

Keep the Whole Process in View

From Stable Cell Lines to Robust Manufacturing Processes

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Mammalian cells have become the dominant system for producing recombinant protein products in clinical applications because of their capacity to properly fold and assemble proteins and add human-like posttranslational modifications. However, when using mammalian host cell systems, cell line and process development are often very time-consuming. For example, to meet regulatory requirements, current state-of-the-art cell culture production processes are performed in well-defined, serum- or protein-free media. Unfortunately, recombinant production cell lines are often developed in serum-containing media. As a result, after clonal selection the cells must be adapted to serum-free conditions. That increases the risk of losing productivity or product quality and significantly extends development time.

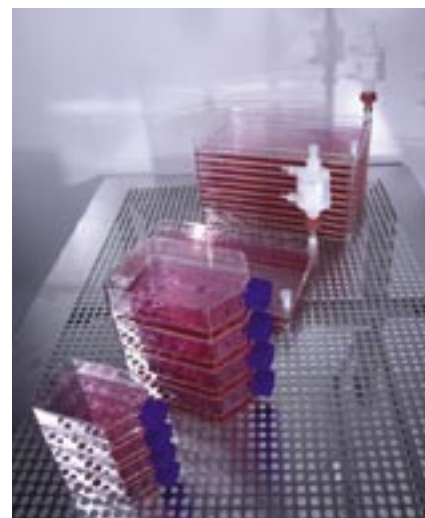
The need to speed up development may lead some companies to compromise the robustness and scalability of their manufacturing processes, often resulting in

suboptimal product yields and/or quality. As a consequence, significant redevelopment may be necessary for pivotal clinical studies and can include extensive comparability studies.

To avoid such additional costs or — often more important — time delays in clinical studies or market launch, Rentschler Biotechnologie pursues a fast-track development strategy for mammalian cell lines that considers all aspects of drug approval right from the start of the process. Beginning with the generation of recombinant cell lines, each stage of development is assessed in view of regulatory and manufacturing considerations with the intention of preventing costly redevelopment efforts.

ESTABLISHING CELL LINES WITHOUT SERUM

Once a promising drug candidate has been identified, a stable cell line must be generated to produce material for preclinical and clinical studies. Rentschler Biotechnologie has established a procedure using CHO dhfr⁻ cells (Chinese hamster ovary cells that are dehydrofolate reductase negative) allowing serum-free execution of all steps essential for development of a recombinant cell line including transfection, selection, amplification, and single-cell cloning. This established procedure significantly speeds up cell line development compared with a traditional serum-containing process while complying with regulatory requirements.



Cell line development: T-flasks and multitray units. RENTSCHLER BIOTECHNOLOGIE GMBH (WWW.RENTSCHLER.DE)

Optimization of Cell Transfection:

A procedure for serum-free transfection of various CHO cell lines (DG44, dhfr⁻, and K1) was developed based on Nucleofection technology, a special electroporation method commercially available from Amaxa Biosystems (www.amaxa.com) that facilitates DNA transpot into cell nuclei. Optimizing electrical parameters as well as the ratio of cell numbers to DNA led to a highly efficient method for serum-free transfection, although transfection efficiencies differed between the cell lines used (Figure 1).

Optimized Medium Formulations:

Single-cell cloning is an essential procedure during recombinant cell line generation for obtaining a uniform and regulatory-compliant production cell line. The process traditionally requires

PRODUCT FOCUS: ANTIBODIES AND COMPLEX GLYCOPROTEINS

PROCESS FOCUS: PRODUCTION

WHO SHOULD READ: MANUFACTURING AND PROCESS DEVELOPMENT, REGULATORY AFFAIRS, AND PROJECT MANAGERS

KEYWORDS: CELL CULTURE, SERUM-FREE, CHO CELLS, CELL BANKING, OPTIMIZATION

LEVEL: BASIC

Figure 1: CHO cells were transiently transfected with an expression vector carrying a GFP reporter gene. Protein expression was quantified by fluorescence measurements. (A) Transfection efficiency for various CHO cell lines (DG44, dhfr⁻, K1, and CHO dhfr⁻ cells cultivated in serum-containing medium); (B) optimizing the transfection efficiency of the CHO dhfr⁻ cell line by applying different pulse programs, and (C) various DNA concentrations.

