

**COMPARISON BETWEEN METHOD OF ONE-FACTOR-AT-A-TIME (OFAT)  
& DESIGN OF EXPERIMENT (DOE) IN SCREENING OF  
IMMUNOGLOBULIN PRODUCTION STIMULATING FACTORS**

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## ABSTRACT

The medium of mouse-mouse hybridoma, 192 producing IgG against 17-OHP was supplemented with seven different inducing agents at different range namely lysozyme, lipopolysaccharides (LPS), potassium phosphate, aldolase, essential amino acid, non-essential amino acid and sodium butyrate. This study aims to compare the statistical design of experiments (DOE) and one-factor-at-a-time (OFAT) method in screening immunoglobulin production stimulating factors. The culture medium supplemented with the inducer agents were screened using OFAT method and Plakett-Burman design method to determine inducer agent that gave positive effect. The effect of inducer concentration towards the antibody production was studied using enzyme-linked immunosorbent assay (ELISA) technique. The optimal concentrations for all the inducing agent were failed to be found in OFAT method. The standard culture medium without addition of inducer yield 0.234 $\mu$ g/ml of MAb. The culture supplemented with the inducer agent that yields MAb close to the standard was non-essential amino acid. Plakett-Burman design gave 12 runs with only the high level (+) and the low level (-) of the inducer were used. Potassium phosphate, LPS and sodium butyrate was found to be the most influential variable using Plakett-Burman design. Plakett-Burman design however indicates that all the point was insignificant and the interaction between inducer was not described. Plakett-Burman under DOE method gave the best time-wise compared to OFAT method in screening monoclonal antibody stimulating factors.

## ABSTRAK

Media untuk sel hibridoma tikus-tikus, 192 yang menghasilkan immunoglobulin (IgG) menentang 17-OHP dibekalkan dengan agen pemangkin pada sukatan yang berbeza iaitu lisozim, lipopolysaccharides (LPS), kalium fosfat, aldolase, amino asid perlu, amino asid tak-perlu, dan natrium butirat. Penyelidikan ini bertujuan membandingkan statistik rekaan eksperimen (DOE) dan kaedah satu-faktor-dalam-satu-masa (OFAT) dalam saringan faktor ransangan ke atas penghasilan immunoglobulin. Medium yang dilengkapi agen pemangkin disaring menggunakan kaedah OFAT dan rekaan Plakett-Burman bagi menentukan agen pemangkin yang memberikan kesan positif. Pengaruh kepekatan pemangkin terhadap pengeluaran antibodi dikaji menggunakan teknik ELISA. Kepekatan optimum untuk semua agen pemangkin gagal ditemui dalam kaedah OFAT. Medium kawalan tanpa penambahan pemangkin mengeluarkan 0.234 $\mu$ g/ml MAb. Kultur dilengkapi agent pemangkin yang menghampiri medium kawalan dalam pengeluaran MAb adalah amino asid tak-perlu. Rekaan Plakett-Burman memberikan 12 percubaan dengan hanya menggunakan kadar maksima (+) dan kadar minima (+). Dengan menggunakan rekaan Plakett-Burman Kalium fosfat, LPS dan natrium butirat didapati menjadi pemngkin utama. Namun rekaan Plakett-Burman menunjukkan bahawa semua nilai tidak relevan dan gagal menunjukkan interaksi antara pemangkin. Rekaan Plakett-Burman berdasarkan DOE memberikan pengendalian masa yang terbaik dibandingkan dengan kaedah OFAT dalam saringan factor pemangkin pengeluaran antibodi monoklonal.

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**LIST OF SYMBOLS/ABBREVIATIONS**

ANOVA	-	Analysis of variance
CCD	-	Central composite design
g	-	Gram
g/L	-	Gram per litre
hr	-	Hour
L	-	Litre
M	-	Molar
mg	-	Miligram
min	-	Minutes
ml	-	Mililitre
mM	-	Milimolar
OD	-	Optical density
OFAT	-	One factor at time method
RSM	-	Response surface methodology
rpm	-	Round per minute

## CHAPTER 1

### INTRODUCTION

Monoclonal antibodies (MAbs) are high in demand nowadays for used in various applications. To use these MAbs, large MAbs must be obtained from hybridoma cells. However, mouse-mouse hybridomas usually produced low level of MAbs (Sato *et al.*, 1988). Therefore, many inducing agent is added to increase the antibodies production. Screening test is the most appropriate experimental procedure to discover suitable inducing agent or screening out insignificant inducing agent among others, in order to identify those that may display significant effect on the antibodies production.

