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Original Article

Robust Experimental Designs for the Development of the N-terminal proB type Natriuretic Peptide (NT-proBNP) Assay Using Europium Chelate

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The natriuretic peptides (BNP - brain natriuretic peptide) and ProBNP are markers with high sensitivity and specificity for heart failure, regulated by intra cardiac pressure. N-terminal pro B type natriuretic peptide (NT-proBNP) is an inactive product of BNP metabolism with a long half-life. BNPs are clinically valuable tools for risk stratification in patients with heart failure. The currently available assays are costly, and we need affordable techniques to make it available to all patients in various economic strata. Lateral flow immunoassay incorporated with fluorescence can be a better tool for quantifying the level of NT-proBNP in blood samples. Developing a point-of-care quantification of NT-proBNP concentration is challenging because of its low levels in blood in normal, healthy individuals. The study aimed to reduce the assay development time and cost by developing a europium chelated-based fluorescence immunoassay using a full factorial design approach. First, the control factors were mapped, and the transfer function was generated using the full factorial design of the experiment (DOE) approach. The control factors were ranked using a Pareto chart. The main effects and interaction effects were determined and plotted. The full factorial DOE also aims to reduce product development time by arriving at an optimum design point.

Introduction

Heart failure (HF) is characterized by the heart's inability to meet the body's circulatory fluid demands and is emerging as a global pandemic. The number of patients is increasing, impacting the health systems in developing and developed countries [1]. Globally, healthcare expenses are alarmingly increasing, and it was reported that the total cost for HF treatment was estimated to be \$30.7 billion in the USA and reported an increase of expenses by \$69.8 billion, amounting to around \$244 for every US adult, in 2030 [2]. According to the global data, India is the country in Southern Asia spending the most on HF, estimating ~\$1186 million (1.1% of total global HF spending). Singh et al. [3] and Inamdar et al. [4] proposed that this enormous cost of HF results from recurrent admissions, diagnosis, multiple drug therapy, device implantations, and various cardiac and non-cardiac co morbidities.

This study focuses on diagnostic expenses and reducing the cost of overall diagnostics for HF management through the indigenization of cost-effective NT-proBNP assay. The Natriuretic peptides (BNP - and brain natriuretic peptide (BNP) and NT-ProBNP are biomarkers with a higher sensitivity and specificity for heart failure, regulated by intracardiac pressure. However, the N-terminal pro B type natriuretic peptide (NT-proBNP) is an inactive product of BNP metabolism production with a longer half-life, which is identified as a blood biomarker for diagnosing cardiac failures. NT-proBNP has a half-life of 120 minutes compared to BNP, which has a life of 20 minutes [5].

Lateral flow Immunoassays (LFIA) are regularly used platform technology for the rapid detection of sample analytes. To enhance LFIA performance, recent reports recommended using amorphous carbon nanoparticles, blue silica nanoparticles, and europium nanoparticles with good analytical sensitivity [6]. Therefore, in this study we used europium chelate to optimize and develop NT-proBNP enhanced LFIA. A computational-based full factorial design approach was used for optimal necessary combinations, or

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Table 1: DoE vs OFAT

OFAT	DoE
Step 1-One factor X1 is varied, and all other factors are held constant	Step 1- Identify factors and levels
Step 2-Find maximum	Step 2- Statistically design the combination of factors and level
Step 3- Hold X1 at the maximum value level, and repeat the process for other factors (Xs)	Step 3- Use the results as direction of your DoE to plan the next experiments

optimal combinations, that will end up with a sensitive optimized process or product. This method supported the Indigenization of the NT proBNP assay, reducing the time and use of costly regents such as NT-proBNP monoclonal antibodies, and europium-chelated based fluorescence reagents for conjugations. Hernandez and Perez-Bernal [7] used the 2^k full factorial design (where is number of levels and k is number of factors) was used to develop the different permutations and combinations of designs and experiments (DOE) for significantly reducing the number of laboratory experimentations.

The DOE approach allows for estimating the main effects and interaction effects in the assay development processes at different levels. Based on Neelakandan et al. [8], the 2^k full factorial design is effectively used in the primary stages of experimental work, especially when the number of process parameters or design parameters (control factors) is minimal. One of the assumptions we make for factors at 2 levels is that the response is approximately linear over the range of the factor settings chosen. 2^k factorial design has two levels, where k indicates the number of control factors under investigation. Hence, this study used full factorial-based design of experiments (DOE) to optimize the europium chelate-based NT-proBNP assay in the lateral flow format to achieve the targeted sensitivity.

