A Hollow Fiber Bioreactor Allows Any Lab to Efficiently Express Recombinant Proteins in Mammalian Cells

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Cultured mammalian cells have become the predominant platform for the production of recombinant proteins for clinical applications due to their capacity for proper protein folding, assembly and post-translational modification. Thus, the quality, bioactivity and efficacy of a protein can be superior when expressed in mammalian cells versus other hosts such as bacteria, insect cells and yeast. Proper glycosylation and post-translational modifications can only be obtained by expression in mammalian cells. This superiority of protein quality is also important in the research laboratory, for proper biological activity and especially for potentially therapeutic and difficult-to-express proteins.

However, the production of recombinant proteins and conditioned medium from mammalian cells in the typical research laboratory can be such a cumbersome process that for many the use of mammalian expression systems is avoided. Large numbers of plates, flasks or roller bottles are required, a large volume spinner flask or the use of an expensive stirred tank vessel may be required for scale-up. Low density suspension cultures or 2-D flask based processes are inherently non-physiologic. While well-understood, robust, and convenient, classical batch-style 2-D cultures are not very biologically relevant systems.

Hollow fiber bioreactors (HFBR) provide a more physiologic, in vivo-like 3-D environment than other cell culture methods, and can also result in improved protein folding and more uniform post-translation modifications over time. Protein concentrations are in the range of 100 µg to 300 µg/mL/day and the production of 10’s of milligrams on up to gram quantities of proteins is possible. HFBRs provide a tremendous amount of surface area for cell attachment and the high cell density permits the use of our CDM-HD protein-free serum replacement. The protein of interest becomes a significant component of the harvested supernatant and the small volumes facilitate purifications. Secreted proteins can be 100 times more concentrated vs. tissue culture supernatant.

ADVANTAGES OF HOLLOW FIBER BIOREACTORS

- Use of protein free CDM-HD facilitates purification.
- Small harvest volume for easy handling and more efficient down-stream processing.
• No splitting of cells required, cartridge maintenance just 15 minutes a day.
• Cultures can be maintained for several months of production.
• Optimal cell culture conditions can result in improved protein assembly and folding.
• Relatively small number of cells required for seeding, no seed reactor required.
• Lower apoptosis, host cells protein and DNA contamination reduced.
• Constant replenishment of nutrients and removal of waste products for homeostatic cell culture conditions. Removal of ammonia is especially important for proper glycosylation.

In order to more closely approximate in vivo cell growth conditions, Richard Knazek developed the HFBR in 1972. The HFBR is a high-density, continuous perfusion culture system (see Figure 1). It consists of thousands of semi-permeable hollow fibers in a parallel array within a tubular housing (or cartridge) fitted with inlet and outlet ports. These fiber bundles are potted at each end so that any liquid entering the ends of the cartridge will necessarily flow through the interior of the fibers. Cells are generally seeded on the outside of the fibers in what is referred to as the extra capillary space (ECS).

Culture medium is recirculated through the insides of the fibers, allowing nutrients and waste products to diffuse across the fiber walls (see Figure 2). Once it has passed through the cartridge, the culture medium is oxygenated and recirculated to the cartridge. Three fundamental characteristics differentiate hollow-fiber cell culture from any other method:

1) Cells are bound to a porous matrix much as they are in vivo—not a plastic dish, micro carrier bead, or other impermeable support.
2) The molecular weight cut-off (MWCO) of the support matrix can be controlled.
3) There is an extremely high surface area-to-volume, 150 cm² to 200 cm² per mL.
CELLS ARE BOUND TO A POROUS SUPPORT MUCH AS THEY ARE IN VIVO

Cells bound to a porous support do not require splitting and passage number becomes irrelevant. Cells in a HFBR maintain viability in a post-confluent manner for extended periods of time—months or longer. A glioma cell line maintained productivity for nearly two years of continuous culture. The lack of passaging and the maintenance of biologically homeostatic culture conditions results in improved folding and complete and uniform post-translational modifications. The more in vivo-like growth conditions and lack of shear within a HFBR also result in significantly reduced apoptosis. The majority of cells become necrotic and do not release host cell proteins, lysozyme nor DNA into the culture medium resulting in a product that is cleaner and easier to purify from the bulk harvest.

THE MOLECULAR WEIGHT CUT-OFF OF THE FIBER CAN BE CONTROLLED

HFBR are available with a 5 kd and 20 kd MWCO. Secreted proteins are retained to significantly higher concentrations, 50-100 X, and the effects of cytokines can also be controlled.

THERE IS AN EXTREMELY HIGH SURFACE AREA-TO-VOLUME RATIO

The small diameter of the fibers (200 µm O.D.) creates a surface-area-to-volume ratio of 150-200 cm²/mL volume. Imagine a film 75 microns thick. When coupled with the high gross filtration rate of the polysulfone fibers, the exchange of nutrients and waste products across the fibers is very rapid. Cell densities of 1-2 X 10⁸ cells per mL are achieved; close to in vivo-like densities.