#### 1.1 Background study

The screening is the way to determine which factors that returns the best possible outcome (response). Generally there are two different strategies for screening: (a) One-factor-at-a-time (OFAT) and (b) response surface methodology (RSM). One-factor-at-a-time, OFAT approach examining one parameter while holding all other parameter constant (Sakkas *et al.*, 2010). In RSM, Design of Experiment (DOE) is employ. Design of Experiment, DOE is a method for detecting interaction between parameter if any and required fewer test than OFAT but require the use of statistics. As the research objective is to compare which analyzing method is more practical in screening and optimizing of MAbs stimulating factor. Most people preferred DOE because it account for interaction

effects between the studied parameter and determine accurately the combination of level that produces the optimum condition.

## 1.2 Choice of design for RSM

The most important part before applying the RSM is the selection of appropriate DOE. Compare to OFAT, DOE method is wider and has many type of design. The common DOEs used in RSM (Sakkas *et al.*, 2010) are:

### 1. Plackett-Burman (two-level fractional design) or full factorial design

Factor takes low (-) or high (+) value only. Its design is based on the first order model which is;  $Z = b_0 + \sum b_i x_i$  where  $Z$  is the response,  $b_0$  is the model intercept,  $b_i$  is the linear coefficient and  $x_i$  is the level of independent. This model is selected because although it does not describe the interaction among the factor, it gives less run compare to other RSM method and only focus to select the factor that influences the response.

### 2. Full three level factorial design

Factor takes low, medium or high only and  $2^k$  or  $3^k$  full factorial design is used to determine number of run. In this design it is preferred if factor less than 5.

### 3. Central composite design (CCD)

Consist 3 type of point: cube point, axial point, and center point .To calculate the number of experiment needed,  $N=2^k+2k+Co$ . It is known as time consuming design with large numbers of factors.

### 4. Box-Behnken design

The design is used for three levels of variable that are evenly spaced. To calculate the number of experiment needed,  $N=2k(k-1)+ Co$ . This design avoids extreme conditions of experiments.

## **1.2 Identification of Problem**

1. Determination of number of component needed to be screen.
2. For OFAT method, screening large amount of inducing agent require long period of time and may miss interaction effect.
3. For DOE method, can only detect interaction if any.
4. For DOE method, if choose too wide range will miss interaction effect and if choose too narrow range won't get the point.

## **1.3 Objectives**

The main objective of this research is to compare the DOE and OFAT method in screening MAb stimulating factors.

## **1.4 Scope of Study**

The scope of study is divided into three parts:

1. OFAT method and Plakett Burman design under DOE method is selected for screening process.
2. The parameter that will be measured is specific growth rate and antibody production.
3. 7 inducer agent is use in screening which are; potassium phosphate, lysozyme, aldolase, lipopolysaccharide, sodium butyrate, essential amino acid, non-essential amino acid.

## **1.5 Significance Study**

The rationale of doing this research is so that DOE and OFAT methods can be distinguish in screening MAb.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

In order to produce antibodies specific for human antigens, cell from mouse or rat has always been the source of production (Mimura et al., 2001). However, mouse-mouse hybridomas usually produced low level of MAbs (Sato *et al.*, 1989). Therefore, many attempts to facilitate immunoglobulin production on hybridomas cell by modification of culture media has been made. Modification of culture media includes addition of inducing agent such as lysozyme. This inducing agent even includes protein, salt solution, and even antigen to be the stimulating factor of immunoglobulin production. In addition to these possibilities, review is needed to determine material that would stimulate the production of human MAbs and the range of concentration of the material. To give a better proves, method for screening the material also needed to be survey.

## 2.2 Technique for cell culture

There are many type of hybridomas cell and not all can tolerate in one condition or has the same doubling time. However, all culture incubation should be in a humidified 37°C, unless otherwise specified and DMEM medium may require CO<sub>2</sub> alteration to maintain pH 7.4 (Phelan, 1998).