Materials and Methods

All major chemicals and consumables were sourced from M/s. Merck, Germany, and M/s. Hi-Media Laboratories, India. The consumables such as filtration devices and essential lateral flow consumables were procured from M/s. MDI Advanced Microsystems, Haryana, India. The Monoclonal mouse anti-human NT-proBNP (HM148, HM 146) Antigen-LA313 were purchased from M/s. East Coast Bio Laboratories, USA. To make the control in the assay, Goat anti-Mouse-IgG was purchased from M/s. Merck, Germany (M8642). The purity and the concentrations were measured and cross checked with manufacturer before conjugation experiments.

Preparation and Conjugation of europium chelate

Europium Chelate-Antibody conjugation kit (Cat. No: ab269889) was procured from M/s. Abcam Biotechnology Company. Based on the literature, the size of the europium chelates selected was 200nm to achieve the targeted sensitivity. The fluorescence emission and excitation of europium used in the kit were measured by spectro-fluorimetry. The Specific monoclonal antibodies of NT-proBNP were coupled with europium chelate as per the instruction manual. The Conjugates were characterized and confirmed using a fluorescence spectrophotometer (M/s. Jasco Inc., Japan. Model No. FP-8200 Spectrofluorometer), Fourier transform infrared spectroscopy (FTIR) (M/s. Perkin Elmer, Model No: L160000F, Spectrum Two FTIR Spectrometer (DTGS Detector), and Transmission Electron Microscopy (TEM) (M/s. Hitachi., Japan. Model No. 7650). The confirmed conjugated Europium Chelate (EuC) with NT-proBNP antibodies were prepared with 2-(N-morpholino) ethane sulfonic acid (MES) buffer (pH 6.5) for developing the strips. The binding efficiency of conjugated europium with NT-proBNP monoclonal antibodies were studied by performing a Bradford assay.

Predictive modelling for assay developments

Predictive modelling is a form of data mining that analyses historical data to identify trends or patterns and then uses those insights to predict future outcomes. Especially, in the product development, the general types of experimentation approaches used are

1. Trial and Error
2. One Factor AT a Time (OFAT)
3. Design of experiments (DOE)
    a. Full Factorial design- Used when number of factors are minimum
    b. Fractional factorial design- Used when number of factors are large
    c. Response surface
    d. Taguchi's method

In this study, a full factorial design-of-experiment method is used to develop a transfer function. The DOE helps to identify the control factors (inputs) that control the system response (outputs) and provides a quantitative measure of their impact on the outputs. The comparison between OFAT and Full factorial DoE is given below in the table 1.

Table 2: Control factors

Factor	Control factors	Units	Lower specification limit (LSL)	Upper specification limit (USL)
A	NC membrane	µm	8	15
B	Concentration of Ab coating	mg/ml	1	2
C	Concentration of Ab Conjugation	mg/ml	0.1	2
D	Running buffer	-	MES	PBS

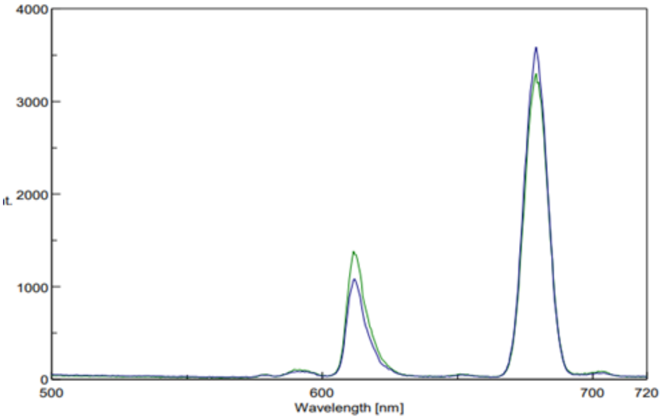


Figure 1a: Spectrofluorimetric analysis shows conjugation confirmations of antibodies conjugated with europium chelate

