Optimization of Cell Culture Media

by Matthew Jerums and Xiaoming Yang

Animal cell-culture media are usually made up of mixed, defined chemicals such as carbohydrates, amino acids, vitamins, minerals, lipids, buffers and proteins such as growth factors. They often contain in addition some undefined or semidefined components known as hydrolysates. The complexity of such composition provides many opportunities to optimize individual components or entire classes of ingredients. Classical culture media developed by Eagle, Ham, and others 40 to 50 years ago (see timeline, Chapter Two) were designed primarily for small-scale and low-density cultures and often required serum as a key nutrient addition. The emergence and growth of the biotechnology industry fueled efforts to improve cell culture media for maximizing product yields and lowering cost of goods. Typically now, media for the biotechnology industry are serum-free and have much higher nutrient concentrations than classical media had because of the need to sustain high cell densities and increase cellular productivity. Medium optimization requires consideration of the product, cell line, and manufacturing process involved.

IRVINE SCIENTIFIC (WWW.IRVINESCI.COM)

CONSIDERATIONS

The products to be made — whether recombinant proteins, DNA plasmids, viruses, metabolic intermediates, or simply overall cell mass — will determine the medium optimization strategies used. For the simple and rapid generation of cell mass, cell growth rate and viability are paramount in the quest to obtain the highest possible cumulative viable cell density. Cell culture media must be able to support maximal cell growth and sustain cell viability at increasing cell densities. Virus production also requires high cell densities to amass enough cells for sufficient viral replication. However, after infection there must be abundant and appropriate nutrients to sustain virus replication and the high levels of metabolic activity that often follow viral infection.

Production of a recombinant protein also benefits from high cell densities and cell viability, but productivity can have an inverse relationship with cell growth. Nutrients required for cell growth can compete with those required for production of proteins as the cells' finite resources are divided between those tasks. Careful process characterization is valuable to determining the maximum cell densities a given medium can sustain for a required level of productivity. In addition, product quality is essential, and changes to the medium during optimization must not negatively affect it.

Different cell lines often show differences in metabolism and nutritional requirements that dictate media optimization methods. The most common cell lines encountered in the biotechnology industry include Chinese hamster ovary (CHO) cells, Vero (African green monkey kidney) cells, baby hamster kidney epithelial (BHK-21) cells, myeloma cells, hybridoma cells, and normal diploid fibroblasts.

Normal diploid fibroblasts, for example, are anchorage-dependent cells that require attachment factors to adhere and spread out on a surface for growth. In contrast to transformed or tumor cell lines, normal diploid fibroblasts grow to much lower cell densities and therefore do not need nutrients in high quantities. Certain cell lines have specific nutritional requirements, such as cholesterol for NS0 myeloma cells. It must be present in sufficient amounts, or the cells must be specially adapted to cholesterol-free media. Hybridoma cells lines are often highly dependent upon glutamine. They typically lack a stationary phase after reaching a peak cell density and then decline rapidly in viability. Medium optimization can

reduce the rate of hybridoma viability decline and improve monoclonal antibody productivity.

The considered manufacturing process mode — whether batch, fed-batch, or perfusion — will affect the choice of cell culture medium as well as approaches to optimization. For batch processes, a single medium is used to sustain cell growth and productivity. It must therefore be rich in nutrients but remain within physiological limits of the cells. In fed-batch and perfusion processes, several different types of media can be used over the course of the cell culture, depending on the stage of the process. A growth medium is designed with lower nutrient concentrations for when cell densities are low (at inoculation) and optimized for high specific cell growth rates to minimize culture lag phase and maintain high rates of cell growth during culture scale-up and early production.

Frequently, a separate production medium may be used in lieu of the growth medium when the culture reaches production stage. Production media usually have an increased nutrient concentration over growth media and may also have relaxed physiologic limits (e.g., osmolality). Feed media differ again from growth and production media by having even further enriched concentrations of nutrients, typically aiming at replenishing those consumed during manufacture of the product. Feed media can be added to a culture either continuously or in bolus form.

Medium Design

Media can be categorized by the degree of complexity of their added undefined ingredients, such as animal sera, protein growth factors, and hydrolyzed animal or plant proteins. Serum-containing media (which encompasses most classical cell culture media) require nutrients and growth factors supplied by animal sera for cell growth. Medium optimization of serum-dependent media focuses on reducing percentages of serum present or substituting less expensive sera to reduce costs. Especially in production media, less expensive newborn calf or calf sera can often replace more

Rational Media Design (1)

(adapted from Fletcher T. "Designing Culture Media for Recombinant Protein Production: A Rational Approach." *BioProcess International* 3(1) January 2005: 30–36.)

