BioProcess International

CELL LINE DEVELOPMENT

FROM GENOME EDITING TO MEDIA ADAPTATION

September 2019

FEATURED REPORT

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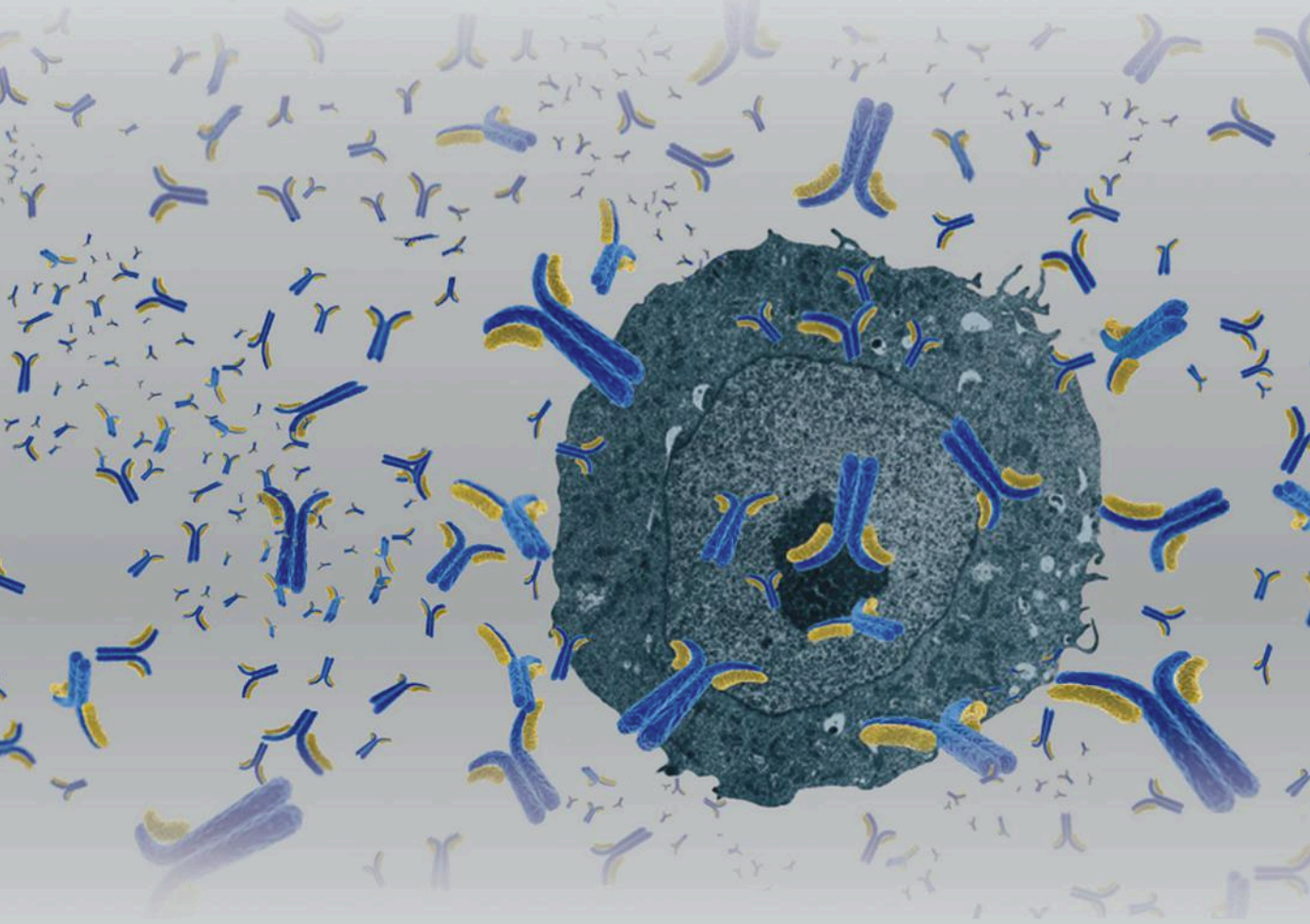

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Introduction

Reporting from the Frontiers of Cell line Engineering at BPI Europe and BPI West

Cheryl Scott

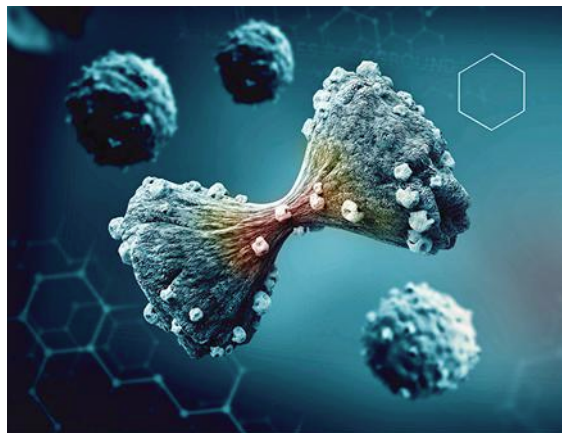
Every biomanufacturing process begins with transfection of recombinant genes into pools of cells — followed by a succession of screenings from which will emerge (ideally) a single progenitor cell of the new production cell line. Cast aside will be those cells that do not uptake the correct genetic material, those incapable of thriving in bioprocess conditions, those that fail to produce recombinant protein at relevant levels, and those without demonstrated clonality and relative genetic stability.

Over the past several years, advancing technologies for genetic engineering, screening, clone selection, analysis, and data management have brought about a revolution in cell line development. Audiences at the BioProcess International (BPI) conferences have watched this field evolve as presenters shared their insights and reported on their experiences. This year's BPI Europe and BPI West events continued in that vein with presentations on technology platforms, clonality, cell line (genetic) engineering, and development of biosimilars and other challenging product modalities.

The Track at BPI Europe

The BPI Europe conference and exhibition in Vienna, Austria, this past April devoted an entire track to cell line engineering and development. Speakers came from across the biopharmaceutical "ecosystem": bioprocess suppliers, drug developers, and academia. They discussed genetics, new technologies, and case studies from the frontiers of the industry.

Genomics and More: Nicole Borth (professor at BOKU University and ACIB) focused on epigenetics of Chinese hamster ovary (CHO) cells in her talk, "Genome Scale Science for CHO." She explained that scientists had spent 20 years hunting for a single gene to boost process performance of mammalian cell lines (whether overexpressed or knocked out/down) by improving productivity, growth, and product quality all in one fell swoop. Unfortunately, she said, such a "miracle gene" is not likely to exist.



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An efficient cell factory, Borth said, results from "a complex interplay of many genes and activities. Productive cells reduce 'unnecessary' functions. They are efficient in providing precursors and energy." Although certain pathways do come up time and again, the precise genes getting regulated differentially within those pathways will vary.