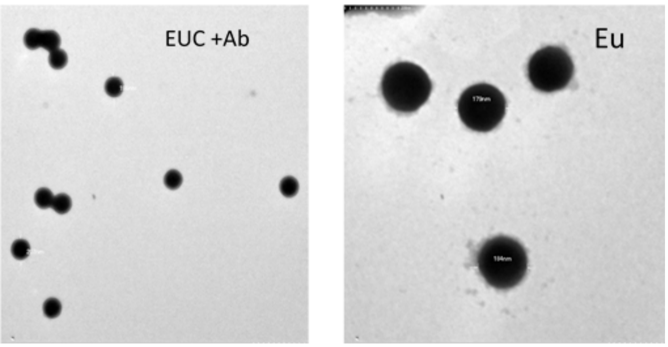


Figure 1b: TEM Images (a) confirms europium chelate particle conjugated with antibody. (b)indicates near spherical morphology with free dispersion

Hence the key applications of DoE when compared with old methods are

1. Screening- Identify which factors are important and rank them

2. Quantify the effect of factors on the response

3. Develop predictive models (transfer function) from real experimental data of *in-silico* data

The benefits of the DOE approach compared with the traditional OFAT approach are

1. Accelerated technology and product development cost

    a. Highly preferred for industrial entity due to less dependence on physical prototypes and experiments

2. Accelerated technology and product development timeline

    b. Quick insight on variations in output and key driving variables

    c. Developing ability to make data driven, analysis driven decision on experiments

    d. Quicker time to market

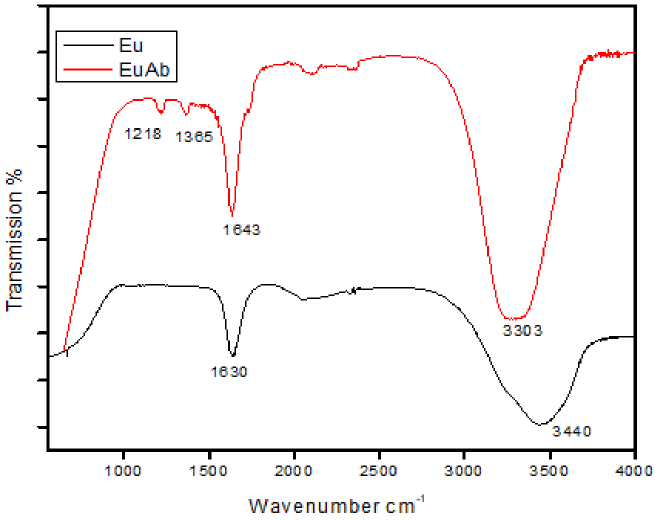


Figure 1c: FTIR analysis shows conjugation confirmations antibody conjugated with europium chelate

3. Quality levels

    e. Reduction of rework and scrap

    f. Reduction in end of line testing

In this study Minitab (Version-18) were used for modelling full factorial-based experiments for achieving optimal control factor specification. Table 2 showing the control factors such as NC membrane, Ab coating concentration, Ab conjugation, and running buffer and its influence on the output grey value (band intensity) were observed using the DOE. Here, Minitab (Version. 18) was used for predicting the main effects, Pareto chart and Interaction plots for various control factors.

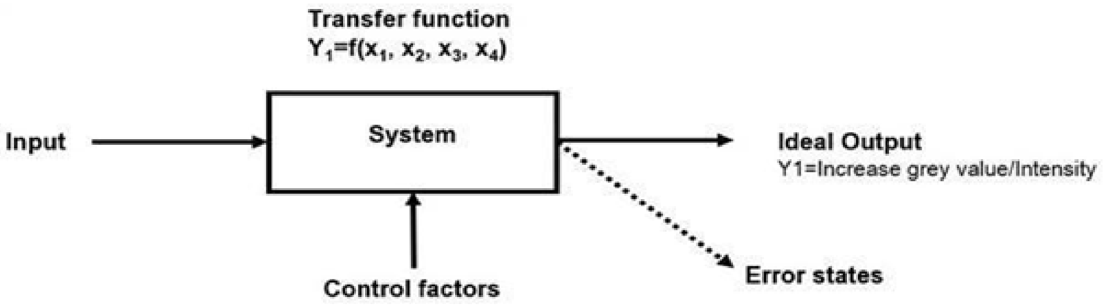
Since multiple factors would influence the output of the process, the significant factors contribute the most defects through Pareto analysis. The full factorial design-based combinations generated using Minitab and 16 experiments were carried out based on these input combinations.

