

Performance-Optimized Hybridoma Medium: Replacing Serum and Other Animal-Derived Components*

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Introduction

The next decade is expected to see a large increase in the use of antibodies as *in vivo* therapeutic agents. Currently there are at least 200 antibodies or antibody fragments undergoing clinical trials.¹ As these biopharmaceuticals are being developed, there is increasing attention focused on the need to produce them using cell culture media that contain no serum or any other components of animal origin.²

Reasons for removing serum and other animal-derived raw materials from media are varied. Usually the focus is on a combination of factors, including component variabilities inherent in biological sources, possible contaminants, the high cost of serum, and down-stream processing issues. Due to the biological nature of serum, both quality and performance of the final products are subject to substantial variation. Serum and animal-derived materials also have the potential for introducing contaminants and toxic elements such as viral particles and endotoxin.³ As a raw material, serum adds a substantial cost to the manufacturing of biopharmaceuticals. In addition, serum often interferes with purification of the final product, which increases the costs of down-stream processing. Furthermore, regulatory issues associated with the use of biological materials in manufacturing processes within the biopharmaceutical industry are problematic; meeting these requirements also raises the final production costs.

We used a three-stage approach in evaluating raw materials and optimizing the formulation of our Serum-free Hybridoma Medium (Product Code: H 4281) and Animal Component-free Hybridoma Medium (Product Code: H 4409). In the first stage, we tested numerous agents for general growth characteristics and gross toxicity limits in static culture using 24-well and 96-well plates. In the second stage, we optimized the complex mix of components for cell growth and antibody productivity using spinner flasks. We tested simple-paired comparisons, concentration profiles, and factorial matrices, using non-subjective numerical methods and graphical techniques to analyze the data. In the third stage, we verified or further optimized these media while comparing them to competitor products and using various cell lines and systems.

Materials and Methods

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

Cell Lines

HFN 7.1 hybridoma cells, from the fusion of a mouse B cell and a P3x63Ag8 mouse myeloma cell, were obtained from the American Type Culture Collection as ATCC num-

ber CRL-1606 (Mannassas, VA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) until frozen at 2×10^6 to 1×10^7 cells in DMSO-containing freezing medium (Product Code: C 6164). During media development, HFN cell stocks were routinely thawed into DMEM/F12 medium (Product Code: D 0547) containing 2% FBS. Next, they were subcultured within 3-6 days into our most current version of Serum-free Hybridoma Medium (Product Code: H 4281) or Animal Component-free Hybridoma Medium (Product Code: H 4409) in spinner flasks. Cells were not allowed to exceed 25 passages and cell densities were consistently maintained at less than 1×10^6 viable cells/ml. The cell density of the stock at the start of an assay was not allowed to exceed 8×10^5 viable cells/ml. Three hybridoma cell lines were obtained from Abbott Laboratories (Abbott Park, IL). Designated as "H" (clone #4-481sc184), "M" (clone #24-168-353) and "P" (clone #H50C95C187), these cells were maintained in a manner similar to the HFN cells.

Weaning of HFN Cells into Other Media

For this set of assays, frozen HFN cells were thawed into DMEM/F12 with 10% FBS. After 3 days the cells were subcultured in T-flasks containing DMEM/F12 with 2% FBS. Over the next ten days the cells were weaned from serum and into the various media by subculturing into each medium using incremental passages. The first weaning step was into 50% DMEM/F12 with 50% other media and 1% FBS. The next passage was into 25% DMEM/F12 with 75% other media and 0.5% FBS. The final passage was into 100% other media (no FBS). Usually at the last weaning passage, cells were also seeded into spinner flasks to begin growing stocks for the assays.

Plate Assays

Sterile polycarbonate 96-well flat-bottom culture plates (Product Code: M 9780) were used in the fluorescence assay. To minimize edge-related anomalies, only the inner 60 wells of each plate were inoculated with 200 μ l of medium containing 5000 cells/well; the remaining perimeter wells contained only 200 μ l of medium/well. The plates were placed in a humidity-controlled incubator at 37 °C and 5% CO₂. After the specified number of days, one-twentieth volume of resazurin dye (Product Code: TOX-8) was added to each well and the plates were incubated as previously described for an additional 30 minutes. Fluorescence was measured using a HTS 7000 Plus™ BioAssay Reader (Perkin Elmer, Norwalk, CT) set at 550 nm excitation and 595 nm emission. Readings from blank wells (containing resazurin but without cells) were averaged and subtracted from each test well to give net relative fluorescence units (RFU).