"Epigenetic regulation determines the transcriptome of each cell and cell type," Borth explained. That includes different layers of regulation both for long-term "memory" and rapid response to changing culture conditions. And this makes epigenetics the second major contributor (after the genome itself) to phenotypic variation. She said that the true secret to control of gene expression will be to use epigenetics to determine phenotype and behavior. "The future of phenotype control will require more detailed understanding of gene expression regulation. Manipulating expression of multiple genes simultaneously requires synthetic biology and epigenetic control tools."

In "Engineering Complex Traits in the Postgenomic Era of CHO Cell Line Development," Nathan Lewis (assistant professor in the departments of pediatrics and bioengineering at the University of California, San Diego) echoed Borth's conclusions. "Many desirable traits require multiple gene edits,"

Upcoming KNect365 Events

Pre-Conference Symposium on Cell Line Development and Engineering at the **BioProcess International Conference and Exhibition** in Boston, MA, on 9 September 2019; https://lifesciences.knect365.com/bioprocessinternational/agenda/1#

Online Academy: Early Stage Upstream Process Development, five weeks starting 1 October 2019; https://lifesciences.knect365.com/early-stage-upstream-process-development

Two-Day Course: Upstream Bioprocess Development in London, UK, on 26–27 November 2019; https://lifesciences.knect365.com/upstream-bioprocess-development

Track on Cell Line Development and Upstream Processing at **BioProcess International Asia** in Kyoto, Japan, on 26–28 February 2020; https://lifesciences.knect365.com/bpi-asia/

Cell Line Development and Engineering, colocated with **BioProcess International US West** in Santa Clara, CA, on 9–12 March 2020; https://lifesciences.knect365.com/cellline

Cell Line Development and Engineering Europe at **BioProcess International Europe** in Amsterdam, The Netherlands, on 28–30 April 2020; https://lifesciences.knect365.com/bpieurope

he explained. "Pathway knowledge and large data sets can guide cell engineering."

As an example, he highlighted the risk of virus contamination of mammalian cell lines and cultures. Profiling virus-resistant cells points to regulators of resistance. In a case study, knocking out combinations of resistance repressors created virus-resistant cells.

Niall Barron (director of the National Institute for Cellular Biotechnology at University College Dublin in Ireland) turned the audience's attention from DNA to RNA epigenetics in "Effect of RNA Epigenetics and Structure on Recombinant Protein Production in CHO Cells." He highlighted the importance of a 5′ untranslated region (UTR) from mouse genetics in enhancing transgene translation. Its structure affects the stability of messenger RNA molecules.

"Manipulation of mRNA epigenetics represents a potential opportunity to manipulate the CHO production platform," said Barron, "but better understanding will be required first."

In "Designing Gene Expression Cassettes for CHO Cell Engineering," Adam Brown (lecturer in DNA engineering for the department of chemical and biological engineering at the University of Sheffield, UK) emphasized the need for control of complex stoichiometric gene expression. He identified four categories of expression cassette design criteria: cell productivity, quality of expressed protein products, design simplicity, and control and stability of protein expression.

Focusing on design space, Brown described a quality by design (QbD) approach to expression cassettes. Designers must answer three questions: What can't we control precisely (gene specificity)? What rates will we keep constant (set points)? and How will we achieve precise stoichiometries (control point)? Brown illustrated the testing phases that follow those decisions, identifying the ultimate goal as a system that enables robust, user-defined manipulation of gene expression stoichiometry.

"Further engineering is required to achieve highly predictable, fine-tuned control," he explained, "and to achieve the objective design criteria."

Advancing Technologies: Anton Bauer (senior scientist at the Medical University of Vienna) highlighted BESTcell technology from The Antibody Lab in "The BEST of Both Worlds — Targeted Integration and Multiple Copies: How Can This Go Together for Improved Cell Line Development?" He identified the challenge of developing highly productive stable mammalian cell clones within just three weeks from transfection.

Common methods for generation of stable mammalian cell lines include random integration of classical plasmid constructs; including chromatin modulators on those plasmids (for random integration of the plasmid constructs flanked by "antirepressors" for chromatin modulation); and knock-in approaches (using highly active host-cell loci for transcription of a transgene). But Bauer pointed out that silencing of the gene of interest (GoI) is a perennial problem to solve.

He pointed out how lessons from transgenic mice have helped scientists learn more about recombinant protein expression. Bacterial artificial chromosomes (BACs) can serve as expression vectors for recombinant protein production in mammalian cells. This is where the BESTcell approach comes in: It targets GoI integration into a "hot-spot" locus in vitro to generate multiple locus copies in CHO cells.

"BESTcell technology outperforms standard plasmid-based expression in stable clones," said Bauer. "Transgene expression correlates with the number of integrations." He showed a standardized process for cell line development without the need for antibiotic selection or amplification. "Multiple target-gene copies are integrated stably into a single integration site."

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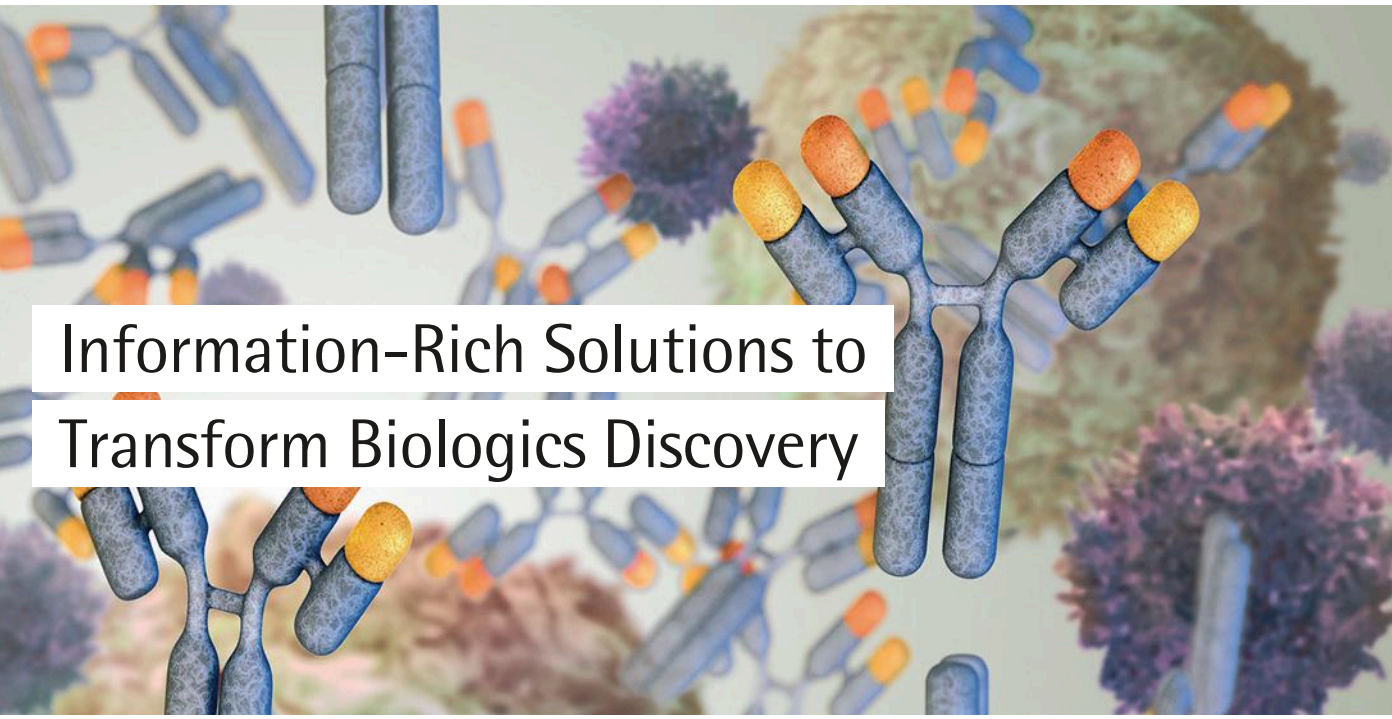