Europium chelates incorporated Lateral flow strips

The different permutations and combinations of NC membrane, antibody concentration, and fluorescence-conjugated antibody strips were prepared based on **Table 3** the strip preparations were done using an antibody coating machine, and fluorescence conjugate was manually sprayed onto the conjugate membranes. All these materials will be appropriately placed on the plastic backing plate with accuracy and skill to ensure that the ends of the components overlap to achieve a continuous flow by capillary action. Finally, the strips were cut with 3mm dimensions using the strip cutter machine. Experiments were performed under controlled relative humidity (RH +/- 30%) for better results.

Fluorescence Imaging

A cooled CCD ImageQuant LAS 500 (M/s. GE Healthcare) was used to image fluorescent test strips. The image taken was converted to a greyscale picture, and the intensity of the antigen concentration was measured. The image and intensity obtained were analysed



Factor	Name
A	NC membrane
B	Concentration of Ab coating
C	Concentration of Ab Conjugation
D	Running buffer

Figure 2: Shows the parameter diagram (P- Diagram)

using the ImageJ software. The optimal combination of components obtained through the DOE was confirmed with known analyte serial diluted based on concentrations and compared with the standard graph.

Results and Discussion

Commercially procured europium chelate-Antibody conjugation kits were used for conjugations. The conjugates were confirmed with spectrofluorimetry and showed a significant fluorescence increment (1100 to 1400) in the europium chelate antibody mixtures (Figure 1A). Further, TEM analysis on the conjugated mixture showed a slight size difference in the conjugated and unconjugated samples (187 nm to 207 nm). Imaging confirms thin, less intense margins of conjugated particles, indicating antibody conjugation. (Figure 1B). Finally, the FTIR spectrum of conjugated europium chelate with antibody the band at 1643cm^{-1} showed the N-H bend primary amine, and 1365cm^{-1}, 1217cm^{-1} showed the N-H stretching and C-N stretching in the aromatic and aliphatic peak of amines (Figure 1C). The conjugation efficiency was determined using the Bradford assay, a colorimetric protein assay. Our study showed that the antibodies used were 100% efficacious in the conjugated mixture.

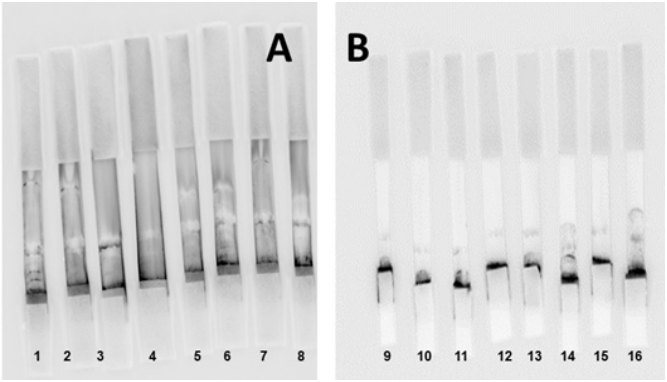


Figure 3: represents ImagQuant pictures of strips developed with 16 combinations

At the most basic level, a transfer function is a relationship identifying how the inputs (control factors) affect output. The general equation often represents this Y=f(X), Y is the dependent output variable and X is the independent input variable. In this study, the Minitab software generated the parameters diagram (Figure 3) showing the control factors and expected ideal output. The outcome of the DOE study, the transfer function of the following form, indicates that

$Y_1=f(X_1, X_2, X_3, X_4)$,

Where X_1, X_2, X_3 and X_4 are variables. X_1 is represented as NC membrane, X_2 is the Antibody (Ab) coating, X_3 is the concentration of Ab conjugation, and X_4 is the running buffer. And Y_1 is the Output grey value (Figure 2). The DOE approach was essential for accelerating product development and expenditure reduction on recurring resources. The control factors varied between the upper and lower specification limits to understand the effect of output grey value.

Based on the DOE analysis, Lateral flow fluorescence test strips were developed with the software-predicted 16 combinations (Table 3 & Figure 3). All these 16 strips developed for optimizations were imaged under ImageQuant LAS. Images taken are converted to measure the band intensity grey values with ImageJ software. These strips were tested with a known concentration antigen (0.1ng/mL) for further optimizations and better combinations. The reduced model of the transfer function derived was by using the formula:

There will be a difference between results from the traditional OFAT method and DOE, which is an advanced method of optimization and experimental development. The reason is that OFAT is disadvantageous in determining the True maximum. So DoE gives the True maximum in optimization, whereas OFAT does not. Therefore, the values will be obviously different, showing the benefit of DoE.

