

Optimization of Culture Medium for a Problematic Hybridoma Cell Line (M2 anti-FLAG) using a Statistical Approach involving Factorial Matrix Designs

Steven C. Peppers, Daniel W. Allison, Damon L. Talley, Heather N. Loke, Terrell K. Johnson and Matthew V. Caple

Sigma-Aldrich Corporation, P.O. Box 14508, St. Louis, MO 63178

The antibody produced by the M2 hybridoma line is used for detection and purification of FLAG fusion proteins. The M2 hybridoma line has been particularly difficult to grow; the original culture medium containing 10% fetal bovine serum (FBS) enabled these cells to attain only 60% -70% viability and a density of 5×10^5 cells/ml, with frequent splitting required. To scale up production of anti-FLAG, a better medium for M2 cells is required. Here we show our matrix-based approach used to optimize a new serum-free hybridoma medium for M2 cells.

Results and Discussion

Recently we developed a serum-free hybridoma medium optimized for both cell growth and productivity of a variety of cell lines (Peppers, et al., 2001). We used the final concentration of secreted antibody (IgG) to measure productivity. For the best single measurement of cell growth, we used the integrated area below the curve of viable cell density vs. days of incubation, which we term "cell-days." The relationship between viable cells and cell-days is depicted in Figure 1.

Relationship Between Viable Cells/Day and Cumulative Cell-Days

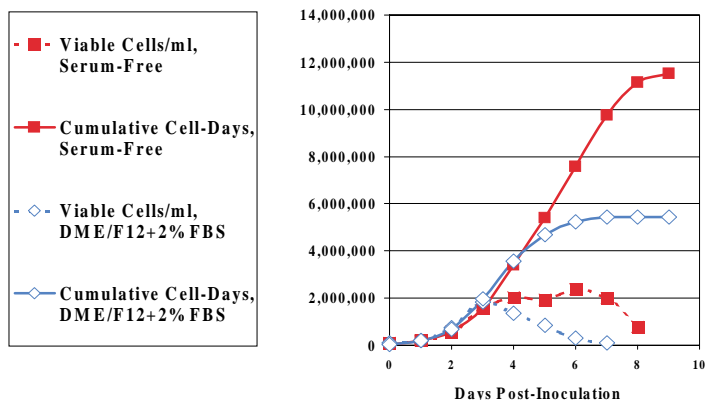


Figure 1. Comparison of the daily plots of viable cells/ml and cell-days with HFN cells in two media. Cells grown in our serum-free medium reach maximum cell densities similar to those grown in DME/F12 medium supplemented with 2% Fetal Bovine Serum (FBS). However, because cell viability was prolonged in our serum-free medium, its final value of cell-days was approximately double that of the serum-supplemented medium. Final cell-days is the primary measurement of cell growth performance reported here.

We have developed two hybridoma media that are optimized for most hybridoma cell lines. Our serum-free hybridoma medium (Sigma product number H4281) contains a small amount of bovine serum albumin (BSA), bovine insulin and human transferrin. Our animal-component-free hybridoma medium (Sigma product number H4409) contains no components derived from animal sources, only a small amount of human recombinant insulin, and no other proteins.

When M2 cells were introduced to serum-free hybridoma medium (H4281), we found that the cells required 2% FBS or cholesterol supplementation. We also found that M2 cells in H4281 containing cholesterol improved further when also supplied with additional insulin, BSA, transferrin, and fatty acids. To identify which of these components significantly improved M2 cell performance and to test for possible interactions, we performed a partial factorial matrix assay.

Design of a 2^{5-1} Partial Factorial Matrix

To determine the factors most important to M2 cells

		EtOH Balanced				
file: M2-1.doe		5 mg/L 10 mg/L Cholest.	.16 ml/L .32 ml/L Fatty A.s	4 mg/L 20 mg/L Insulin	100 mg/L 900 mg/L BSA	3 mg/L 10 mg/L Transf.
Spinner #	Test	A	B	C	D	E
1,2	T	-	-	-	-	+
3,4	C	+	-	-	-	-
5,6	F	-	+	-	-	-
7,8	CFT	+	+	-	-	+
9,10	I	-	-	+	-	-
11,12	CIT	+	-	+	-	+
13,14	FIT	-	+	+	-	+
15,16	CFI	+	+	+	-	-
17,18	B	-	-	-	+	-
19,20	CBT	+	-	-	+	+
21,22	FBT	-	+	-	+	+
23,24	CFB	+	+	-	+	-
25,26	IBT	-	-	+	+	+
27,28	CIB	+	-	+	+	-
29,30	FIB	-	+	+	+	-
31,32	CFIBT	+	+	+	+	+
33,34	Center Point A	cp	cp	cp	cp	cp
35,36	Center Point B	cp	cp	cp	cp	cp

Table 1. Experimental design of a 2^{5-1} partial factorial assay to explore interactions and effects of cholesterol, fatty acids, insulin, BSA, and transferrin. Lower concentrations of the factors are shown in the table, indicated by "-," while higher concentrations are indicated by "+." Center points ("cp") are at the midpoint of each of the factors. In this assay duplicate spinner flasks were prepared for each test condition.