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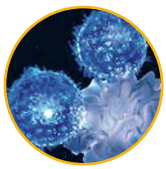
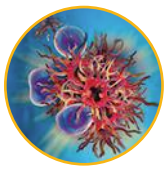
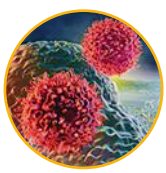


Immunophenotyping



Immune Cell Killing



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Genetic stability and high yield (e.g., of human IgG1) with BESTcell clones have been demonstrated and maintained in long-term cultures. The system also has been proven with multiple types of proteins. Its capability has been tested in >50 case studies: standard and alternative IgGs, secreted polypeptides, "difficult-to-express" proteins, and IgAs. Bauer pointed out that BESTcell technology is used by Polymun for its EAVI2020 consortium work (http://www.eavi2020.eu) in good manufacturing practice (GMP) production of HIVgp140 (currently in a phase 1 clinical trial).

Whereas Bauer had focused on genetic "software," Kerensa Klottrup-Rees (scientist of cell culture and fermentation sciences at AstraZeneca) touted new hardware in "Cytena Single Cell Printer (SCP): Advancing from Implementation to Robust Process." In an evaluation study at her company, the instrument enabled progression to a single round of cloning with a six-week reduction in time lines. She said that it also was easy to use.

Side-by-side analysis of two projects comparing ClonePix and SCP processes showed at least equivalent titers, cell growth, and product quality despite the challenges of longer pool passaging, a lack of subcloning, and fewer data points available for review.

"SCP cloning has been implemented on five additional projects within the past year," said Klottrup-Rees, "including novel molecules." Quality and titer of the final clones in ambr microbioreactors (Sartorius Stedim Biotech) have been as good as or better than expected.

Subcloning of the host cell line and creation of new host pools has improved clone recovery. Evaluation of pooled hosts and clones is ongoing, but she said that data so far (up to shake-flask scale) show equivalent performance.

"Changing a single instrument has a massive implication for the whole process workflow," she cautioned. But her company has been pleased enough with its evaluation of this new instrument that it will be worth the trouble. "Our future focus is on better use of the data to monitor trends and identify areas for improvement."

Hannah Byrne (product manager at Valitacell) turned our attention to measurement of cell line properties in "Novel Analytical Solutions for Cell Line Development." Her company offers semiautomated, microplate-based assay platforms (ChemStress and fluorescence polarization, FP) for a number of protein characteristics including titer.

"Our suite of simple, quantitative assays can assist in transforming the complex manufacturing process," Byrne said. "Our patented technology allows us to create an assay applicable for any target using the FP platform. Migration toward in-line analytics is the future."

Anke Mayer-Bartschmid (senior scientist of cell and protein sciences at Bayer) focused on data-driven decision-making using databases and analysis in "Making the Most Out of the Data Collected Along the CLD Process." Such decisions enable selection of the best clones as well as continuous improvement through identification of highly productive clones, finding the right vector for a given system, and so on.

"Relevant and high-quality data must be stored in a searchable database," said Mayer-Bartschmid. "Our database forces the input of structured data." She explained that information should be made readily available for decision makers. "A database should enable fast analysis. The data have to be representative of the questions being asked."

Analytical information must be engineered and stored, she explained, and then connected correctly. Accessibility through a central storage system and interfaces is important to allow for data collected over years to show correlations (e.g., certain glycosylation patterns dependent on cells, media, or the combination of both).

Case Studies: Sophie Broussau (cell line development specialist at Canada's National Research Council in Montreal) reported on use of a new instrument in her case study, "Novel HEK 293 Cell Line Development for Upstream Production of a Viral Vector Using the CellCelector Nanowell Cloning System." Lentiviruses are complex, enveloped retroviruses, she explained, best exemplified by the human immunodeficiency virus (HIV).

Broussau's team used a CellCelector nanowell-based cloning system from Automated Lab Solutions (ALS) for cell line development of stable human embryonic kidney (HEK 293) cells to produce lentiviruses. The automated single-cell and colony-picking system takes and saves photos of every colony picked. It uses an ALS Flowbox incubator to maintain stable environmental conditions for living cells in culture.

Broussau concluded with a list of advantages her team found in using the CellCelector system: high levels of cell survival during cloning; complete documentation proving monoclonality; rapid clone analysis; straightforward workflows; and reduction of both time and materials needed to get results.

Martin Bertschinger (deputy director of cell sciences at Glenmark Pharmaceuticals) offered

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another case study in "Highlights from the Process Development of GBR 310, Glenmark's Biosimilar for Xolair." He emphasized the QbD approach: "process development with predefined objectives — beginning with the end in mind."

Biosimilar developers must begin with a quality target product profile (QTPP) based on knowledge of the reference product. They generate a list of potential and identified critical quality attributes (CQAs). This requires development of an analytical strategy and analysis of innovator batches to provide target CQA ranges.

Bertschinger's company used those development targets to guide in cell line development. Drug product CQAs were used to derive ranges for cell-population screening and selection. "We classified the CQAs roughly into three categories," he said, "CQAs impacted by cell population, by process, and by both."

The resulting production process was scaled to a 2,000-L bioreactor. Both physicochemical and functional biosimilarity were demonstrated using a tiered approach and appropriate statistical tools. Remaining differences were understood and considered to be clinically insignificant. And a phase 1 bioequivalence study has been successfully completed.

Talks at BPI West

Unlike BPI Europe, the conference in Santa Clara this past March did not include a dedicated track on cell line development and engineering. BPI West is organized differently: by product/process development stage (**1**). However, some speakers in the "Early Stage Process Development" track inevitably focused on cell line engineering topics.

Speed to IND: Yves Durocher (mammalian cell expression section head in the Human Health Therapeutics Research Center at the National Research Council of Canada) highlighted the need for speed in getting a product to the investigational new drug (IND) phase. "Are stable pools or transient gene expression viable manufacturing options?" was the main question of his presentation.