The experimentally measured intensity values for the 16 combinations of DOE are recorded, and the factors indicated in the Pareto chart (Figure 4A) are used to rank and identify areas to focus on first in process improvement. The concentration of Ab conjugation (control factor C) has a major impact on grey value, or rank 1, followed by the interaction effect between the NC membrane

Table 3: Two-level and four-factor based DOE combination generated by Minitab

No	NC Membrane(μm)	Concentration of Ab coating(mg/ml)	Concentration of Ab Conjugation (mg/ml)	Running buffer
1	8	1	2	MES
2	15	2	2	MES
3	15	1	2	PBS
4	8	2	2	MES
5	8	2	2	PBS
6	15	2	2	PBS
7	8	1	2	PBS
8	15	1	2	MES
9	8	1	0.1	PBS
10	8	2	0.1	PBS
11	8	1	0.1	MES
12	15	2	0.1	MES
13	15	1	0.1	MES
14	8	2	0.1	MES
15	15	2	0.1	PBS
16	15	1	0.1	PBS

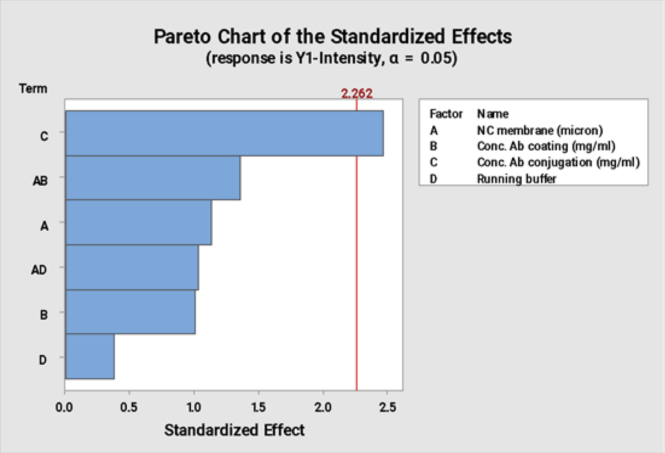


Figure 4a: Pareto chart of the standardised effects

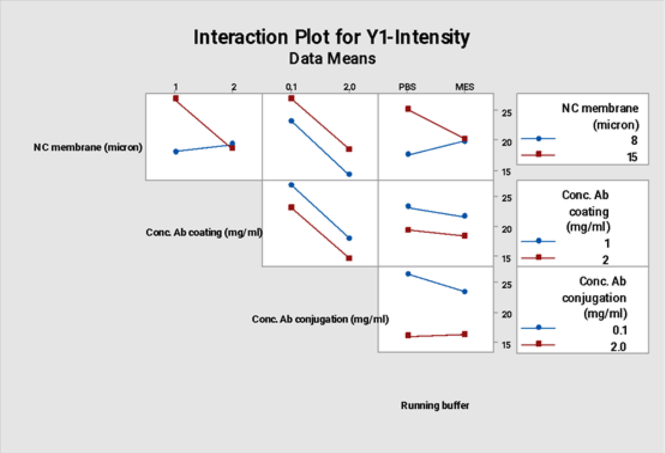


Figure 4c: Shows the interaction plots of the Assay

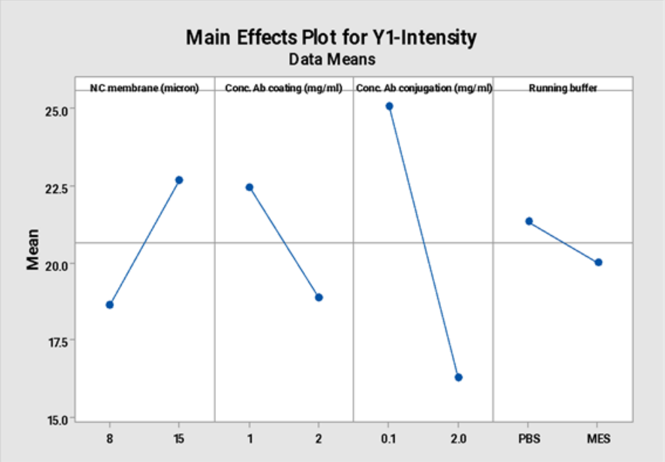


Figure 4b: Shows the main effects of the Assay

and the Concentration of Ab coating (control factor AB). When an interaction effect term (AB and AD) is present in the transfer function, the output (grey value) changes upon the level of mutual interaction between the control factors (A, B, and D). Based on the transfer function coefficients, AB is the most significant interaction effect, followed by AD.