Based on the protocol for mammalian cell culture from Phelan (1998), before passaging or subculture the cell into new flask to multiply the culture, cell must be maintained in culture by feeding every 2 to 3 days until they reach confluency. One third of the media need to be discarded before fresh medium is top up into the media. The fresh media is also reminded to be warm before the replacement process. This is to ensure the low temperature does not interrupt the cell growth or have cold shock since the old medium is warm already.

When a new cell is needed to be thawed, proper aseptic technique is needed depending on the source of the freezer. If the cell is taken out from liquid nitrogen tank, a quick thaw in the 37°C water bath with duration less than 60 second is needed to prevent formation of ice crystals that can cause cell lysis. Then, cell is gently resuspend in small amount of complete medium and centrifuge at 150g before discard the supernatant to remove residual DMSO. Culture is checked after 24 hour to ensure that cell have attached to the plate.

### 2.3 Selecting of inducing agent

Mouse-mouse hybridoma, 192 is one of the animal cells that can secrete IgG against various antigen, for these case against 17-OHP. Yusuke *et al.* (2001) use CHO-K1 cells from Chinese hamster ovary and NS0 from mouse myeloma as subject of study. Although the cell use is different, but the responses anticipated is the same which is IgG. The inducing agent use to stimulate IgG production is sodium butyrate with 0-5 mM concentration. In the study, 13 days is choose as time of cultivation because the doubling time during exponential growth phase up to 95.5 hour. The antibody titers are measured by ELISA which used NIP-ovalbumin during coating stage and IgG3/WT in standard loading stage. The effect of 5 mM sodium butyrate is reported to increase the production of IgG up to 2-4 folds. This is a very convincing result as the sodium butyrate did increased IgG production.

However, sodium butyrate is reported to be dissolved in PBS 0.4 M and stored at -20°C for only 2 weeks as stock because further storage will gives changes in its effectiveness. This result does not resemble the stimulating effect of sodium butyrate on H192 cell as it might differ from CHO-K1 cells. So, certain changes need to be made such as antimouse IgG needed to be use in coating stage instead of NIP-ovalbumin. Cultivation time for inducing agent also needed to be reduced less than 13 days because H192 does not have high doubling time and could achieve death before even reach to day 13.

Sodium Butyrate is also being use in Yamamoto et al (1997) to enhance production of antibody in murine splenic B cell. Various concentrations from 0-3 mM are use and the expressions were compared using two-dimensional electrophoresis. It is reported that 3 mM can increased antibody up to 2 fold.

Sato *et al.* (1989) analyzed stimulating effect of sodium and potassium phosphate toward monoclonal antibody production in human hybridoma cells. Before stimulating effect of sodium phosphate and potassium phosphate is being the scope of the study, a research on additive for Rat Dermal Fibroblast (RDF) and Dulbecco Modified Eagle (DME) basal medium is reported done. The research reported that result of IgG stimulating only valid if insulin, transferin, ethanolamide, selenium and yolk lipoprotein (ITES/YLP) is use as an additive for basal medium because only basal medium with the additive is further use to study the stimulating effect of sodium phosphate and potassium phosphate.

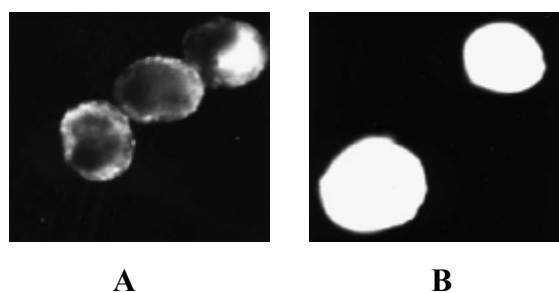
Various concentration of sodium and potassium phosphate is used in  $1 \times 10^5$  to  $5 \times 10^5$  cells/ml of HB4C5 cells according to Sato *et al.* (1989). It is reported that, potassium phosphate was more effective compare to sodium phosphate. Cell seeded at  $5 \times 10^5$  cells/ml only gives maximum production of MAbs. Control media only produced  $0.08 \mu\text{g/ml}$  -  $0.42 \mu\text{g/ml}$  MAbs titer while addition of 15 mM potassium phosphate can produced MAbs up to  $1.8 \mu\text{g/ml}$ . Although potassium phosphate is just a salt, but it gives pH effect when added to medium. It is stated in the research to maintain the potassium phosphate solution at pH 7.4 before adding into medium although no chemical addition is stated to maintain the pH.