Focusing on CHO cells as the dominant mammalian expression system to manufacture biologics, Durocher highlighted the FDA's assurance-of-clonality requirement. Without that, he explained, "additional control strategies would be needed on cell lines, product characterization, and processes to ensure product quality and homogeneity."

However, accessing state-of-the-art technology platforms for generating CHO clones represents a "stark financial barrier" for small biotechnology companies. Beyond the initial licensing of a cell line, a master cell bank must be generated under GMP compliance. Otherwise, developing a CHO platform in-house requires time, specialized equipment and materials, and expertise.

Durocher described the investment in time and money required to make a clone for generating GLP toxicological materials as a significant obstacle to bringing new biotherapeutics into clinical testing, especially for smaller companies. They want to know whether other, faster manufacturing methods might be available.

Durocher pointed to three viable alternatives: transient gene expression (TGE), stable pools, and targeted (site-specific) gene integration — which he referred to as *recombination-mediated cassette exchange* (RMCE). In recent years, all three options have shown significant improvement in productivity (some achieving similar expression titers to those of stable clones), robustness, and product quality (again equivalent to that of material produced by stable CHO clones).

"TGE, stable pools, and RMCE do accelerate production of biologics," Durocher said. Because their quality attributes can match those of stable clones, they can be used to generate material for toxicology (and even early clinical) studies. "The emphasis should be on product quality, safety, and process robustness," he concluded, even when speed is the goal. These technologies for faster and more cost-effective manufacturing should facilitate the early stage development of biologics at small companies.

Getting Biosimilars Started: Michael Brem (senior scientist in cell line development at Cytovance Biologics, a Hepalink company) offered a case study to demonstrate successful development of a cell line expressing a biosimilar antibody. In particular, he reported on the use of a Gibco Freedom CHO-S kit from Thermo Fisher Scientific. In this collaboration between the two companies, Cytovance used mostly Thermo Fisher products to take a MAb-based biosimilar product "from DNA to toxicology."

The goal was to use the Freedom kit to produce a stable cell line that would meet or exceed productivity targets (1–2 g/L). Brem's team wanted to shorten the time line as follows:

- DNA construction (weeks 0–5)
- stable pool selection (weeks 5–10)
- single-cell cloning and scale-up (weeks 10–14)
- clone evaluation (weeks 14–16)
- working cell bank for top eight clones (weeks 16–17)

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• process development and stability studies on top four clones (weeks 17–27)

• toxicology-scale production campaign (weeks 23–27).

"Clone 9" was identified as the most solid and high-producing stable clone. It exceeded the productivity target (to >3 g/L) after process development in an ambr15 microbioreactor system (Sartorius Stedim Biotech). A timeline that used to take about a year was reduced to 27 weeks for this biosimilar monoclonal antibody. Brem said that using current Thermo Fisher catalog products was sufficient for development of this cell line. And he said that Clone 9 is a viable candidate to be used in future evaluations of Thermo Fisher products (new base and feed media).

Moving on to Perfusion: Finally, David Busch (senior scientist in preclinical development at Merck Research Laboratories, Kenilworth, NJ) looked toward the next step in "Bridging Clone Screening Platforms with Perfusion Bioreactors for Continuous Manufacturing." Continuous manufacturing is of increasing interest in the biopharmaceutical industry.

Busch said that "mock perfusion" using deep-well plates allows for high-throughput clone selection in an emulated perfusion process. His team screened MAb-expressing clones in 24 deep-well plates and compared the results with those of TPP TubeSpin minibioreactors. "MAb-expressing clones have similar growth characteristics, productivities, and quality attributes using deep-well plates and TPP spin tubes in the mock-perfusion screening platform," he explained.

Data showed that viable cell density and viability were comparable across media exchange volumes over five days. Exchange volume had no effect on accumulated titer or cell-specific productivity. The Merck team compared MAb-expressing clones from mock-perfusion cultures with those at a similar growth phase in 2-L perfusion bioreactor cultures and again found similar titers and cell-specific productivities, as well as quality attributes.

"The mock-perfusion deep-well–plate (DWP) format provides a high-throughput method for clone screening," Busch concluded.

In This Insert

The remaining articles in this BPI featured report expand discussion of genomics and cellular adaptation — the "nature" and "nurture" approaches to cell line development . First, Harvard professor Masahito Yamagata highlights the potential of advanced genetic engineering techniques for fine-tuning the capabilities of production and bioassay cell lines. Following that, authors from an Italian biopharmaceutical company show how they rapidly adapted a cell line to culture in defined media. Doing so allowed them to start with a relatively inexpensive cell line for their production process.

With speed and precision taking center stage, biotechnologists are looking to instrument suppliers and academic scientists in genetics and cell biology to help them push the envelope in cell line development ever further. See the "Upcoming Events" box for suggested live and online venues where you can take part in the future discussions.

From the BPI Archives

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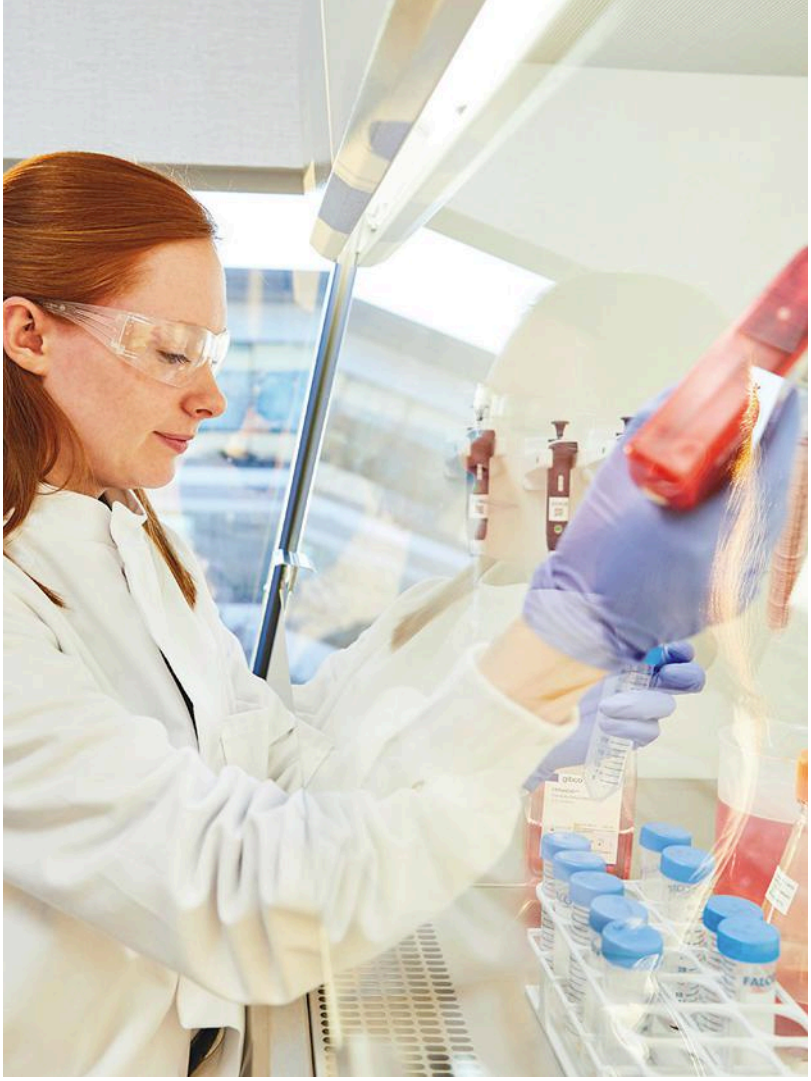