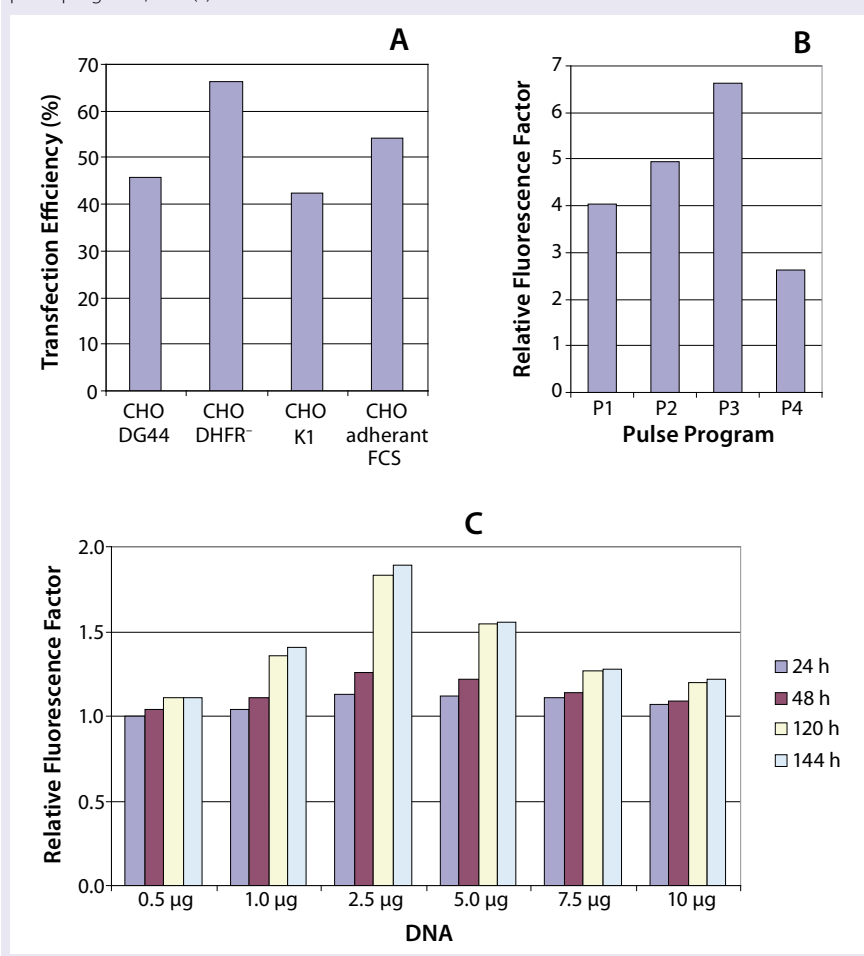
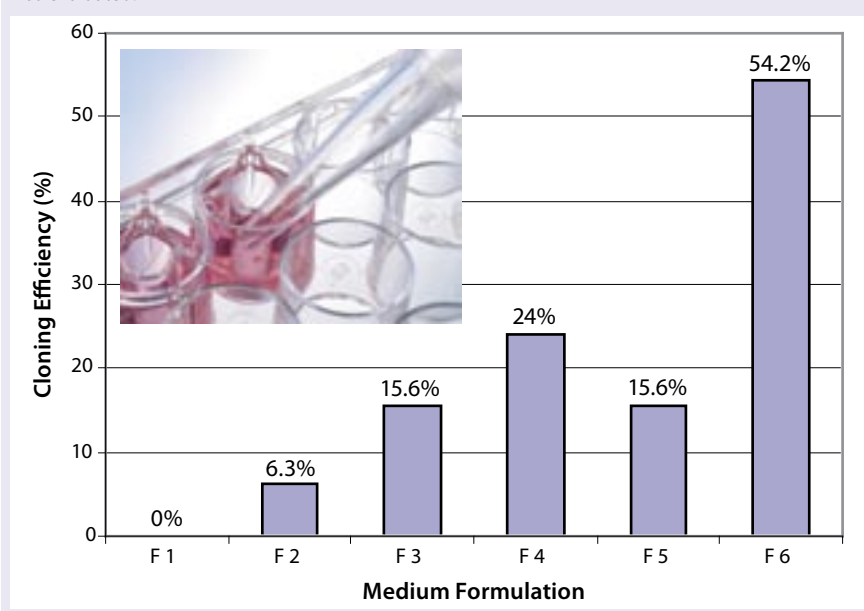