There are four main immunoglobulin (Ig) that is secreted in human body as defense against pathogen and antigen which are IgG, IgM, IgA and IgD (McKee *et al.*, 2003). But not all inducing agent that stimulate one type of Ig can stimulate another type of Ig. Sugawara *et al.* (1982) reported that Phorbol Myristate Acetate (PMA) only induced polyclonal IgM production on human peripheral B.



Sugahara *et al.* (1998) on the other hand reported that aldolase from rabbit muscle can enhance both IgM and IgG by different fold respectively.  $1 \times 10^6$  cells/ml of HBC5 cell is seeded into insulin-transferrin-ethanolamide-selenium (ITES-ERDF) medium and added with  $100 \mu\text{g/ml}$ - $1000 \mu\text{g/ml}$  aldolase. MAbs titers are only being reported analyzed after 4 days and 8 days. IgM is reported to increase about 5.3 folds while IgG up to 1.4 fold. The research shows that aldolase still can be use to enhance IgG production although it enhance IgM better. However, PMA use in Sugawara *et al.* (1982) is not applicable to be use to enhance monoclonal IgG. Although only day 4 and day 8 IgG titer data is recorded in data, but still increasing of IgM production after 4 hour is recorded in graph. This is why research needed to be done to determine whether aldolase can increase IgG less than 4 days.

Sugahara *et al.* (2000) on the other hand study the mode of actions of lysozyme as an immunoglobulin production stimulating factor. Human-numan hybridoma, HB4C5 cell was cultured in ITES-ERDF medium supplemented with  $380 \mu\text{g/ml}$  of lysozyme. The cell also was reported to be treated with actinomycin D to suppress transcription, treated with cycloheximide to suppress translation and treated with monensin to suppress post-transcription. The figure 2.1 clarify fact that monensin cause high IgM content in cell but inhibit secretion of IgM. IgM production is reported to increase up till  $501.1 \mu\text{g/ml}$  and lysozyme also can increase the production of IgG up to 2.3 fold. So, in order to use lysozyme to increase IgG secretion, no monensim treatment is needed.



**Figure 2.1:** Intracellular content of HB4C cell with addition of lysozyme and:  
(A) without monesim treatment; (B) with monesim treatment,  
(Sugahara *et al.*, 2000)

Shinohara *et al.* (1993) study the effect of some constituents' egg yolk lipoprotein (YLP) on the growth and IgM production of human hybridoma cells. HB4C5 cells were plated at cell density  $5 \times 10^5$  cells/ml and supplement with various concentrations of constituents' YLP and after 4 days, YP-1 fraction at concentration of 500  $\mu$ g/ml produced highest cell number and IgM concentration compare to YP-2, YP-3, and YP-4. Sugahara Takuya *et al.* (1999) study the effect of aldolase from rabbit muscle towards immunoglobulin production in human hybridoma cells. HB4C5 cells were inoculated at  $1 \times 10^5$  cells/ml in ITES-ERDF medium supplemented with r-aldolase at different concentrations. After 6 hour cultivation, aldolase concentration at 9000  $\mu$ g/ml increase the IgM concentration. Although all of them has tested to induced IgM, this however only been done to human hybridoma cell and not to mouse hybridoma cell. The effect might be varied if the cell used in culture is different. To improve their research, this research will use as many inducing agent in mouse hybridoma cell culture and try to screen using OFAT method and Plakett-Burmen under DOE to see which inducing agent give the most effect towards MABs production. Then, compare which method gives the best result toward optimizing MABs production.

A model based on the stoichiometric nutritional demand has also been developed (Xie and Wang, 1994) to avoid toxic waste accumulation in the culture. The importance of balance supplementation has been stressed especially amino acid to avoid excess toxicity from certain vitamin (Baker *et al.*, 1988). This toxic will suppress antibody production and growth rate of cell. Therefore, Ducommun *et al.*, (2001) has done research to study the formulation of medium and concentration of amino acid on murine hybridoma cell, Zac3. Medium use for cell nutrition supply is supplemented with formulation of 13 types of amino acid. The amino acid range is reported varied from 0.06 mM-25 mM and cell is seeded at  $5 \times 10^4$  cells/ml. After 70 hour, it is recorded that IgA concentration is increased from 26.6 to 100.2 mg/L. This shows that addition of amino acid did increase the production of MABs. However, many factor needed to be tested again such as the range of effectiveness because the range is only being study to increase IgA not IgG. Cell density seeded is also need to take into consideration if amino

acid is used as an inducing agent because the cell uses it differently in type and probably in doubling time too.