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Creating Novel Cell Lines By Genome Editing

Simplifying Cell-Based Assays and Improving Production of Biomolecules

Masahito Yamagata

Cultured cell lines have a diverse range of applications. They are used broadly by cell biologists, clinicians, tissue engineers, biotechnology scientists, and bioengineers. The most important uses of cell culture are in the cell-based assays and production of biologically active recombinant proteins. In recent years, genome editing has been used widely to study the structure, function, and localization of endogenous proteins in cultured cells. However, applying the same genome editing techniques to cell lines also could improve the propagation of cells and help scientists devise novel assays for discovering bioactive molecules. Below, using a cadherin adhesion molecule as an example, I explain a simple approach to genome editing for generating novel cell lines to achieve those goals.

Case Study

Cadherins are cell-surface transmembrane proteins that mediate cell-to-cell adhesion in animals. In 1987, Masatoshi Takeichi and colleagues at Kyoto University introduced cDNA of E-cadherin (CDH1) into fibroblastic cells lacking intrinsic cadherin (**1**). The transfected fibroblasts stuck together and aggregated. That simple experiment proved that cadherins are molecules required for intercellular adhesion.

Human embryonic kidney (HEK293) cells are one of the most widely used cell lines in biomedical research as well as industrial production of therapeutic molecules and viral vectors. The cell line is easy to transfect efficiently and grows well in culture. HEK293 cells have strong intrinsic cell–cell adhesion mediated by cadherin-2 (CDH2, *N*-cadherin). By applying CRISPR/Cas9 technology (see box, right), we recently deleted the cdh2 gene from HEK293 cells. The resulting cdh2-deficient HEK293 cells do not aggregate (Figure 1) (**2**). By overexpressing the

CRISPR/Cas9 Genome Editing

Clustered, regularly interspaced, short palindromic repeats (CRISPR) is a family of DNA sequences from the genomes of prokaryotic organisms. Originating from DNA fragments from viruses that infect bacteria and archaea, they can be used to detect and destroy DNA from similar viruses during subsequent infections. So these sequences play a key role in prokaryotic antiviral defense.

CRISPR-associated protein 9 (Cas9) is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. CRISPR-Cas9 technology can be used to edit genes in a wide range of cell types. This editing process has a number of applications, from basic biological research to development of biotechnology products and potential treatment of diseases.

From the BPI Archives

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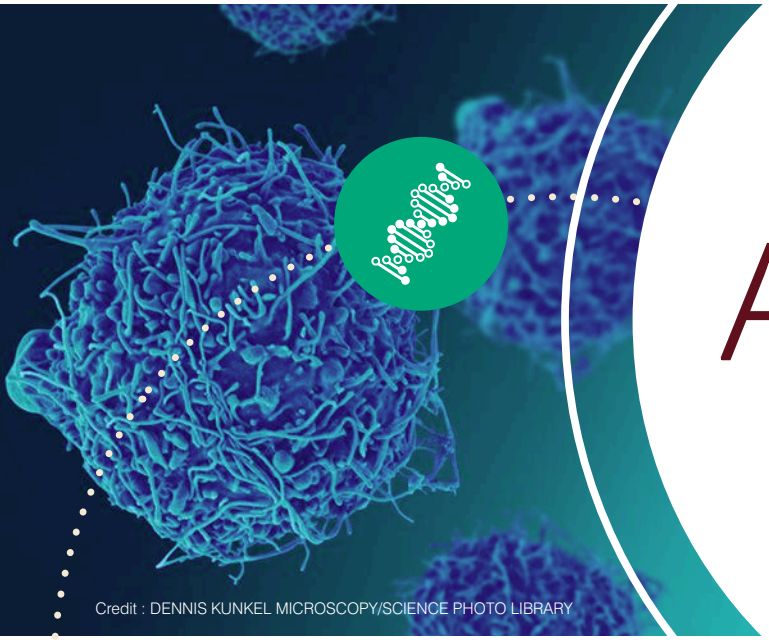

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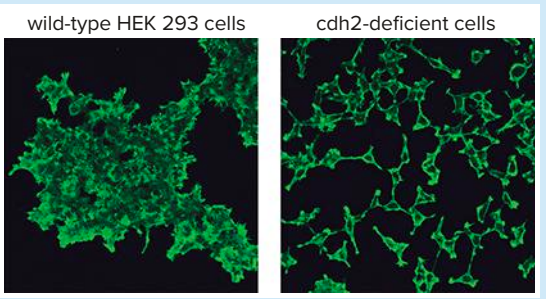
Figure 1: HEK293 wild-type and cdh2-deficient cells were stained with fluorescein-conjugated phalloidin (**2**).



wild-type cdh2 gene, then CDH2-mediated adhesion can be rescued.

Here's the advantage of removing cell–cell adhesion from cultured cells: Passage and maintenance in culture of cdh2-deficient HEK293 cells become effortless without the need for trypsin to dissociate them. It could be possible to culture them under unique conditions (e.g., adherent but higher density culture, or suspension culture) because they do not aggregate.

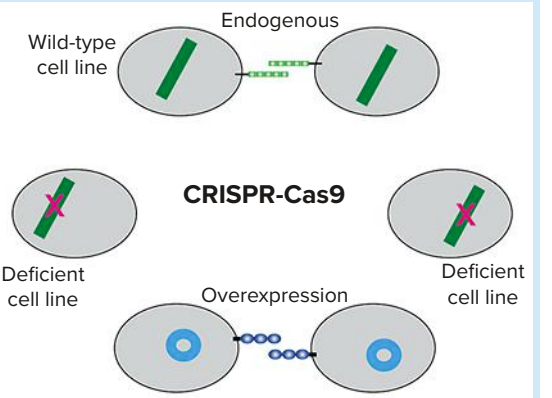
Novel HEK293-cell–based assays could be created for discovery and study of protein–protein interactions. This cell line is suited particularly well for such experiments because it is highly transfectable with exogenous DNA. For example, the specificity of cell-adhesion molecules can be tested in cdh2-deficient cells that lack cell–cell adhesion activity. The cell line can be used as a reporter system for monitoring protein–protein interactions on cell surfaces that otherwise would be hampered by CDH2-mediated adhesion. We showed that the structure and function of several synaptic-adhesion molecules can be studied by transfecting this cell line (**2, 3**).