Finding a good cell culture medium is important, first of all, because it affects process performance. After the cell line, there is perhaps no other factor that more greatly influences process performance. The chosen medium must also meet applicable quality standards and regulatory requirements. Consider what a critical role a culture medium plays in providing the primary source of raw materials that actually end up in the drug itself. It is believed that most amino acids making up the primary structure of a therapeutic protein produced in cell culture are derived directly from the culture medium. The importance of media development to overall process development is indicated by the fact that, once a successful process has been developed, the culture medium formula and details of how it was developed are often two of the most closely guarded secrets of the manufacturer.

The Challenge of Media Development

Cell culture media technology has advanced tremendously during the past few decades. The "Media Examples" box lists some early media, which were all developed for use with blood serum supplements that supply the highly complex mixture of once-unknown nutrients necessary for in vitro culture (1). As our understanding of cellular nutritional requirements improved, serum-free media were developed in the 1970s and 1980s to provide a more defined culture environment. The box lists a few.

When the risk of previously unknown pathogens became a concern after discovery of prions in the 1980s (2), efforts were initiated to completely eliminate all animal-derived components from the culture media used to produce human therapeutics. The latest step in this advancing technology has been to develop culture media that are not only free of animal-derived components, but are also chemically defined (3). Many first-generation animal-component–free media relied on vegetable-derived protein hydrolysates or other undefined components to achieve successful performance.

Today's challenge is to develop sophisticated media that can be individually optimized for a range of processes within a short amount of time. Difficulty comes from the diversity of cell lines and production processes in use, the large number of media components involved, and the fact that many of those components are interdependent on others because of the complexity of cellular metabolic pathways.

Media requirements for two different processes may differ greatly even when all other aspects of those processes are very similar. Even cell lines derived from a common parent often exhibit different nutritional requirements. For medium manufacturers, the diversity of requirements is increased even more by the number of different cell line varieties and processes in common use today. Thus, for example, we wouldn't expect a medium designed for the batch culture of Chinese hamster ovary (CHO) cells to be the best medium for a fed-batch culture of mouse myeloma (NS0) cells. The nutritional requirements of those different cell lines are dissimilar, as are the requirements of different processes (batch and fed-batch).

The complex problem of determining the best blend of media components for a given cell line used in a particular process deserves a carefully devised strategy. This becomes immediately apparent to anyone familiar with the complexity of cellular metabolism and the large number of components in media. And not only will a typical cell line require 50–75 different ingredients to perform best in culture, but the optimum concentrations of many components are interrelated. By varying the concentration of one component, you often change the requirement for another.

(continued)

RATIONAL DESIGN *CONTINUED*

The next greatest factor affecting most culture media development projects is time. Experienced process engineers often practice the art of finding the best balance of tradeoffs to meet a particular deadline. Choosing an effective media development strategy often comes down to knowing how to design the right experiments that will quickly deliver the best information. That's where rational culture media design comes in.

What It Isn't . . .

Rational culture media design can best be understood if it is first explained in terms of what it is not. Using a rational design approach means being careful not to overcommit to any single method of media development. Each method has its merits, but each suffers from particular weaknesses as well. None alone can meet the challenge as defined above.

Component titration is the classic approach to media development. It involves performing a series of experiments to determine the "dose response" of a cell line to various media components by adding each one in varying amounts to individual cultures.

Media blending rapidly generates many new media by simply blending existing formulations. By evaluating these combinations and then iteratively performing additional blending and testing of those that perform well, process developers can rapidly zero in on the best blend.

Spent media analysis can provide important information using chemical analysis to measure how a medium changes during the culture process. By comparing spent medium with a fresh sample, process developers can make calculations describing both nutrient depletion and metabolite accumulation.

(continued)

expensive sera (such as fetal bovine sera) when cell growth rates are less important than they are with growth media.