Individual Unsorted Conditions in Factorial Assay, M2 Cells

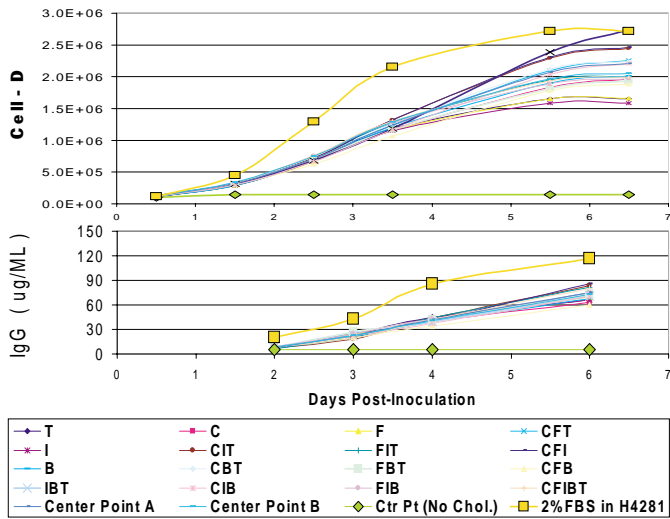


Figure 2. Unsorted data from the factorial assay. Two control conditions (no cholesterol and 2% FBS) show that M2 cells are absolutely dependent on cholesterol and that they grow most rapidly with serum supplementation. The other conditions require sorting and analysis before conclusions may be drawn.

Summary ANOVA Tables						
Cell-Days			IgG Production			
Source	F-Value	Prob > F	Source	F-Value	Prob > F	
Model	18.30	< 0.0001	Model	8.16	< 0.0001	
Cholesterol	A	25.76	< 0.0001	A	3.43	0.0781
F.Acids	B	3.20	0.0864	B	11.30	0.0030
Insulin	C	22.71	< 0.0001	C	35.56	< 0.0001
BSA	D	6.22	0.0200	D	4.19	0.0534
Transferrin	E	16.18	0.0005	E	14.79	0.0009
Interactions Included in Model			Interactions Included in Model			
AD	46.03	< 0.0001	AB	1.99	0.1728	
AE	3.15	0.0884	AC	8.12	0.0096	
BD	32.91	< 0.0001	AD	4.82	0.0394	
BE	8.56	0.0074	AE	2.33	0.1419	
			BC	4.48	0.0464	
			BD	4.48	0.0464	
			CD	2.38	0.1376	
R-Squared	0.8728		R-Squared	0.8234		

Table 2. Analysis of Variance (ANOVA) tables for growth and productivity from the factorial assay. Using the Design-Expert® software package, we evaluated the data first by creating ANOVA tables to look for significant effects ($p \leq 0.02$), printed here in **bold red**. With regard to cell-days, the effects of boosting the medium levels with cholesterol (A), insulin (C), BSA (D) and transferrin (E) were each significant, as were the interactions of A with D (designated as AD), BD, and DE. With regard to IgG, there were significant effects with fatty acids (B), insulin (C) and transferrin (E), as well as the interaction AC, and there were borderline significance ($p \leq 0.05$) with several other interactions (printed in **bold black**). The negative interactions between BSA and fatty acids warranted a close examination before further analysis.

Final Cell-Days

Final IgG Concentration

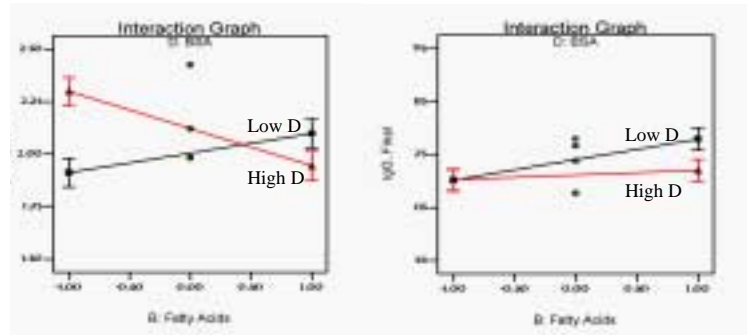


Figure 3. Negative interaction between BSA and fatty acids. The substantial boost in growth resulting from high BSA was reversed by high fatty acids, while any boost in productivity resulting from high fatty acids was reversed by high BSA. To determine the best conditions for both growth and productivity with M2 cells, we performed a numerical optimization procedure to chart surface contours from the mathematical model.