Getting Rid of Annoying Genes

Figure 2 shows how the idea can be extended. Overexpression once was considered to be predominantly a method for increasing the productivity of cultured cell lines. By introducing plasmids or riboproteins to generate CRISPR/Cas9 complexes into such lines, their genomes can be edited at any desired position (**4, 5**). This genome-editing technique has been used widely to study the structure, function, and localization of endogenous proteins both in vivo and in cultured cells. By applying genome editing to cell lines, we can design them simply by eliminating troublesome or annoying genes.

For example, the genes that mask particular protein–protein interactions can be eliminated from

Figure 2: Elimination of endogenous genes that potentially hamper overexpressed genes reveals the activity of overexpressed genes.



a given cell line. This enables the creation of novel cell-based assay systems for studying exogenous proteins by overexpression using the resulting mutant cell line. If a cell line expresses endogenously either inhibitors or activators of a protein of interest, you can eliminate them by using this straightforward technique. It might sound simple, but it opens up a whole new avenue for the future use of cell lines.

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Streamlined Serum-Free Adaptation of CHO-DG44 Cells

Using a Novel Chemically Defined Medium

Noemi Moroni, Annarita Di Nunzio, Adele Perli, Roca Puca, Kimesha Hammett, Graziella Piras, James Brooks, and Leonardo Sibilio

Monoclonal antibodies (MAbs) have radically transformed the treatment of many chronic diseases, mainly in the fields of oncology and autoimmunity. The overwhelming majority of therapeutic MAbs are manufactured from recombinant Chinese hamster ovary (CHO) cell lines. The original CHO cell line was isolated in the 1950s, and since the early 1980s, it has become the workhorse of the biopharmaceutical industry.

The CHO-DG44 strain was generated after several rounds of mutagenesis that deleted both copies of dihydrofolate reductase (dhfr) genes by ionizing radiation (**1, 2**). The DHFR enzyme catalyzes reduction of dihydrofolic acid to tetrahydrofolic acid, making it central to the biosynthesis of purines, thymidylic acid, and certain amino acids (**3, 4**). For that reason, CHO dhfr$^{-/-}$ strains require supplementation of hypoxanthine and thymidine (HT) in their cell culture media. Several MAbs currently are produced by recombinant CHO-DG44 cell lines to allow for consecutive rounds of genomic amplification of a gene of interest mediated by methotrexate (MTX) (**5, 6**).

Upstream process development depends strictly on the availability of chemically defined (CD) media and feeds that can encourage high cell densities and MAb expression titers (currently averaging several grams per liter of culture). This is particularly important for production of biosimilars, for which the pressure to reduce cost of goods (CoG) is demanding. Development of specific CD media is a time- and resource-consuming activity that generally involves statistical design of experiments (DoE) (**7**). Nevertheless, use of media without components of animal origin is recommended highly by all regulatory agencies as a means to allay safety concerns regarding the possibility of infective-agent transmission. Agents of concern include bovine/porcine viruses, mycoplasma, and transmissible spongiform encephalopathies (TSEs). Biotechnology companies typically follow one of two approaches to develop their media platforms:

- use internally generated data from spent media analysis and adjust formulations accordingly
- codevelop tailored media in collaboration with specialized media companies.

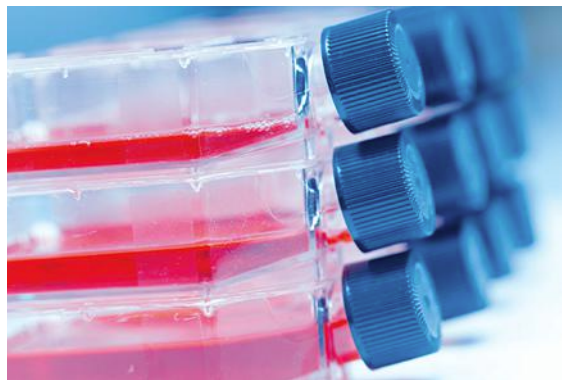

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Table 1: Stepwise new medium adaptation protocol for Chinese hamster ovary (CHO) DG44 cells using HyCell or CD-DG44 media

Day	α-MEM Medium	Fetal Bovine Serum	CD Medium
0	100%	10.00%	0%
3	100%	10.00%	0%
7	100%	5.00%	0%
10	100%	5.00%	0%
13	100%	2.50%	0%
17	100%	2.50%	0%
20	100%	1.25%	0%
24	100%	1.00%	0%
28	100%	1.00%	0%
31	50%	1.00%	50%
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38	10%	1.00%	90%
42	0%	1.00%	100%

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We believe that this new protocol is suitable **PARTICULARLY** for biotechnology companies wishing to develop their own CHO-DG44 expression platforms.

The choice between those approaches depends on a number of factors, with time and resource availability playing pivotal roles. Choosing the second option in the context of a media panel assessment project, we developed a streamlined protocol to adapt a CHO-DG44 cell line from adherent, serum-supplemented culture to suspension culture in a fully chemically defined medium (Advanced Bioprocessing medium #27 from Thermo Fisher Scientific) over just five weeks. That reduced our early process development timeline significantly. And we believe that this protocol is suitable particularly for biotechnology companies wishing to develop their own CHO-DG44 expression platforms.

Materials and Methods

Cell Cultures: Our CHO-DG44 cells were not adapted to CD media. Actually, they had been stored for a long period in liquid nitrogen after having been cultivated and frozen in α-MEM medium containing fetal bovine serum (FBS). We thawed and initially cultivated these cells into α-MEM medium (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific), 200 mM L-glutamine (Sigma-Aldrich), and HT supplement (Thermo Fisher Scientific).

Cells were thawed in a water bath at 37 °C and centrifuged at 1,100 rpm for five minutes at room temperature. After discarding the supernatant, we resuspended the pellet in prewarmed medium in a 175-cm^2 T-flask (TF) from Corning Life Science. Every three to four days, we removed spent medium, washed the cells with sterile phosphate-buffered saline (Thermo Fisher Scientific), detached them by a short incubation with recombinant trypsin (Life Select Tryple Select from Thermo Fisher Scientific) at 37 °C, and reseeded them at 0.1–0.3 × 10^6/mL in 40 mL of fresh medium.

We screened several CD media to adapt the cells for serum-free conditions: CD DG44 (Thermo Fisher Scientific), HyCell CHO medium (GE Healthcare), and Thermo Fisher 's Media Assessment Panel system. The latter comprises a total of 20 different CD media named by number (e.g., medium #27).