For serum free media, replacement of expensive protein growth factors and carrier molecules (e.g., transferrin or albumin) are key optimization targets for ultimately designing a medium that is better defined and, increasingly, protein free. When sera are omitted, a true animal-free composition can be accomplished by substituting plant- and recombinant-derived components for animal-derived ones. Media free of animal components are increasingly sought as concern grows over potential contamination with transmissible spongiform encephalopathies (TSEs) and adventitious viruses. As a medium becomes more chemically defined, the emphasis shifts to a balance of the small-molecule nutrients supplied. Undefined protein hydrolysates can be replaced to some extent with higher concentrations of individual amino acids or defined peptides. Ideally, the evolution of medium design during optimization should be a balanced approach toward higher cell densities, viabilities, and productivities.

OPTIMIZATION STRATEGIES

A useful start to medium optimization is to focus on fundamental classes of components that comprise mammalian cell culture media. An initial basal medium contains carbohydrates, amino acids, vitamins, minerals, lipids, and growth factors that can be evaluated one at a time or in concert. Screening experimental designs with these components at low and high concentration ranges are useful for determining which components have significant effects on cell growth, viability, and productivity. By narrowing the large number of components in media to those that exert significant effects, culturists can conduct more in-depth experiments and realize considerable savings in time. (The "Rational Culture Media Design" box goes into more detail.)

Following initial screening, more component concentration ranges can be evaluated and interactions among significant components assessed. Substitute components not typically found in culture media — such as galactose to supplement glucose or lipid emulsions to complement fatty acids and other lipid precursors — also can be evaluated for their effects on culture performance. The ability of cultures to grow and produce without expensive growth factors also should be assessed, especially after several rounds of media optimization have been completed.

Complex ingredients (e.g., sera and protein hydrolysates) are still very often used to optimize performance. Evaluating different brands from different vendors and multiple lots from various manufacturers ensures selection of the highest-performing material (Figure 1). Use of statistical experimental designs minimizes the numbers of experiments needed. Software such as the Design-Expert program from Stat-Ease, Inc. (www.statease.com) can help.

Protein hydrolysates vary in their percentages of total protein as well as free amino acids, small peptides, and polypeptides depending on the hydrolysis conditions used by the manufacturer and source of the protein. As with screening experiments, the use of statistical experimental designs enables economical determination of the best protein hydrolysate and determination of optimal concentrations.

Mixture experiments for analyzing combinations of different defined media are another useful empirical means to optimize culture media. This method was used in the early development of classical culture media to create such mixtures as Dulbecco's Modified Eagle's Medium (DMEM) F-12 and Roswell Park Memorial Institute 1640–DMEM/F-12 (RDF), where dissimilar media complement each other both qualitatively and quantitatively with respect to their compositions. Through evaluation of the performance of various media mixtures, insight can be gained on favorable concentration ranges of the various medium components. This method is most effective when each medium is significantly different from the others to widen the concentration range of components investigated.

Media Examples (1)

Early Media

Ham's F-10 nutrient mixture was formulated for serum-free growth of Chinese hamster ovary (CHO) cells and growth of various mammalian cell lines with serum supplementation.

Ham's F-12 nutrient mixture was formulated for single-cell plating of near-diploid Chinese hamster ovary (CHO) cells.

Minimum Essential Media (MEM) are are well suited for the growth of a broad spectrum of mammalian cells.

Dulbecco's Modified Eagle Media (D-MEM) are well suited for supporting the growth of a broad spectrum of mammalian cell lines.

RPMI Media are enriched formulations with extensive applications for mammalian cells. They were formulated for suspension cultures or monolayer culture of human leukemic cells.

Later Media

Iscove's Modified Dulbecco's Media (IMDM) are highly enriched synthetic media well suited for rapidly proliferating, high-density cell cultures.

CMRL Medium is especially useful for cloning monkey kidney cell cultures and for growth of other mammalian cell lines when enriched with horse or calf serum.

MCDB 131 Medium was developed in 1987 as a reduced serum-supplemented medium for the culture of human microvascular endothelial cells.

NCTC-109 is a synthetic medium suitable for general use in generating and maintaining hybridoma cells. Like MCDB, this medium typically requires the addition of a growth factor cocktail or serum.

Table 1: Summary table comparing four methods of media development **(1)**

	Component Titration	Media Blending	Spent Media Analysis	Automated Screening
Throughput	Low	High	Medium	High
Modeling	Good	Good	Good	Poor
Instructive	Good	Poor	Good	Fair
Scope	Unlimited	Limited	Limited	Unlimited

Another approach is to use suboptimal media that are deficient in one or more components and thus compare culture performance to determine which nutrients have significant impact. Here, too, statistical experimental mixture designs can aid in interpretation of results and help economize the number of experiments required.