Spinner Assays

Spinner flasks of 125-ml or 250-ml capacity from Bellco Glass (Vineland, NJ) or Techne Inc. (Princeton, NJ) were used. Only one manufacturer, size, and style of spinner was used in any given experiment. Cells were seeded into spinner vessels at either 5×10^4 or 1×10^5 cells/ml on day zero. Total cells/ml in each spinner were 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. Unless otherwise stated, all data points are averages of two spinners. The average percent difference between each spinner in a replicate pair was approximately 12% for cell growth data and 9% for antibody productivity.

IgG Concentrations

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

Contour Plots

Mesh plots of data from full arrays (96-well plates) were generated using SigmaPlot® version 5.0 (SPSS Science, Chicago, IL). Response surface plots of data from central composite design experiments were generated using Statistica™ Release 5.1, Experimental Design Module (StatSoft Inc., Tulsa, OK).

Results and Discussion

Using resazurin, a fluorescent indicator, we found that increasing relative fluorescence correlated well with increasing cell growth. As an example in this stage, Figure 1 shows the results obtained from the titration of two vitamins (components B and T) in an orthogonal array (two-component matrix). This method was used to screen certain agents or mixtures for interactions in toxicity or growth. In this example, components B and T each appeared to be somewhat toxic at their highest concentrations (approximately 250 times that in DMEM/F12 medium); individually B and T inhibited HFN cell growth by approximately 45% and 20%, respectively. Combined, they showed an additive toxicity of approximately 65%. In addition, a slight rise in RFU was seen as T increased from the base level, but no such effect was seen for B. Subsequent trials focused on concentrations of B and T that fell within the relatively broad plateau of high activity (data not shown).

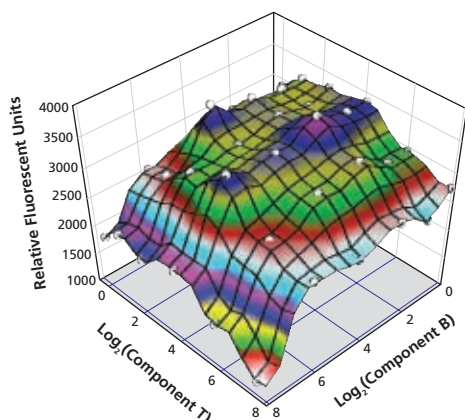


Figure 1. Two-Component Plate Matrix. Example of method used to screen two vitamins for interactions with respect to general growth and upper toxicity limits. Each data point embedded in the mesh plot represents the average fluorescence of four replicates using resazurin in 96-well plates.

The second phase of optimization involved testing media using 125-ml or 250-ml spinner vessels to identify the best combination of components for cell growth and antibody productivity. The final concentration of antibody, usually IgG, was chosen as the best single parameter for measuring hybridoma productivity. The best single parameter for measuring cell growth is less straightforward. Figure 2A illustrates how DMEM/F12 medium containing 2% FBS appears to

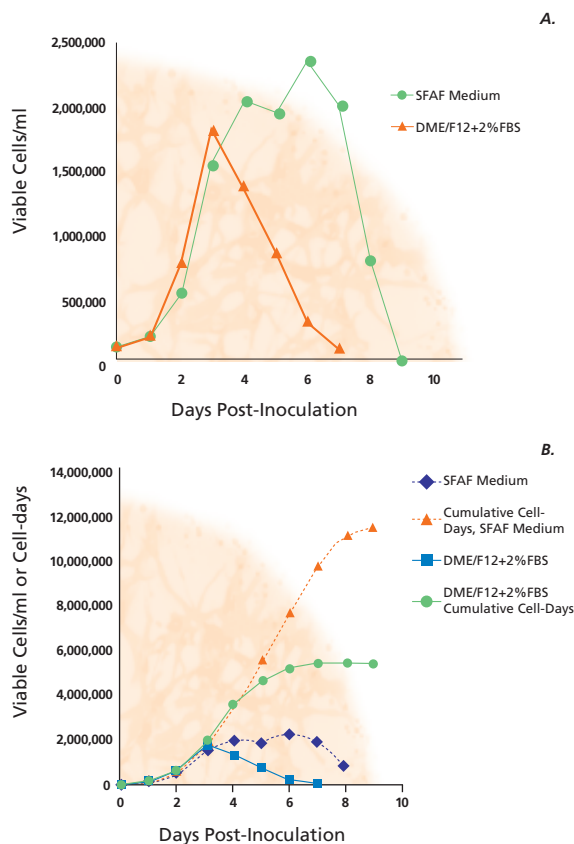


Figure 2. Derivation of “cell-days” as a primary measure of cell growth capacity during media optimization. (A) Viable cells/ml vs. time after inoculation of HFN cells into spinner flasks containing DMEM/F12 with 2% FBS or Sigma’s Animal Component-free Hybridoma Medium. (B) Relationship between viable cells/ml and cumulative “cell-days” over the time course of the experiment.