Flasks were incubated in a static CO_2 incubator (Forma Steri-Cult, Thermo Fisher Scientific) at 37 °C, with 5% CO_2 and 80–90% relative humidity. Agitated cultures used 125-mL Erlenmeyer shake flasks (Corning Life Science) in an Infors HT Multitron cell stirrer incubator at 110 rpm and 50-mm shaking throw at 37 °C, with 5% CO_2 and 80% relative humidity. Sterile operations were performed under the hoods of vertical laminar biosafety cabinets (Faster BH 2004 Biohazard).

Only after the cells were adapted completely to the new media were they seeded in shake flasks and grown in the agitated incubator, subculturing every three to four days. Once completely adapted to the new medium in suspension-agitated cultures, small cell banks were frozen in 45% spent medium, 45% fresh medium, and 10% dimethyl sulfoxide (Sigma-Aldrich). Briefly, we centrifuged cell suspensions at 1,100 rpm for five minutes at room temperature, then discarded the supernatant and resuspended the pellet in cold freezing medium at a concentration of 10^7 viable cells/mL in 2-mL cryovials (Corning). The cryovials were frozen in a Mr. Frosty (Thermo Fisher) container at –80 °C for two days, then transferred to a Dewar liquid nitrogen freezer.

Cell Culture Parameters Measurements: Next we measured cell density and viability using a ViCell-XR cell counter (Beckman Coulter). We monitored metabolic parameters (pH, glucose, lactate, glutamine, glutamate, and ammonia) using a YSI 2950 biochemical analyzer.

Results

Stepwise Serum Reduction Followed by New Media Adaptation (Standard Protocol): Standard protocols for adapting CHO cells from adherent, serum-containing cultures to suspension growth in serum-free media usually follow a stepwise serum reduction with gradual introduction of the CD medium. Generally, toward the end of such a protocol, some cells begin to grow in suspension and can be transferred to shake flasks. Obtaining robust cell growth in agitated systems usually is the main goal of upstream process development because MAb manufacture normally requires stirred-tank or wave-mixed bioreactors, so the cells must be "educated" to grow in suspension.

Initially, we adopted a standard protocol to induce our CHO-DG44 cell line to grow in suspension: Cells were revived and subcultured in static T flasks with complete α-MEM medium, as described above. Table 1 shows the percentages of α-MEM medium, FBS, and the new medium that we adopted during these adaptation steps. After two

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Figure 1: CHO-DG44 adaptation to CD DG44 + 1% FBS (Condition B, green) and HyCell + 1% FBS (Condition C, orange); Condition A (blue) is the control medium (α-MEM + 1% FBS). The arrow identifies the day of the switch to suspension growth in shake flasks. FBS = fetal bovine serum

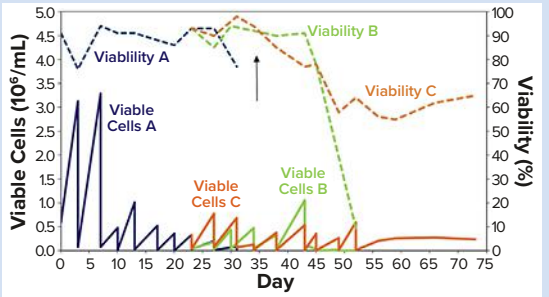

Figure 2: Cell density and viability over time of the stepwise adaptation protocol in Table 2; (A) 100% α-MEM; (B) 100% HyCell medium; (C) 100% CD DG44 medium; (D) 100% medium #27; SF1 = shake flask from Condition D switched to suspension growth from day 25; SF2 = shake flask from Condition D switched to suspension growth from day 36

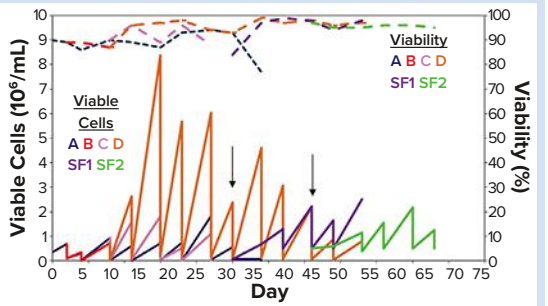

Table 2: New adaptation protocol for CHO-DG44 cells using HyCell, CD DG44, or medium #27

Day	Fetal Bovine Serum	CD Medium
0	10.00%	0%
4	10.00%	0%
8	5.00%	50%
12	5.00%	75%
15	2.50%	90%
18	1.25%	100%
22	1.00%	100%
25	0.75%	100%
29	0.30%	100%
32	0.15%	100%
36	0.00%	100%

consecutive passages using 10% FBS-containing medium, we halved the FBS percentage approximately at each passage from 10% to 1%. That serum-reduction step lasted 28 days.

Starting from day 28, we split the culture into three conditions to substitute the basal medium gradually with two commercial CD media. At this stage, we had three culture conditions: α-MEM + 1% FBS (Condition A, the control condition), CD DG44 + 1% FBS (Condition B), and HyCell + 1% FBS (Condition C). Each step took three to four days.

Figure 1 shows that Condition A (blue line) experienced a rapid drop in viability and was discarded. This demonstrates that α-MEM + 1% FBS is insufficient to maintain a constant cell growth. Conditions B and C showed sufficient adaptation until about day 35 (Figure 1, black arrow), when the cells started to show suspension growth. Then we transferred those cells into two 125-mL shake flasks (30-mL working volume) and incubated them in the shaker incubator, then repeatedly subcultured them. At day 45, when the new media had substituted the α-MEM medium, both Conditions B and C showed rapid decline in viability (Figure 1 dashed lines). They never recovered fully.

We speculated that these failures probably resulted from switching to suspension growth too early or from suboptimal adaptation to the two CD media. Moreover, the whole process took a long time: over two months from thawing to the end of adaptation. We emphasize here that the serum was not completely eliminated with this protocol; it was kept constant at 1% throughout the adaptation steps.

Contemporary Serum Reduction and New-Media Adaptation (New Protocol): To resolve those issues with an intent to reduce the adaptation time significantly to serum-free, suspension-growing cell cultures, we modified our adaptation protocol. In the new protocol, we used low-serum adaptation that gradually introduced the CD media. Using this combined approach, we sought to adapt our cell line to both CD medium and serum-free conditions simultaneously. We also included a new CD medium (medium #27) in this evaluation trial, having selected it from the 20 CD media in a panel from Thermo Fisher Scientific Advanced Bioprocessing after initial screening (data not shown).