Solving Model for Optimized Conditions

Factor	Constraint
Cholesterol	Held constant at Midpoint
Fatty Acids	Allowed to roam within full range (-1 to +1)
Insulin	Allowed to roam within full range (-1 to +1)
BSA	Allowed to roam within full range (-1 to +1)
Transferrin	Allowed to roam within full range (-1 to +1)

Measurement	Goal	Importance Level
Cell-days, Final	Maximize	5
IgG Conc, Final	Maximize	5
Cells/ml day4	Maximize	2

Table 3. Numerical optimization schedule. Model constraints for all factors except cholesterol were free to roam between “-1” and “+1.” Cholesterol was set to the midpoint in this model because of concerns about solubility limitations in future development. The goals were chosen to maximize both growth and productivity (each set to an importance level of “5”). Maximizing cell/ml at day 4 was also selected (importance level of “2”) as a tie-breaker to favor conditions that would grow cells more quickly. A formula described by Myers and Montgomery (1995) was used to calculate the “desirability function,” a value between zero and one. At any given combination of factors within the selected constraints, the generated desirability function would indicate the relative suitability of the medium for meeting the selected goals (in this case, maximizing productivity and cell growth). Desirability function results were then graphically viewed as a desirability response surface

plot, a two-dimensional representation with each curve depicting a constant level of desirability, as printed on that curve.

Interaction of Fatty Acid Supplement with BSA

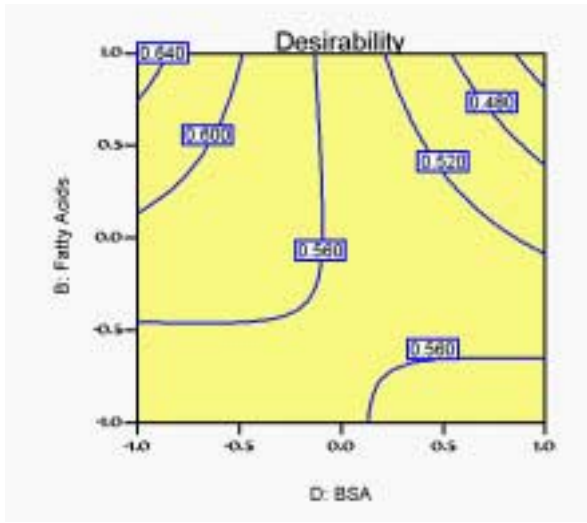


Figure 4. Desirability response surface for the interaction of BSA and fatty acids. For this plot, all other factors were set to their midpoint values. The saddle-shaped contour showed that maximizing fatty acids and minimizing BSA gives the highest desirability function for M2 cells. Therefore, plots of the other three factors were generated with BSA set to its lowest concentration and cholesterol at its midpoint.

Another factorial assay was performed with higher levels of insulin, fatty acids and transferrin. Results (not shown) indicated that further increases in these factors have little effect on the desirability. We chose the optimal mix for M2 cells to be H4281 medium at 20 mg/L insulin, 10 mg/L transferrin, 0.3 ml/L fatty acid mix, and 0.1 mg/L BSA.

We also found that diluting the supplemented H4281 medium by 25% in Hanks buffered saline solution (HBSS) resulted in a larger than expected decrease in medium performance with M2 cells. This suggested that one or more H4281 components were still suboptimal for this cell line. Twenty one of the H4281 components were selected for screening, based on their likelihood of influencing cell performance. These were “bundled” into six groups, which were then tested in a 26^{-1} factorial matrix assay. Concentrations of all components were first reduced to 75% of H4281 levels by dilution with HBSS. Grouped components were then spiked back into the medium to reach 125% of H4281 original levels. ANOVA performed on growth and productivity data (not shown) revealed a significant interaction between groups B and F.