outperform the serum-free hybridoma medium in the initial days of testing when measuring only viable cells/ml. Since the serum-free medium is clearly superior in total growth support, comparing cell densities alone is not an adequate single parameter to gauge cell growth. Instead, we have estimated the integral (area under the final growth curve) and combined cell density and longevity into a single measurement we term “cell-days.” Figure 2B shows that the serum-free medium was more than twice as effective in supporting cell growth than the serum-containing medium.

Because serum is such a complex mix of undefined components, replacing it involves introducing or changing concentrations of many different interacting components. The traditional method of medium development has been to optimize each component in a linear sequence and then repeat this cycle until the medium is declared optimized. This method does not efficiently address the highly interactive nature of many components in a complex medium. While simultaneously looking at all possible combinations of all components would certainly reveal their interactions, it would be too complicated and require a prohibitive number of experimental conditions. We employed a statistical matrix to screen for interactions and main effects of several components. Full-factorial designs were used to examine these interactions in more detail.⁴

For testing the effects of three supplements (two concentrations each) on HFN cells, Figure 3A shows a “2³ plus center-point” design with a center-point at mid-concentration for each of the supplements. Numbers shown are

final IgG concentrations normalized to the basal level (Figure 3A; lower left corner with 0.1X amino acid supplement and without vitamin or trace metals supplements). The data shows that antibody productivity decreased consistently when moving vertically from the lower square to the upper square of the cube, indicating that the additional trace metals were not beneficial. We therefore chose to ignore the effect of the trace metals supplement to gain greater statistical power in our data. The vertically linked pairs of data from the cube were then averaged and re-normalized to the pooled basal level. These values were re-plotted as a square with center-point to examine only the amino acid and vitamin supplements. The squares in Figure 3B show changes in cell-days and IgG concentrations at each of these five points. Raising only the level of vitamin supplement resulted in an increase in cell-days without affecting antibody productivity. However, raising only the level of amino acid supplement resulted in an increase in antibody productivity without affecting cell-days.

Simultaneously increasing both supplements gave substantial boosts in both cell growth and antibody productivity. The center-point values also suggested a nonlinear response to changes in these supplements.

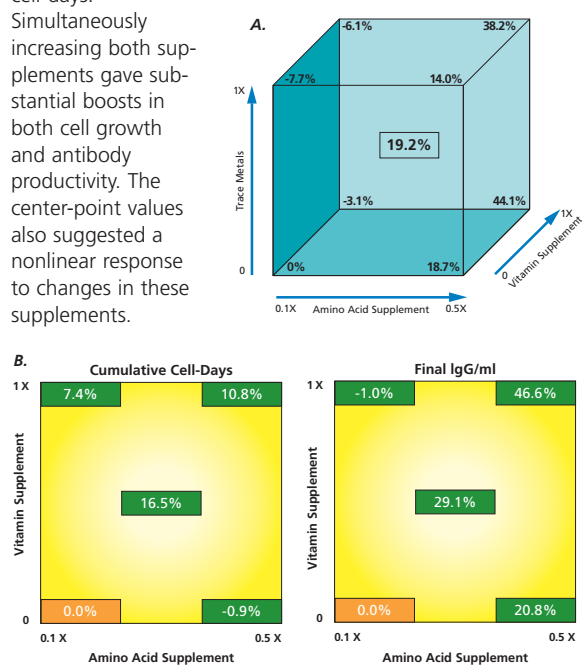


Figure 3. Three-Component Matrix for HFN cells. (A) Final IgG concentration (% change from original) when testing the effects of trace metals supplement, amino acid supplement, and vitamin supplement in spinner flasks. **(B)** Cumulative “cell-days” and final IgG concentrations after removing the effect of trace metals supplement. The percent increases in IgG within pairs of high and low trace metals were averaged and then renormalized to the origin.