We thawed a new vial of CHO CD44 cells into 40 mL of α-MEM containing 10% FBS in a 175-cm^2 TF. After two passages (eight days), we reduced FBS to 5%. Then we gradually adapted the cells to the CD media (Hycell, CD DG44, and medium #27) — and to low-serum conditions according to the protocol described in Table 2. Figure 2 shows that after two weeks (on day 22), when FBS was still 1.0% and the media had been substituted completely, cells growing in medium #27 (medium D, orange line), reached ~8.0 × 10^6 cells/mL. Media A, B, and C

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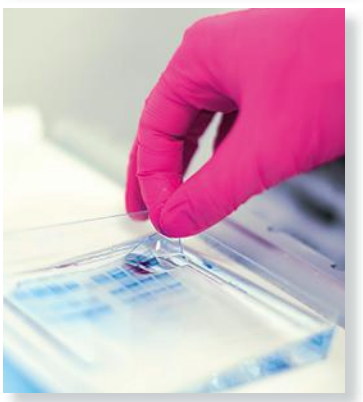

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Figure 3: Streamlined, seamless protocol for adaptation of DG44 cells from serum-containing media to chemically defined medium #27

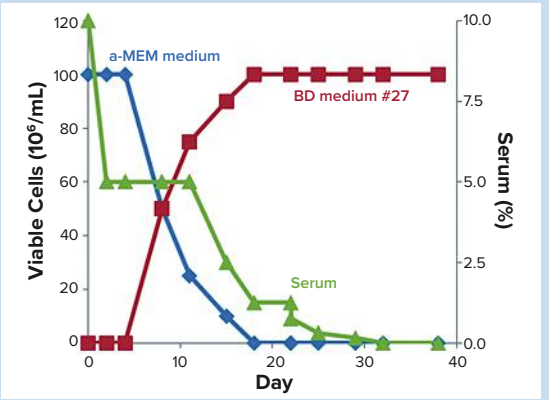
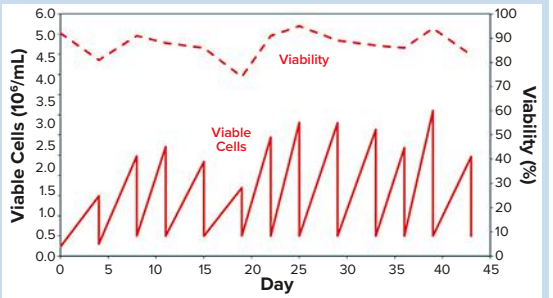


Figure 4: One vial of CHO-DG44 cells adapted to medium #27 over 12 consecutive subculturing passages was thawed in 125 mL SF (30 mL of medium #27) and expanded in 500 mL SF (150 mL of medium) over 12 passages.



sustained no substantial cell growth. Thus, we eliminated those media at day 29 for poor growth and low viability.

From days 25 and 36 (Figure 2, black arrows), we shifted two aliquots of cells grown in medium D either with 0.75% serum or serum-free) to suspension growth in two 125-mL Erlenmeyer flasks (Figure 2, SF1 violet line and SF2, green line). Figure 2 shows that the suspension-adapted cells cultivated in serum-free medium D were highly viable, both in static and agitated cultures, after repeated passages. These data demonstrate that, in slightly over a month, we were able to adapt CHO-DG44 cells from a 10% serum-containing medium to a serum-free CD medium with a seamless protocol of simultaneous serum reduction and medium adaptation. The same cells did not adapt to other media under the same experimental conditions.

Figure 3 recapitulates this streamlined, combined protocol of serum reduction and contemporary adaptation to CD medium. To demonstrate that adaptation was consistent, we created a small cell bank (10 vials) of the adapted cells as described above. Figure 4 shows that those cells displayed consistent growth and viability, once they were thawed, for more than 10 consecutive subculturing passages in shake flasks using the CD medium. This demonstrates that the adaptation protocol was efficient, and the frozen cells could be used as parental cells to generate recombinant products.

Discussion

CHO cells are the standard expression system for production of MAbs and other recombinant proteins for therapeutic use. Several commercial sources offer good manufacturing practice (GMP)-banked CHO cells and related expression plasmids, often coupled with optimized CD media that allow users to obtain stable clones with high productivity. Moreover, much effort is devoted to understanding the nutrient requirements of CHO clones and then optimizing media formulation and feeding strategies accordingly (**8**).

Although such expression kits can facilitate selection of high productivity clones greatly, the associated licensing fees can be expensive. Sometimes adoption of such ready-to-use solutions includes payment of royalties at defined steps in the clinical development of molecules derived from them and even during the commercial stage of resulting biopharmaceuticals. Those costs can be especially burdensom for small biotechnology companies, so the need remains for less expensive ways to establish stable CHO cell lines.

Reasonably priced CHO cells from commercial distributors normally come in basal media containing FBS. To limit the use of animal-origin components, regulators generally recommend that biopharmaceutical developers switch to CD media during the earliest phases of cell line development. Adopting a CD medium during early process development — ideally before DNA transfection and clonal selection — enables a company to save substantial time and resources. But standard protocols for adapting CHO cells to serum-free conditions are tedious and time-consuming, with no guarantee of positive results. Standard protocols for adapting cells to serum-free CD media can take over three months to produce fully adapted, suspension-growing cells. The usual critical point is the step from the presence of serum at a low percentage to completely serum-free conditions, when the absence of growth signals from serum-soluble factors often arrests growth.

We adopted the Thermo Fisher Scientific Advanced Bioprocessing Media Assessment Panel

(MAP) program, which includes evaluation of 20 proprietary CD media and six feeds, then selected one CD medium that enabled adaptation of an adherent-growing CHO-DG44 cell line from basal medium containing 10% serum to suspension growth in about a month. Using this CD medium, we remarkably reduced the length of the serum-free adaptation process over three different commercial media. We propose a seamless serum-reduction and new-medium–adaptation protocol that saves significant time relative to standard approaches.

With our protocol, which combines reduction of serum and gradual adaptation from basal media to a CD medium, we obtained fully adapted, suspension-growing cells in a streamlined workflow starting from adherent cultures. We speculate that the contemporary reduction of serum and gradual increase of CD medium enables cells to adapt to the deprivation of growth signals from the serum. But that was possible only with medium #27, not with other CD media we tested. This suggests that the medium formulation fits particularly well with our CHO-DG44 clone's nutrient requirements. It would be worth testing with other CHO-DG44 clones and different strains (e.g., CHO K1).

The medium also displayed flexibility in replacing other established CD media/feed platforms without the need to preadapt cells (data not shown). In fact, when we used it to inoculate cells directly in our Sartorius Stedim Biostat Univessel 2L production-phase bioreactor, we observed significant increase of cell density and MAb titers regardless of the medium used during the seed-train expansion (data not shown). This suggests that medium #27 could be used as a production medium as well, at least with our CHO clone, although its effects on product quality attributes should be evaluated carefully. Such flexible use of this medium in production could contribute to further reducing the length of our process development.

We have concluded that

• The Thermo Fisher Scientific Advanced Bioprocessing Media Assessment Panel system is a valuable tool for media screening and quickly establishing proprietary CHO-DG44 expression platforms

• Medium #27 is a flexible and valuable alternative to commercial CD media for CHO-DG44 cells, particularly when our seamless adaptation protocol is applied.

This is particularly relevant in the early phases of recombinant product development, when it is paramount to find a good media/feed platform to advance quickly in process development. To be more specific, our streamlined protocol is applicable to generation of proprietary CHO-DG44 starting from inexpensive commercial sources of such cells.

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