Figure 2: Different serum-free medium formulations (F1–6) were tested for cell cloning suitability. Five cells per well were seeded into 96-well plates, and the number of wells showing cell growth was evaluated.



addition of 10–20% fetal bovine serum (FBS) to a cell culture medium. But the presence of sera during single-cell cloning carries a potential risk of contamination with animal viruses or other adventitious agents such as prions. We have performed substantial medium screening and medium optimization to identify serum-free formulations that facilitate single-cell cloning of CHO cells. Those efforts yielded specific media formulations that are suitable for supporting single-cell cloning by limiting dilution without the addition of serum at any step. Figure 2 illustrates cloning efficiencies in different media.

Single-Cell Cloning: To show the productivity of single-cell clones, we transfected a growth-factor-encoding gene into CHO dhfr⁻ cells and subsequently selected among them using hypoxanthine and thymidine deprivation. After single-cell cloning, numerous cell clones were obtained and assessed with respect to growth characteristics and specific productivities to identify those most suited for development of an economically feasible cell culture process. We identified two clones that showed good growth behavior as well as a fivefold increase in cell-specific productivity compared with the cell pool obtained after stable transfection (Figure 3). A further increase of cell-specific productivity — up to 400% — followed additional optimization of the serum-free culture medium (Figure 4). Comparable results were obtained for several other proteins including monoclonal antibodies.

FROM ROBUST CELL LINES TO PHARMACEUTICAL PRODUCTION

In accordance with current good manufacturing practices (CGMPs), a cell bank referred to as the *master cell bank* (MCB) is established subsequent to selecting the most promising cell clones for production purposes. MCB cells must be suitable for culture, offer high-yield production of the recombinant protein, be genetically stable, and provide integrity of the protein product, which is assessed by a battery of protein and carbohydrate analytical methods.



USP development. RENTSCHLER BIOTECHNOLOGIE GMBH (WWW.RENTSCHLER.DE)

Figure 3: Productivity of single cell clones compared with the original cell pool; the pool was obtained by transfection of a CHO dhfr⁻ cell line with a gene encoding a human growth factor and subsequent selection by hypoxanthine–thymidine deprivation. After single-cell cloning, several hundred clones were screened for productivity (insert). Cell-specific productivities for selected clones are shown in relation to the starting cell pool (which is set to 1).