#### **2.4 Selecting of method use in screening inducing agent**

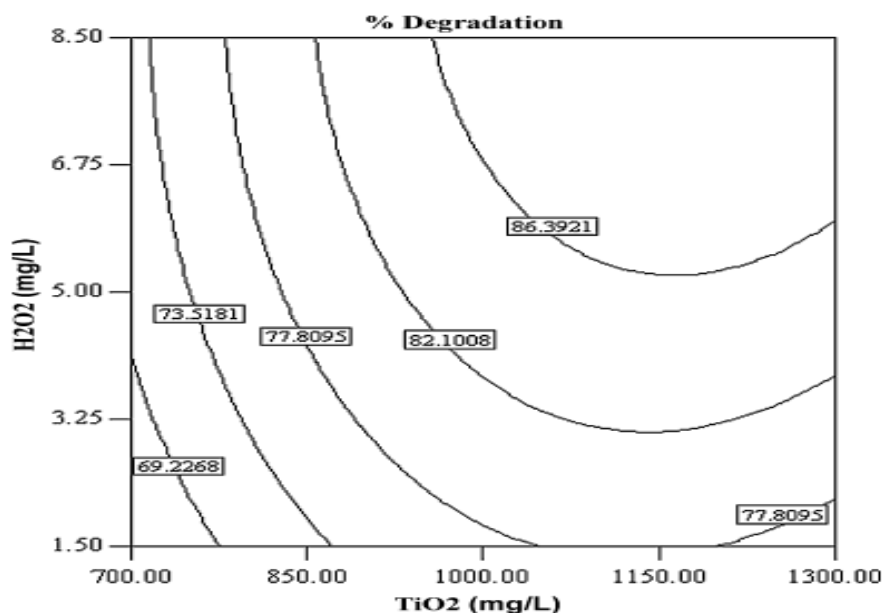
One-factor-at-a-time, OFAT is an unvaried analysis method which often shows inadequate optimization toward responses. There is now increasing recognition that OFAT method ought to be replaced by chemometric methods such as response surface methodology based on statistical DOEs (Sakkas *et al.*, 2009).

Some researcher has done a comparative study between OFAT and DOEs method while other just employ DOE method for optimizing the product or effect. Based from the Plackett Burman model of Bari *et al.* (2009) research, the main effect is recorded in bar graph and it is reported that 5 out of 8 independent variables give effect on production of citric acid. Then both central composite design (CCD) and OFAT is employed in the optimization of production of citric acid from oil palm empty fruit bunches (EFB). By using results gained from Plackett-Burman design, percent of glucose, minerals and inoculums is analyzed using OFAT and CCD because only these three are the most significant factors influencing citric acid production. Production of citric acid from screening gives 128.9g/kg-EFB, optimization using OFAT reported 218.6g/kg-EFB while optimizing using CCD is 335.4g/kg-EFB. The result clearly shows that DOE is more efficient compared to OFAT method toward citric acid production.

Plackett Burman is reported to be time-wise in screening method but does not show interaction (Bari *et al.*, 2009). This comparative study is only done for chemical production not for MAbs in living cell production. Furthermore, the comparative study is only done toward optimization and not screening. Different scope could give different effects toward OFAT and DOE application. That is why this research will be done in a living animal cell to see whether it affects the performance of DOE and OFAT.

Only optimization step using DOE can show interaction among parameters. CCD which is one of the Research Surface Methodology (RSM) under DOE itself function to hunt efficiency for the optimum value of the parameter such that response is maximize by giving linking interaction between parameters (Tanyildizi *et al.*, 2005).

Sakkas *et al.* (2010) only employed CCD to analyze the simultaneous effect of  $H_2O_2$ , and  $TiO_2$  in the photocatalytic degradation of the Congo red (CR) in aqueous solution. Experimental data were fitted using artificial neural network (ANNs) for optimization. The findings indicate the influence of each variable, with  $TiO_2$  being the most significant factor followed by  $H_2O_2$ . The software optimized degradation of CR up to 90.22%. The optimum response can also be seen in 3D response and 2D contour plot based from RSM design. Elliptical contours are only obtained when there is perfect interaction between the independent variable (Muralidhar *et al.*, 2001).