To explore in more detail the relationship between the two supplements and cell growth or antibody productivity, we used a rotatable central composite design (Figure 4). This design was chosen for the generation of second-order, three-dimensional, contour plots because of its inherent minimization of statistical bias.⁵ A test condition at the basal level was also included for reference. HFN cells with four replicate spinners were included at the center-point condition, with two replicates for each of the other test conditions. The entire experiment with HFN cells was repeated, except an additional 4 spinners were included at the center-point. Contour plots were generated from the averaged data of the combined HFN experiments using Statistica™ software. For optimizing media performance and reliability, our goal was to identify the region on the contour plots that showed both high productivity and growth (maximal region) as well as low variability (most level region).

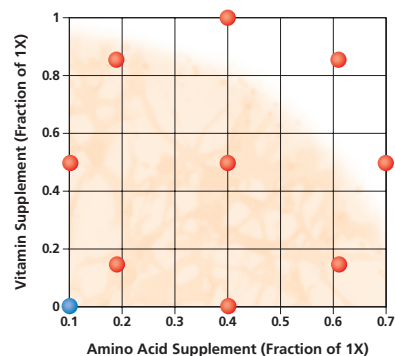


Figure 4. Two-Component Central Composite Design. Schematic showing the concentrations of the vitamin supplement and amino acid supplement used to generate the 2nd order best-fit response surfaces. The blue point represents an additional control at the original medium composition.

Figure 5A demonstrates that cumulative final cell-days for HFN cells increased sharply as both the vitamin and amino acid supplements were simultaneously raised, reaching a relatively broad maximal response near the center-point.

Figure 5B shows that the response surface for IgG production appears to be slightly saddle-shaped for HFN cells. Similar to cell-days, IgG concentrations also rose in response to increases in both the vitamin and amino acid supplements and became somewhat level toward the center-point. The highest concentrations of vitamin supplement showed even greater IgG production, perhaps due to a general stress on the cells similar to the reported effect of high osmotic concentration.⁶

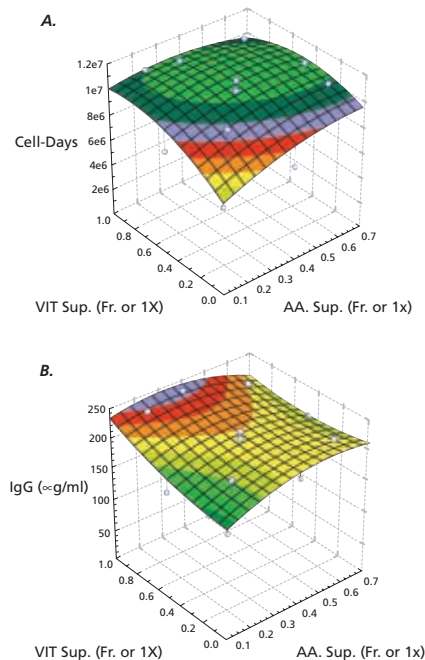


Figure 5. Contour plots for HFN cells as functions of vitamin and amino acid supplements. (A) Cumulative “cell-days.” **(B)** Final IgG concentration.

Another hybridoma cell line, “H” (clone #4-481sc184), was also examined by the same central composite design and with identical concentrations of the two supplements (Figure 6). Four replicate spinners were included at the center-point and two replicates for each of the other conditions. With respect to both cell growth and antibody productivity, cell line “H” was more responsive to increases in the vitamin supplement than the amino acid supple-

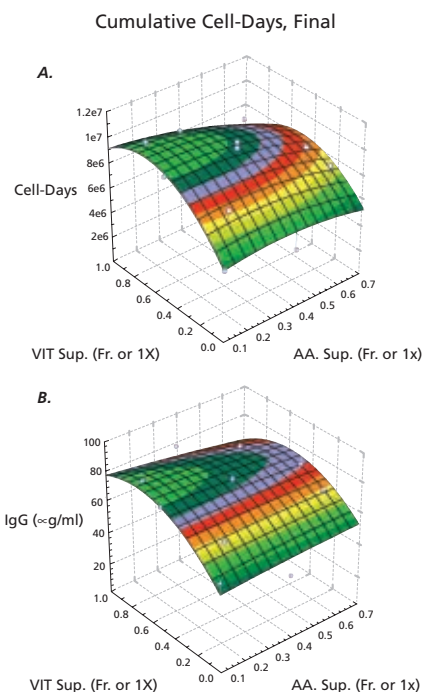


Figure 6. Contour plots for "H" cells as functions of vitamin and amino acid supplements. (A) Cumulative "cell-days." (B) Final IgG concentration.

ment. After considering the growth and productivity contours for both cell lines (HFN and "H"), we chose an optimal concentration near the center-point of this series for the formulation of our serum-free medium. Figure 7 shows that the newly optimized hybridoma medium is superior to its earlier version in growth and productivity of HFN cells throughout the assay time-course. During optimization of the animal component-free medium, transferrin was replaced by an iron-chelation system that gave equivalent performance in several tested cell lines (data not shown).