The stoichiometric approach to medium optimization uses nutrient consumption as a foundation for medium formulations. Spent-medium analysis from a time course experiment can identify which components are depleted or in excess over the course of the culture. Their specific rates of consumption can be calculated and the concentrations of each medium component adjusted in the formulation to reflect their actual use. Theoretical metabolic models have also been used to determine nutrient concentrations based on estimated consumption from modeling equations. Alternatively, the composition of a recombinant protein product or host cell line can also be considered for insight on the balance of amino acids and other nutrients. In a fed-batch or perfusion process, a key nutrient such as glucose or glutamine is tracked and the concentrations of other nutrients linked to it stoichiometrically based on their individual consumption rates relative to the key component.

The chief limitation of this approach is the availability (or lack thereof) and in some cases sensitivity of assay methods used to analyze spent medium. In addition, peptides from serum and/or protein hydrolysates also

Rational Design *continued*

Automated screening focuses primarily on throughput. The use of robotic fluids handling and multiwell plates can rapidly create and analyze large numbers of candidate media. So this method generates a large dataset of performance results obtained in parallel conditions.

Comparing the Methods: How useful are those methods? When should each be used, and when should it be avoided? What are their strengths and weaknesses? A brief evaluation might consider the following important characteristics:

- Throughput — how many samples are evaluated in a given time?
- Modeling — how well does a method represent a real application?
- Instructive — does the method effectively reveal useful information about cell culture process requirements? Does it contribute to a knowledge base?
- Scope — how well does the method consider all possible solutions to the problem?

Table 1 summarizes the results of such a survey. None of the methods is best in every way, and each has its own particular weakness. So depending on any method alone would not be an effective way to develop a new medium rapidly.

What It Is . . .

So what is rational culture media design? It can be described as a multidimensional approach: Instead of relying on a single technique, rational culture media design makes use of several complementary methods. A media development project is generally divided into phases that guide its progression from screening through optimization to verification.

All the methods described above are used in rational design, but they must be chosen for use with careful regard to their strengths and weaknesses.

(continued)

The combination is carefully chosen for each project to fit the goals of each particular phase and provide results that will be relevant to those goals. Project management tools (such as Gantt charts) are used to facilitate efficient planning and coordination of project tasks. That becomes especially important when operating under accelerated timelines that require performing many experiments concurrently.

As would seem fitting for its use in solving industrial problems, rational culture media design can be termed an applied science because it is concerned with effectively solving "real-world" problems by properly combining tools that fit each specific problem. Before any experiments begin, development goals must be carefully defined in terms of not only process performance, but also regulatory and any other requirements. The same care is applied to choosing appropriate models for the actual experiments — in regards to both scaled-down cell culture methods and using appropriate end-points.

Finally, the rational culture media design approach does not allow you to avoid performing cell culture work during media development. But it is both rapid and unbound by the limitations inherent in any single-method approach. Appropriate software tools help design an efficient discovery process. For example, by using a statistical Design of Experiments (DoE) program — such as ECHIP (ECHIP, Inc., www.echip.com), Design-Ease and Design-Expert (Stat-Ease, Inc., www.statease.com) — you can perform experiments that capture the complex interactions of multiple components without testing the full factorial number of combinations. Statistical tools help you choose the lowest possible number of conditions for your experiments without sacrificing statistical confidence in your results.

By using several carefully chosen methods that are effective in the rapid development of culture media optimized for specific processes, rational culture media design can help you meet the stringent challenges presented by today's cell culture processes.

References

1 Jayme D, Watanabe T, Shimada T. Basal Medium Development for Serum-Free Culture: A Historical Perspective Cytotechnology 23(1–3) 1997: 95–101.

2 Prusiner SB. Novel Proteinaceous Infectious Particles Cause Scrapie. Science 216(4542) 1982: 136–144.

3 Broedel S, Papciak S. The Case for Serum-Free Media. BioProcess International 1(2): 56–58. c

***Tom Fletcher** is research and development director for industrial cell culture at Irvine Scientific, Irvine Scientific, 2511 Daimler Street, Santa Ana, CA 92705-5588; tfletcher@irvinesci.com; www.irvinesci.com.*

contribute to cellular nutrition, and it is difficult to model their effects on metabolism compared with free amino acids. Nutrient use can vary considerably, depending on the type of process: batch, fed-batch, or perfusion. If a process evolves from fed-batch to perfusion or from perfusion to fed-batch, then repeated analyses of nutrient consumption rates may be necessary to ensure that the medium is again optimal to support a robust and predictable operation.