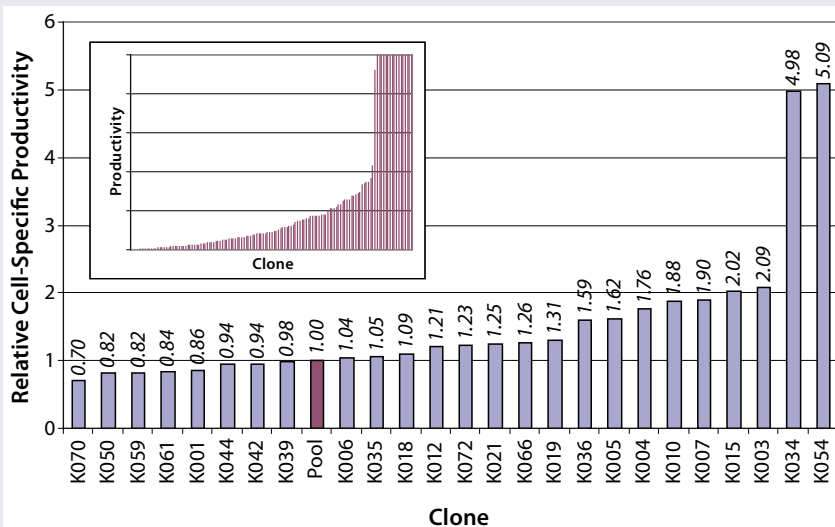
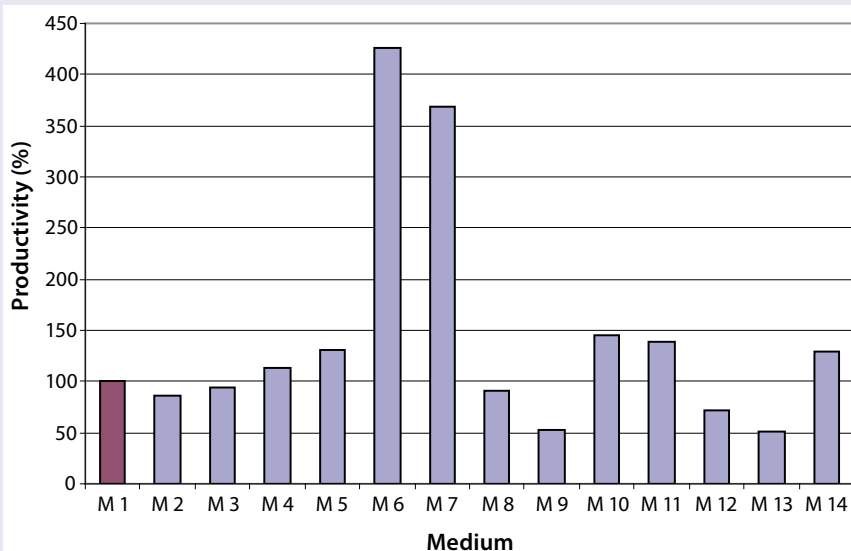


Figure 4: Cell-specific productivity of a human-growth-factor–producing single cell clone grown in different medium modifications (M1–14). M1, the medium formulation used to generate the recombinant cell line and single cell clones, was set to 100%.



Robust cell lines that are optimally suited for cell culture processes guarantee a rapid scale-up for pharmaceutical production. The yield and integrity of protein products is of vital importance for costs and further development of their production processes. Stable cell lines that show no genetic variation throughout development and production are an important regulatory requirement. Following selection of a starting clone, we use MCB cells to develop culture procedures and downstream processes, which saves time in process development.