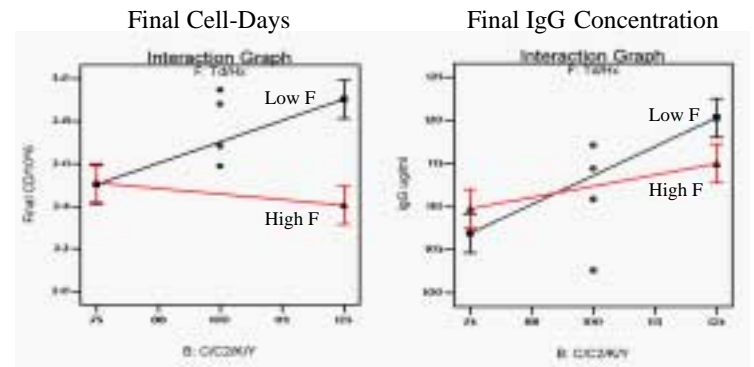


Figure 6. Interaction between group B (Cysteine, Cystine, Lysine and Tyrosine) and group F (Thymidine and Hypoxanthine) with M2 cells. Group B alone strongly enhanced cell growth and productivity. Group F alone had little or no effect on growth or productivity, but it prevented much, if not all, of the effect of group B. Clearly there are at least two factors within the groups that need to be identified and optimized.

Another 26^{-1} factorial matrix assay was performed with the six components present in groups B and F. This time, the H4281 original levels were the lower concentrations of all components. The components of group B were tested at 150% of H4281 levels and those of group F were tested at 125% of H4281. ANOVA performed on the growth and productivity data (not shown) revealed a significant interaction between cysteine and hypoxanthine and a significant single effect of cystine (cys2).

Desirability Response Surfaces and Cube Graph

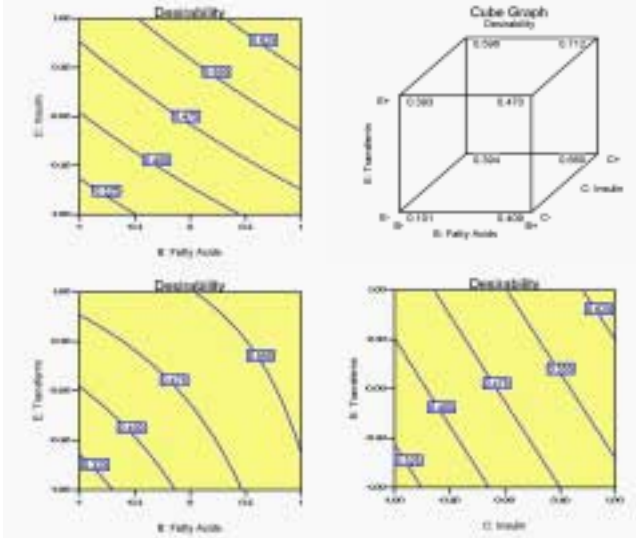


Figure 5. Response surfaces and cube graph showing desirability functions for combinations of fatty acids, insulin and transferrin. Each of these two-factor response surfaces were generated with the third factor set to its midpoint concentration. Therefore, each response surface depicts desirability functions along a plane that intersects the center of the cube. Desirability functions at the vertices of the three-factor cube are also shown. The highest desirability function was computed to lie at the vertex representing maximum insulin, fatty acids and transferrin.

Conclusion

We have successfully met the challenge of removing all FBS from the medium for M2 cells. We are in the process of testing our final medium using stirred tank perfusion bioreactors for scaling up the production of the anti-FLAG product of the M2 cells. Elimination of serum will result in greater purification efficiency and lower production costs.

Using a factorial matrix-based process of finding an optimal set of complex variables is an efficient approach to a complex problem. The traditional linear process of optimized medium is to titrate each component individually, adjusting the optimal mix as progress determines, and then to repeat this titration cycle again and again until the medium is considered suitably optimized (McKeehan, et al., 1981). While such an approach may ultimately result in adequate optimization, it is slow, fails to identify important interactions, and has no clear endpoint. A factorial matrix-based approach is efficient, identifies interactions early in the process, and has a well-defined endpoint. The reduced amount of time spent in R&D equates to significant cost savings.

While developing H4281 and H4409, we have used several different cell lines to ensure that the medium is optimized for most hybridoma cells. However, the unique genetic combination of each clone suggests that no single medium will be optimal for all hybridoma cell lines. A factorial matrix approach could be used to customize medium for any particular cell line, thereby improving product yield and efficiency.