**Figure 2.2:** Contour plot for CR catalytic degradation showing interaction (Sakkas *et al.*, 2010)

Joelsson Daniel *et al.* (2008) on the other hand employed only statistical DOE to optimize the assay precision over the linear part of the dose response curve. Two separate factorials are created, one for each set of factors. Incubation times are

investigated in the plate factorial, and reagent concentrations are investigated in a separate factorial that is replicate in each plate. Changing the assay condition to those identified in the DOE studies significantly decrease the assay variability by a 3.5% average.

Both of the researchers only used DOEs method toward optimization without even considering OFAT method or consider screening the parameter to see which gives best responses. It will be a waste if the OFAT method is more effective in the study because DOE use more software and a lot of statistical which might give an error. That is why OFAT and DOEs should be run along and compares to see which method give the best toward screening. There is other method that has been tried by other researcher that does not involve DOE method to screen or optimize responses. Although it does not involve Design Expert software but still uses a lot of statistical data.

Kallel et al., (2003) used Taguchi's methods as a basis to optimize hybridoma cell line growth and antibody production. 23G11 murine hybridoma cells is culture in spinal flask and L8 orthogonal experimental design was use to study different culture component, stirring speed, nature of serum and nature of media. The experiments were conducted using two levels for each factor studied and a direct ELISA test was used to estimate the level of antibody production. The specific growth rate,  $\mu$  (h) was estimated by the following equation:

$$\mu = (\text{Ln } X_2 - \text{Ln } X_1) / (t_2 - t_1)$$

where X represents the viable cell density per ml, t represent the time points of sampling expressed in h; the subscripts 1 and 2 stand for two succeeding sampling points.

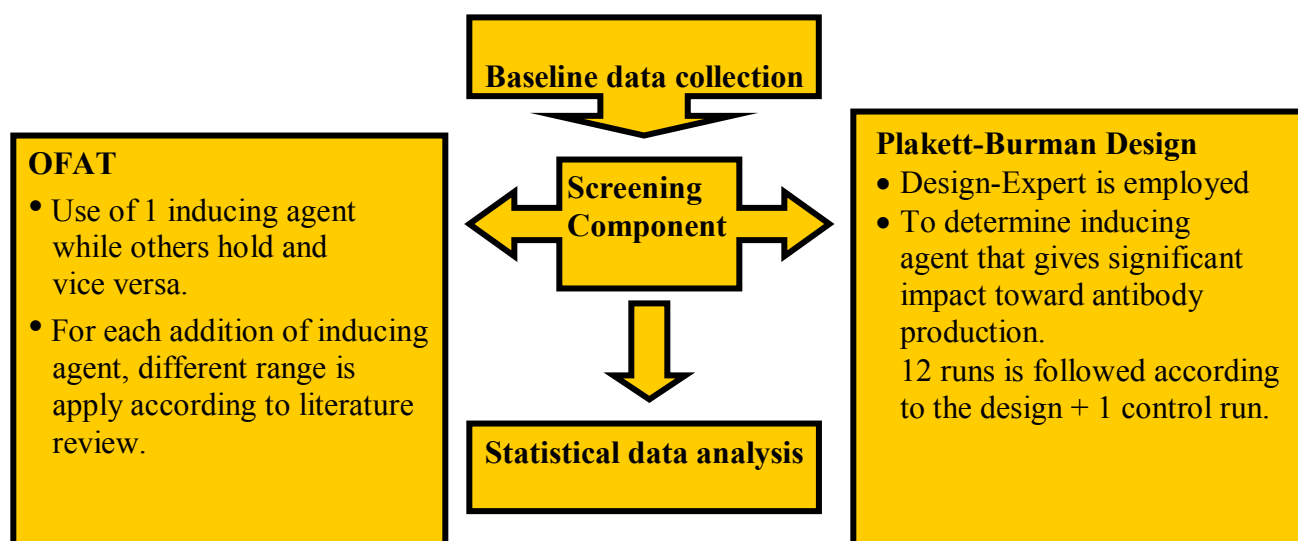
This study emphasizes the value of using Taguchi's methods as a basis for optimization of mAb production from a hybridoma culture, in cost effective and significantly less labor intensive ways. Daigo et al., (2005) on the other hand suggested a

simple hybridoma screening method for high-affinity monoclonal antibodies using the signal ratio obtain from time-resolved fluorescence assay. However both of the method is impractical to be use since uses more time, equipment and involve lot of equation and data analysis that can lead to high error compare to simple OFAT and Plakett Burman under DOE method. Both of the method is only ideal to be use if complex analyze and further study is needed to support result from simple screening and optimizing method.