To compare the performance of our media with competitors, we obtained 16 commercially available hybridoma media that are marketed as being serum-free, protein-free, or animal component-free. During the weaning procedure, one-half of the media products failed to support the HFN cells, leaving ten cell populations weaned into eight competitor media products (Figure 8; A-H) and our two media (Product Codes: H 4281 and H 4409). A spinner assay was begun using the surviving populations and determining viable cells and IgG concentrations as described in Materials and Methods. Eight days later a second spinner assay was begun using cells maintained in their respective

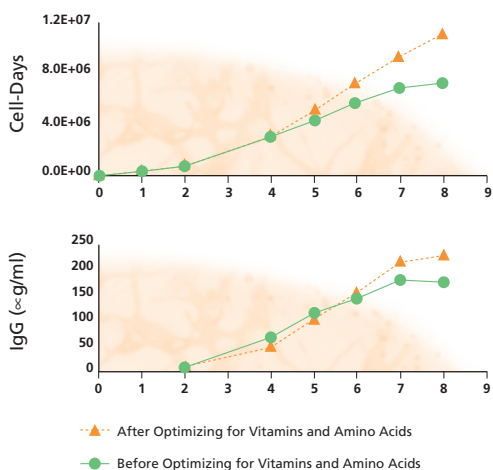


Figure 7. Optimizing Sigma media with vitamins and amino acids. Graphs show the improvement from first-generation media (before optimization) to new media (after optimization) plotted against the time course of the experiment.

media. An additional weaning and assay was later performed with the same eight competitor media. Figure 8 shows the average growth and antibody productivity resulting from the three experiments with standard error bars (n = 3 for H 4281, H 4409, A, B, C and E; n = 2 for F, G and H; and n = 1 for D). The complete weaning procedure was followed twice for each media of n ≥ 2. In these experiments our Serum-free Hybridoma Medium (Product Code: H 4281) outperformed all other "serum-free" competitors tested. Our Animal Component-free Hybridoma Medium (Product Code: H 4409) essentially matched one competitor product (E) and outperformed all other tested "protein-free" or "animal component-free" competitor products.

We also compared our media with the top three competitor products (products A, E, and F) using three hybridoma cell lines from Abbott Laboratories. Figure 9 shows that our serum-free medium (Product Code: H 4281) outperformed the leading competitors' products for all cell lines. Our animal component-free medium was similar to one competitor product (E) and outperformed the other competitor products (A and F) for these cell lines. Similar results were obtained in fixed-batch operated bioreactors with HFN cells, comparing our serum-free and animal-component free media with these top competitors (data not shown). Altogether, our two hybridoma media products have shown excellent growth and productivity performance. The unique genetic combination of each clone suggests that no single medium will be optimal for all hybridoma cell lines. During media development, we chose HFN as our model cell line because its growth characteristics were typical of many

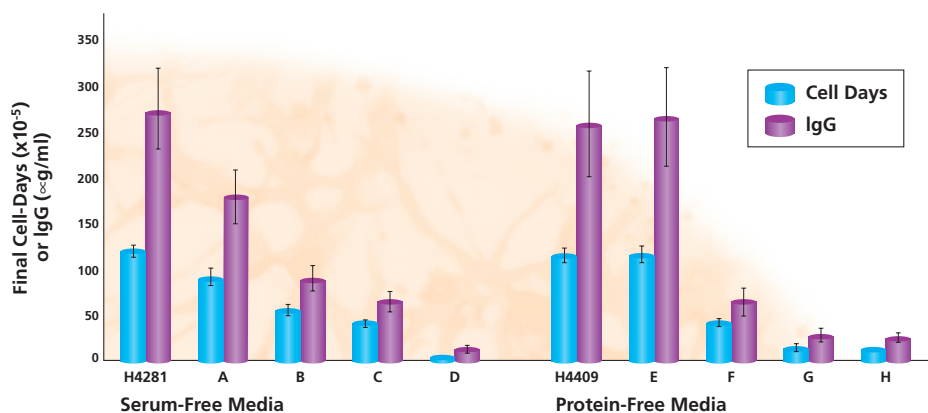


Figure 8. Sigma's hybridoma media vs. competitor hybridoma media using HFN cells. Comparison of final "cell-days" or final IgG concentration when using Sigma's optimized hybridoma media (Product Codes: H 4281 and H 4409) and competitor hybridoma media products (A-H). All products were either serum-free or protein-free. Error bars represent mean ± SEM.