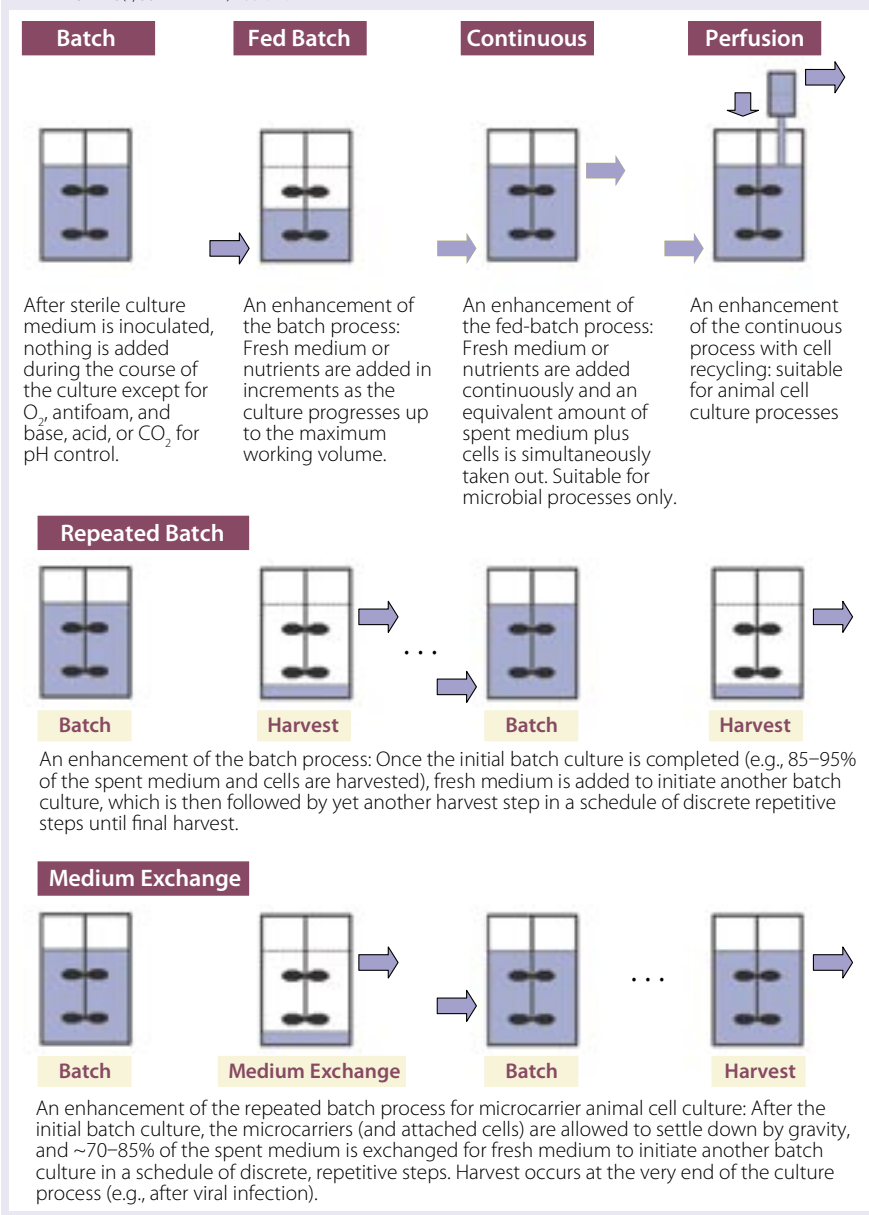
Culture in a stirred-tank bioreactor is the most widespread method for production of recombinant proteins by animal cells. Whereas the production of antibodies is mostly carried out in fed-batch mode, sensitive glycoproteins such as growth factors or proteins (e.g., proteases) whose accumulation in the culture medium can inhibit or be toxic to cell growth require perfusion or repeated-batch culture.

The Repeated-Batch Method:

Rentschler Biotechnologie has developed a repeated-batch culture process that combines the advantages of fed-batch and continuous modes. Repeated-batch culture is a discontinuous process that allows repeated harvesting (Figure 5). The repeated-batch process can be quickly adjusted to cell- and product-specific requirements, which saves time in process development. Due to the prolonged production period associated with this mode, repeated-batch culture provides an increased final product yield over that of a standard fed-batch process. Thus, smaller culture vessels can be used for production. In addition, media consumption is often lower here than in perfusion processes.

During the production phase of a repeated-batch culture, spent medium is replaced with fresh medium at preset intervals using a flow-through centrifuge (we use a Carr centrifuge from Pneumatic Scale Corporation, www.pneumaticscale.com). Intervals for medium replacement (and protein harvesting) are defined according to cellular growth and productivity as well as product stability. Repeated

Figure 5: Classification of bioreactor operational modes — batch culture is the only closed culture mode. Fed-batch, continuous, and perfusion culture are semicontinuous or continuous modes of operation as compared with repeated batch and medium exchange, which are discrete culture modes of operation. JULIEN C, WHITFORD W. BIOREACTOR MONITORING, MODELING, AND SIMULATION. *BIOPROCESS INTERNATIONAL* 5(1, SUPPLEMENT) 2007: 11.



harvesting prevents extracellular degradation (e.g., of glycoprotein oligosaccharides by accumulated glycosidases), which provides a more homogenous starting material for downstream processes and thus contributes to high yields and high-quality products.

During medium replacement, cell density can be adjusted, which allows for controlled and constant culture conditions over the whole production period, thus minimizing batch-to-batch variation (Figure 6). This helps us prevent inhibition of cell growth and productivity or reduced product

quality due to the accumulation of toxic by-products such as lactate or ammonia. Cellular growth, viability, and productivity as well as product quality remain constant throughout the overall culture process. Because of a constant high cell viability, only low amounts of residual host cell proteins (HCPs) and DNA are found in our harvests, which significantly facilitates downstream processing. We have applied the repeated-batch process for a broad range of different CHO cell lines and products under both non-GMP and GMP-compliant conditions at batch scales from 30 L to 250 L.