Single Factor Plots (Cys2, Cys, HypoX) and Interaction Plot (Cys, HypoX)

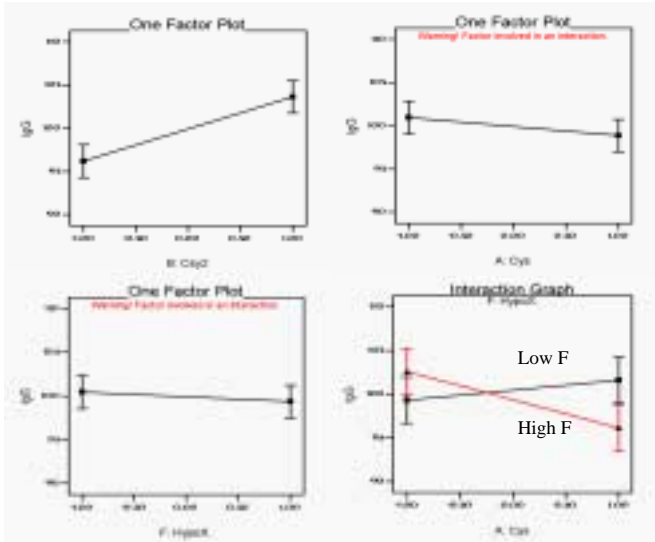


Figure 7. Effects and interactions among Cysteine (cys), Cystine (Cys2) and Hypoxanthine (HypoX) with M2 cells. Cystine by itself strongly promoted M2 cell productivity and showed no significant interactions with other factors in this assay. Neither cysteine nor hypoxanthine showed significant single factor effects, but they displayed a pattern of negative interaction similar to what was seen between groups B and F from the previous experiment. It was concluded that cystine was likely responsible for the positive gain caused by group B and that the interaction of cysteine and hypoxanthine was likely responsible for the negative interaction between groups B and F.

Improved Performance of M2 Hybridoma Cells

Culture Media in Spinner Flasks

Growth Attribute	DMEM	H4281	H4281
	+10%FBS	+2%FBS	M2-Optimized
Lag from frozen state	3-4 wks	1-2 days	n.d.
Viability During Log Growth	60-70%	80-90%	75-85%
Growth rate (pop.doublings)	0.8 pd/day	1.6 pd/day	1.5 pd/day
Maximum Viable Density	5×10^5 /ml	1.2×10^6 /ml	1.0×10^6 /ml
Final Cell-Days in Spinner Flask	0.9×10^6	3.1×10^6	4.0×10^6
Final IgG Conc. in Spinner Flask	40 ug/ml	130 ug/ml	160 ug/ml

Table 4. Progress chart in optimizing H4281 for M2 cells. Switching from DMEM with 10% FBS to H4281 with 2% FBS resulted in substantial improvement in performance with M2 cells. Current data show that our component-adjusted H4281 without serum now outperforms the serum-supplemented H4281 in cell-days and productivity.

Materials and Methods

Materials. All materials were from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Spinner Assays. Spinner flasks (125-ml capacity) from Bellco Glass (Vineland, NJ) or Techne Inc. (Princeton, NJ) were used. Only one manufacturer of spinner was used in any given experiment. Cells were seeded into the spinner vessels at either 5×10^4 or 1×10^5 cells/ml on day zero. Total cells/ml in each spinner was determined daily using a CASY®-1 cell counter (Scharfe Systems, Reutlingen, Germany). Percent viability was determined using trypan blue and a hemacytometer. The concentration of viable cells/ml was then calculated as percent viability times total cells/ml. Cell-days, the integral area under a cell growth curve, was approximated using a trapezoidal point-to-point summation method.

IgG Concentrations. The concentration of immunoglobulin G secreted into the medium was determined by HPLC using a Protein-A binding column for capture and 280nm absorbance for quantitation.

Analysis Software. Matrix analysis and graphical representation of results were generated with Design-Expert®, version 6.0.2 (Stat-Ease, Inc., Minneapolis, MN, USA).

Acknowledgements

We wish to thank Thomas W. Nolan for his assistance in our experimental designs.

References

Peppers, Steven C., Daniel W. Allison, Terrell K. Johnson, Damon L. Talley, and Matthew V. Caple. "Replacing the Animal Component in Serum: Evaluating Raw Materials for Inclusion in Optimized Hybridoma Media." *BioPharm* 14.5 (May 2001): 22-30.

McKeehan, Wallace L., Kerstin A. McKeehan, and Richard G. Ham. "The Relationship Between Defined Low-Molecular-Weight Substances and Undefined Serum-Derived Factors in the Multiplication of Untransformed Fibroblasts," in *The Growth Requirements of Vertebrate Cells in vitro*, Chapter 15, (C. Waymouth, R. Ham and P. Chapple, eds.), Cambridge University Press (New York), 1981, pp. 223-243.

Myers, R.H., and Douglas C. Montgomery. *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*. Wiley, New York, (p. 244) 1995.