## CHAPTER 3

### METHODOLOGY

#### 3.1 Flow Diagram



### **3.2 Material**

Mouse hybridoma cell H192 was obtained from Universiti Malaya. The varied inducing agent such as LPS, sodium butyrate, lysozyme, potassium phosphate, aldolase, essential amino acid and non-essential amino acid were purchased from Sigma. *Dulbecco's Modified Eagle's Medium* (DMEM) is also purchased from Sigma for media preparation. Other chemicals were available in UMP chemical laboratory.

### **3.3 Baseline data collection**

Mouse-mouse hybridoma cell are seeded at  $2.5 \times 10^5$  cells/ml volume in complete medium and cultured in T-75 flask 250 ml and incubate at  $32^\circ\text{C}$  under humidified 5%  $\text{CO}_2$  -95% humidified air. The cell was subcultured for several times to ensure its continuous growth rate. As the cell reaches its maximum growth rate which at  $1.35 \times 10^6$  cells/ml the cell is spun at 5000 rpm for 5 minutes and the supernatant was kept for ELISA analysis. ELISA analysis was done to determine baseline MAb titer for control medium.

### **3.4. Screening using OFAT**

The cell was seeded at  $2.5 \times 10^5$  cells/ml for each 7 ml new tube. Six-well plate is use instead of T-75 flask for cultivation of the cell. Each run was triplicate making one run require 7 ml of medium seeded with cell. Inducing agent was added at various concentrations shown in Table 3.1. The control medium without addition of inducing agent is run concurrently. This is important for identifying whether medium with addition of inducing agent produce high antibody concentration by comparing it with control MAb titer. The culture medium is cultivated for 72 hour at  $37^\circ\text{C}$  under humidified 5%  $\text{CO}_2$ -95% air.



**Table 3.1:** Range of inducing agent use in OFAT screening method.

Inducing agent	Control	Concentration range
Potassium phosphate	0µg/ml	1020 - 4080µg/ml
Lysozyme		250 - 500µg/ml
LPS		25 - 50µg/ml
Aldolase		200 - 400µg/ml
Essential amino acid		0.5 - 3.0mM
Sodium butyrate		550 - 8000µg/ml
Non-essential amino acid		0.5 - 3.0mM

### 3.5 Screening using Plakett-Burman design

The purpose of the first step of screening was to identify the significance of the inducing agent for the production of antibody. The Table 3.2 below show the design with an output of 12 experimental runs to seven inducing agent used. It was based on the first order model which is given by:

$$\text{IgG conc.} = 0.174207.77700 \times 10^{-6}(A) - 3.56373 \times 10^{-4}(B) - 1.44749 \times 10^{-4}(C) - 0.077243(D) + 2.47932 \times 10^{-3}(E) + 2.57416 \times 10^{-5}(F) + 5.74667 \times 10^{-3}(G)$$

**Table 3.2:** Plakett-Burman design for the screening media

Run	A, µg/ml	B, µg/ml	C, µg/ml	D, mM	E, µg/ml	F, µg/ml	G, µg/ml	IgG conc., µg/ml
1	550	400	250	0.5	25	4080	1	0.108121
2	550	200	500	1	50	1020	1	0.082701
3	550	200	250	0.5	25	1020	0.5	0.079066
4	550	200	250	1	50	4080	0.5	0.205622
5	8000	200	250	0.5	50	4080	1	0.41528
6	8000	400	500	0.5	50	4080	0.5	0.119427
7	8000	400	250	1	50	1020	1	0.097064
8	8000	400	250	1	25	1020	0.5	0.11089
9	550	400	500	0.5	50	1020	0.5	0.173336
10	550	400	500	1	25	4080	1	0.084819
11	8000	200	500	0.5	25	1020	1	0.128116
12	8000	200	500	1	25	4080	0.5	0.21052