FOR FURTHER READING

Repeated-batch fermentation is more common in industrial biotech settings (microbial fermentation) than in biopharmaceutical production so far. Here are some potentially useful citations for more information on the concepts discussed herein, arranged from most to least recent:

Lattenmayer C, et al. Protein-Free Transfection of CHO Host Cells with an IgG-Fusion Protein: Selection and Characterization of Stable High-Producers and Comparison to Conventionally Transfected Clones. *Biotechnol. Bioeng.* 96(6) 2007: 1,118–1,126.

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Dempsey J, et al. Improved Fermentation Processes for NS0 Cell Lines Expressing Human Antibodies and Glutamine Synthetase. *Biotechnol. Prog.* 19(1) 2003: 175–178.

Gramer MJ, et al. Removal of Sialic Acid from a Glycoprotein in CHO Cell Culture Supernatant By Action of an Extracellular CHO Cell Sialidase. *Bio/Technology* 13, 1995: 692–695.

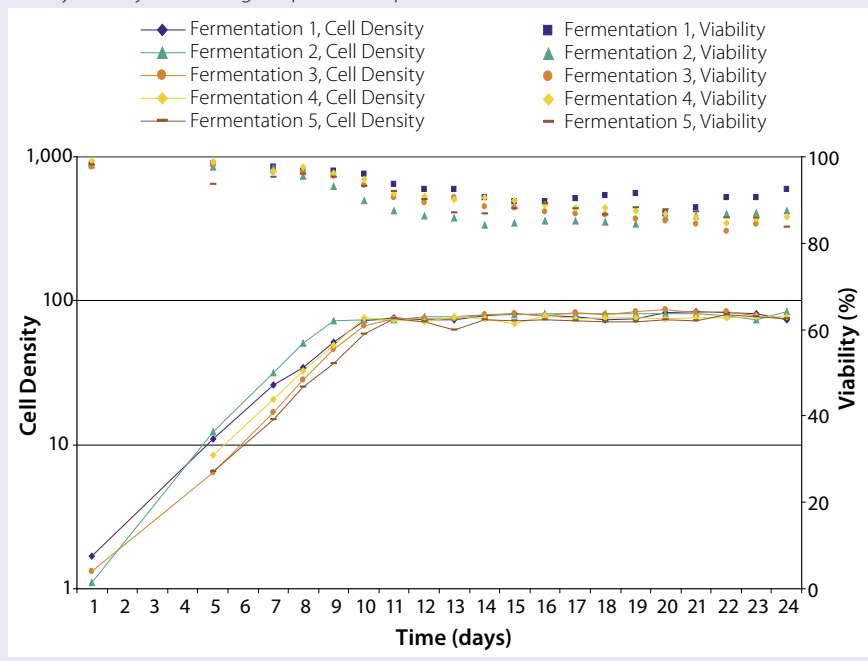
Gramer MJ, Goochee CF. Glycosidase Activities in Chinese Hamster Ovary Cell Lysate and Cell Culture Supernatant. *Biotechnol. Prog.* 9, 1993: 366–373.

FAST-TRACK, HIGH-PERFORMANCE, HIGH-QUALITY DEVELOPMENT

Time to clinic is of vital importance in the development of biopharmaceutical products. The need to speed up development times is always urgent, but the search for quick fixes often leads to compromises in scalability, productivity, and/or quality. Consequently, the development process results are not always feasible for manufacturing. Costly and time-intensive redevelopment is often necessary.

Instead, development experts can work closely together, considering all

Figure 6: To evaluate cell growth and viability during repeated batch fermentations, five fermentation runs were performed successively in the repeated-batch mode. A growth period of seven days was followed by a production period of 17 days. Daily harvests were made, and cell density was adjusted during the production period.



aspects of drug approval right from the start of the development process. At each stage, activities should be evaluated in view of regulatory and manufacturing considerations with the aim of making costly redevelopment efforts unnecessary. This integrated development concept — involving recombinant protein expression, product development, and overlapping activities from the start — permits fast-track but economically and technically feasible product development. 🌐

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