New approaches to medium optimization look at cellular metabolism using metabolic flux analysis and/or gene expression profiling to give a big-picture overview of the metabolic state of the cells. In metabolic flux analysis, major biochemical reactions are rendered as mass-balance equations involving carbon and nitrogen. Usually only carbohydrate and amino acid catabolic reactions are considered, along with formation of biomass and product. Through comparison of changes in flux rates of metabolic intermediates during medium optimization, insight can be gained from medium formulation changes and their individual effects on cell metabolism, growth, and productivity. However, the use of simplified metabolic networks in flux analysis combined with the difficulty in accounting for hydrogen and oxygen balances (and complex nutrients such as sera) limit the results to more of an approximation showing general trends.

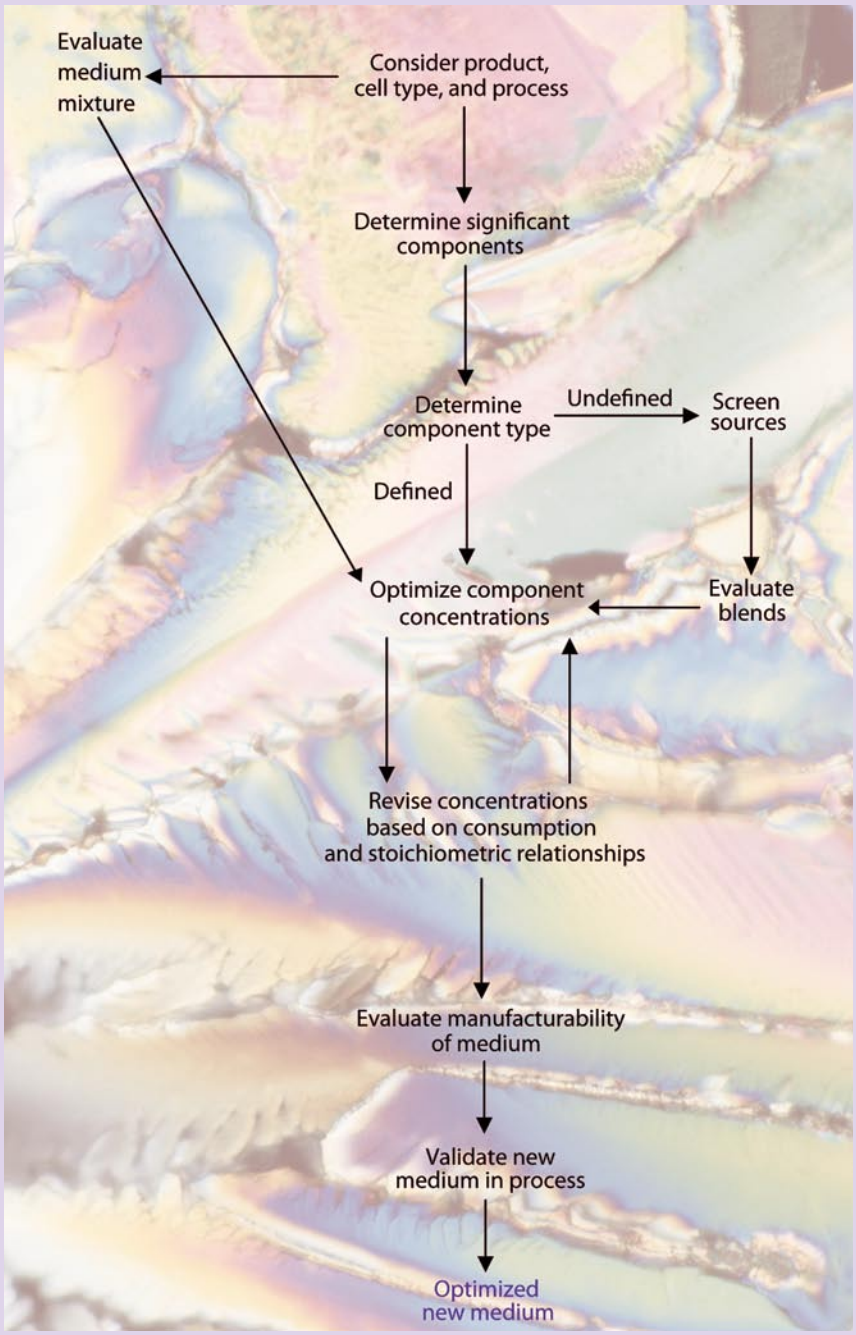
Gene expression profiling involves monitoring messenger RNA (mRNA) levels for select genes. This is commonly accomplished using microarrays with oligonucleotides, complementary DNA, or expressed sequence tags spotted onto chips and then allowed to hybridize with mRNA isolated from cells. Through comparison of mRNA levels in response to medium formulation changes during optimization, information can be obtained about certain key enzymes (and their metabolic pathways) that are up- or down-regulated during growth and/or production. Nevertheless, mRNA levels may not always correlate to protein levels, and small unrecognized changes in certain message levels may cause large changes in metabolic rates. Both metabolic flux analysis and gene expression profiling benefit if the results can be viewed graphically on a metabolic map to better visualize changes in cellular metabolism.