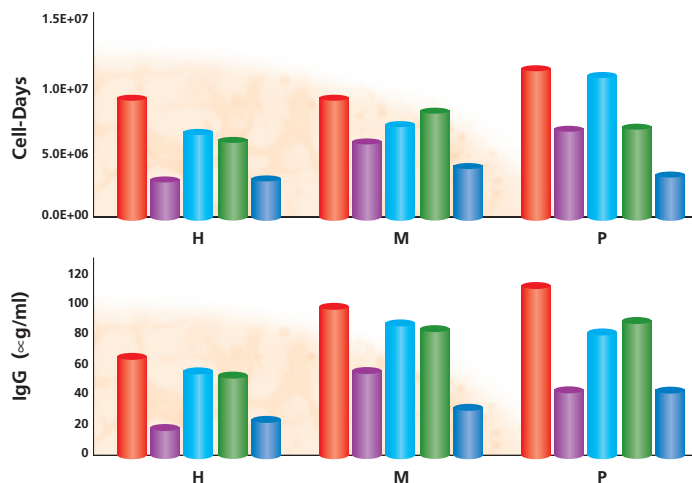
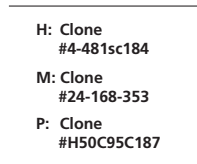
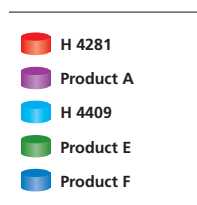


Figure 9. Sigma's hybridoma media vs. competitor hybridoma media products using different clones. Comparison of "cell-days" and IgG concentration using Sigma's optimized hybridoma media (Product Codes: H 4281 and H 4409) and competitor hybridoma media products (A, E, and F). All comparisons were performed using three different hybridoma clones, H, M, and P.

hybridoma clones used in the biopharmaceutical industry. Additional hybridoma cell lines and growing systems (e.g., fed-batch bioreactors, perfusion bioreactors, and hollow-fiber bioreactors) are currently being tested. Results to date support the conclusion that our media rank at the top of all commercially available media products in supporting cell growth and productivity of a variety of hybridoma cell lines and growing systems (unpublished data).

Conclusion

By a systematic method involving three stages of development, we have eliminated serum and other animal-derived agents from hybridoma culture medium and have replaced it with a complex mixture of components from non-animal sources. This medium formulation has been optimized for cell growth and productivity using multiple hybridoma cell lines and has been tested against many competitor products. Both final products, a serum-free low-protein hybridoma medium (Product Code: H 4281) and an animal component-free hybridoma medium (Product Code: H 4409), show excellent cell growth and antibody production characteristics and rank at the top of commercially available hybridoma media.

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ORDERING INFORMATION

Product Code	Product Name	Unit	Price
H 4409	Hybridoma Medium, Animal Component-free	1 liter	\$43.60
H 4281	Hybridoma Medium, Serum-free	1 liter	\$37.60

For more information, check Hybridoma Medium on the enclosed business reply card.

Life Science Catalog 2000-2001, page 345.

Acknowledgements

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References and Note

- Morrow, K.J., Jr., Monoclonal antibody production techniques. *Gen. Eng. News*, **20(14)**, 21 (2000).
- Wrotnowski, C., Cell culture media trends mirror bioindustry. *Gen. Eng. News*, **20(8)**, 8 (2000).
- Merten, O-W., Safety issues of animal products used in serum-free media, in *Animal Sera, Animal Sera Derivatives and Substitutes Used in the Manufacture of Pharmaceuticals: Viral Safety and Regulatory Aspects*, Dev. Biol. Stand. Basel, Karger, **99**, 167-180 (1999).
- Moen, R., et al., *Quality Improvement through Planned Experimentation*, 2nd Ed. (McGraw Hill, New York, 1999).
- Montgomery, D., *Design and Analysis of Experiments*, 4th Ed., (John Wiley & Sons, New York, 1997).
- Cherlet, M., and Marc, A., Hybridoma cell behavior in continuous culture under hyperosmotic stress. *Cytotechnology*, **29(1)**, 71-84 (1999).

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