optimized using Taguchi design.

CONCLUSION

This work illustrates the potential of Taguchi design for the rapid and efficient Ab microarray optimization with concurrent identification of the significant and nonsignificant factors that helps focusing efforts to optimize and controlle the critical factors. Moreover, the quantitative results of the Taguchi method allow rationalizing the choice of the optimal, consensus level for a series of factors for a multiplicity of assays in consideration of both the performance observed for each individual assay and of external constraints, such as cost of reagents and time to result. For example, a lower-than-optimal concentration of cAb may be chosen to save on reagent expenses. A cost analysis can be conducted by balancing savings vs (i) the loss in signal intensity, (ii) the overall significance of the factor, and the (iii) overall assay performance in consideration of the intended application and, thus, the overall optimal levels selected for each particular case. High assay sensitivity is desirable for many applications and is critical for protein biomarker discovery and validation from clinical samples, such as blood, because early detection of disease implies detecting biomarker proteins at low concentration within a background of other proteins at high concentration. In our study, remarkable improvements of signal intensity and LOD were achieved after Taguchi optimization, despite the fact that the assays had already been preoptimized using conventional methods, indicating that many multiplex assays might benefit from additional, rigorous optimization experiments, and that it may allow extending the LOD of these assays and help detect low-concentration biomarker proteins.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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