Figure 4B shows the main effect plot and 5C shows the interaction effect plot. Main effect plots show the effect on the output (Y) when a control factor (X) is changed from its low to high level. Interaction plot shows non parallel lines for strong interaction and parallel lines for weak interaction. When an interaction is present between factors the magnitude of the effect on the output (Y) when a factor changes from its low to high level depends upon the level of one or more other factors.

The main effect plot shows the variation of output Y1 intensity by control factors; the main parameters for calculating the Y1 intensity include Nitrocellulose membrane, coating antibody, conjugated

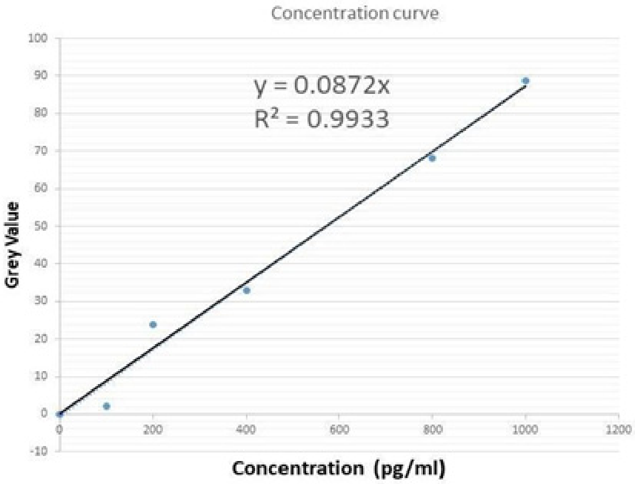


Figure 5: Shows the OFAT experimental results of the optimized linearity profile of the developed assay

antibody, and running buffer. The main plot suggested that conjugated and coating antibody concentrations should be minimized for maximum Y1 intensity. At the same time, the size of the NC membrane should be maximum for getting the Y1 intensity. Interaction plot revealed that the combinations with NC membrane (15 microns), coating antibody concentration (1mg/mL), conjugated antibodies (0.1mg/mL), and Buffer (PBS) showed higher sensitivity detections of the analyte.

The mutual interactions between the control factors are shown in Figure 5C. The converging or diverging plots show strong mutual interaction, whereas parallel plots show weak interactions between control factors (Figure 4C).

Validations of the software predicted combinations were evaluated at laboratory by developing the lateral flow strips. Concentrations known antigens (NT-proBNP) were serially diluted from 0.1 ng/mL to 1ng/mL range and tested in thus developed strips**.** The graph showed comparable linearity when plotted with concentration and grey values (Figure 5). The repeatability and reproducibility were also checked with these combinations thrice. This indicates that the predicted DOE model was beneficial for optimizing the experiments with less time and reducing the reagent usage.

Conclusion

The significant challenges of developing these LFIA technologies are the cost of consumables such as antibodies, fluorescent nanoparticles, monoclonal antibodies, and specialized consumables used for the developments. The design of the experiment approach is widely used with varied applications to reduce the developmental cost of experiments incurred due to one factor at a time. DOE helps in technology developments to reduce the time of product and/or process developments, lab-to-market transitions (product assimilation), scale-up and commercialization, etc. This study established the link for optimizing an effective assay with the DOE. The design of the experiment approach helps accelerate the product development of an effective fluorescence immunoassay. This method can be used effectively in multiple applications related to technology developments, such as drug developments, assay optimizations, and combinational device developments.

The major advantages of this study are:

> The transfer function derived from experiments using a full factorial design gave an idea about the control factors or inputs, quantitative relationship between the inputs (X) and outputs (Y), and ranked the control factors based on the order of effect on output.
>
> The interactions between factors were detected and estimated through full factorial DOE approach, which is impossible through OFAT.
>
> Information is obtained about a broader region of design space. Rather than conducting cost intensive physical experiments, now this transfer function may be used for determining the output for any combination of inputs in the low and high levels used in this study.
>
> Fewer resources are required to obtain a given amount of information for IVD translational research.

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