FACTOR IN ALL THE DETAILS

Combined with vector and cell line development, medium optimization has proven to be another valuable avenue toward process yield and performance improvement. Evolution of media formulations through analysis of consumption rates, screening of different components, and comparison of mixtures can significantly improve cell growth, viability, and product titers during process development. Improved productivity and reduced cost of goods achieved by replacement of expensive components are the ultimate outcomes of a successful optimization strategy.

Consideration must also be given to product quality and potential changes induced by different media formulations. Changes to formulations that lead to improved cell growth and productivity must not come at the expense of deleterious or unacceptable

Figure 1: Medium optimization flow chart (CRYSTAL BACKGROUND FROM WWW.PHOTOS.COM)



changes in product quality such as changes in glycosylation, increased protein aggregation, or proteolytic cleavage.

Prototype medium formulations need to be further assessed under realistic manufacturing conditions — whether at full scale or with suitable scale-down models that accurately reflect a production environment. Moreover, cell culture medium development also needs to stress the manufacturability of media formulations at large scales. Changes in formulation should not adversely affect solubility/filterability or otherwise compromise stability through precipitation or degradation of medium components. Medium compounding protocols and standard operating procedures should be established to achieve consistent and reproducible performance of every newly developed and optimized cell culture medium.

Reference

1 Fletcher T. Designing Culture Media for Recombinant Protein Production: A Rational Approach. *BioProcess International* 3(1) 2005: 30–36.

For Further Reading

Buntemeyer H, Lutkemeyer D, Lehmann J. Optimization of Serum-Free Fermentation Processes for Antibody Production. *Cytotechnology* 5, 1991: 57–67.

Heidemann R, et al. The Use of Peptones as Medium Additives for the Production of a Recombinant Therapeutic Proteinin High-Density Perfusion Cultures of Mammalian Cells. *Cytotechnology* 32(2), 2000: 157–167.

Jayme D, Watanabe T, Shimada T. Basal Medium Development for Serum-Free Culture: A Historical Perspective. *Cytotechnology* 23, 1997: 95–101.

Keen MJ, Rapson NT. Development of a Serum-Free Culture Medium for the Large Scale Production of Recombinant Protein from a Chinese Hamster Ovary Cell Line. *Cytotechnology* 17, 1995: 153–163.

Martial-Gros A, et al. Amino Acid Metabolism by VO 208 Hybridoma Cells: Some Aspects of the Culture Process and Medium Composition Influence. *Cytotechnology* 37(2) 2001: 93-105.

Tholudur A, et al. Using Design of Experiments to Assess *E. coli* Fermentation Robustness. *BioProcess International* 3(9) 2005: in press.

Whitford W. NS0 Serum-Free Culture and Applications. *BioProcess International* 1(12) 2003: 36–47.

Xie L, Wang DIC. Stoichiometric Analysis of Animal Cell Growth and Its Application in Medium Design. *Biotech. Bioeng.* 43(11) 1994: 1164–1174.

Yandell C, et al. An Analogue of IGF-I: A Potent Substitute for Insulin in Serum-Free Manufacture of Biologics by CHO Cells. *BioProcess International* 2(3) 2004: 56–64.

Matthew Jerums *is senior associate scientist, and corresponding author* ***Xiaoming Yang*** *is principle scientist in cellular process development at Amgen Inc., 18S-1-A, Thousand Oaks, CA 91320; 1-805-447-4076, fax 1-805-499-6819; xyang@amgen.com.*