A 20 mL cartridge will support as many cells as a 2 L spinner flask or 20-40 roller bottles. High cell densities produce more protein per milliliter volume than standard cell cultures, and also facilitates the adaptation to lower serum concentrations or a simplified, protein-free serum replacement such as CDM-HD from FiberCell Systems. The high densities allow the cells to auto-support using their own cytokines and conditioning factors. The use of protein-free media results in much cleaner harvests of products and simplified purification. Yields can be improved by reducing the number of purification steps required.
CONCENTRATED PRODUCT FOR HIGH YIELDS AND EASIER PURIFICATIONS

The above features of hollow fiber cell culture result in protein concentrations that can be 100 times higher than those found in flask or spinner culture, with no contaminating proteins from the cell culture medium and reduced host cell protein contamination. The more in vivo-like cell culture conditions result in improved protein folding and more uniform and complete post-translational modifications over time. Since it is a continuous perfusion system, the amount of protein produced is determined both by the length of time of culture and by the size of the cartridge. Harvest volume is 20 mL and protein concentrations can range from 50 µg/mL to 300 µg/mL per day. The C2011 or C2008 cartridge will typically consume one liter of medium every two days and produce 2-5 mg of protein every day, while the C2018 and C2003 cartridges will consume two liters of medium per day and produce between 5 and 20 mg of protein every day.

CDM-HD SUPPORTS MAMMALIAN CELLS UNDER HIGH DENSITY HOLLOW FIBER CELL CULTURE CONDITIONS

CDM-HD is a chemically defined, protein free, animal component free, cGMP compliant serum replacement optimized for high density culture. The specific high density cell culture conditions inside a hollow fiber bioreactor are different enough from standard cultures that a cell culture medium can be simplified, specifically designed for, and optimized to take advantage of these conditions.

CDM-HD contains specific micronutrients, amino acids, free iron, and additional buffering capacity as part of its proprietary formulation. CDM-HD does not contain surfactants. It is supplied as a dry powder to make one liter, and is used at 10% with standard basal media such as DMEM. It can be used as a serum replacement for most cell types, except those that are cholesterol dependent. CDM-HD does not work well in flask or spinner culture as it contains no protein attachment factors and the cells need to be at high density in order to be supported by it.

The use of CDM-HD in a hollow fiber bioreactor system eliminates contaminants such as lipids, endotoxin, proteins, intracellular DNA, viruses and other adventitious agents. Lack of these contaminants can simplify regulatory compliance and reduce the purifications steps required, improving overall yields as well. CDM-HD is more than a serum replacement. It is a direct manifestation of the unique cell culture environment provided by a hollow fiber bioreactor.
A hollow fiber bioreactor from FiberCell Systems is the ideal method for the \textit{in vitro} production of 25 mg on up to gram quantities of a recombinant protein. The following cartridges are available from FiberCell Systems.

### Cartridge Specifications

<table>
<thead>
<tr>
<th>Catalog No.</th>
<th>Size</th>
<th>Surface Area</th>
<th>Fiber Type</th>
<th>Packing Density</th>
<th>ECS Vol</th>
<th>MWCO 50%</th>
<th>Max. Cell#</th>
</tr>
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<tbody>
<tr>
<td>C2003</td>
<td>Large</td>
<td>1.2 m²</td>
<td>low flux PS</td>
<td>50%</td>
<td>70 mL</td>
<td>5 kd</td>
<td>$5 \times 10^{10}$</td>
</tr>
<tr>
<td>C2008</td>
<td>Medium</td>
<td>3000 cm²</td>
<td>low flux PS</td>
<td>50%</td>
<td>20 mL</td>
<td>5 kd</td>
<td>$10^9$</td>
</tr>
<tr>
<td>C2011</td>
<td>Medium</td>
<td>3000 cm²</td>
<td>high flux PS</td>
<td>50%</td>
<td>20 mL</td>
<td>20 kd</td>
<td>$10^9$</td>
</tr>
<tr>
<td>C2018</td>
<td>Large</td>
<td>1.2 m²</td>
<td>high flux PS</td>
<td>50%</td>
<td>70 mL</td>
<td>20 kd</td>
<td>$5 \times 10^{10}$</td>
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### CASE STUDIES

**Production of a Hexeramized Recombinant Protein from CHO Cells**

Production of a hexeramized IgG consisting of 6 IgG1 subunits held together by 3 IgA tails. When grown in flask culture (top trace) about 40% of the protein is expressed as an incompletely folded monomeric sub-unit. When the same cells are culture in the FiberCell Systems hollow fiber bioreactor module, more than 90% of the protein is expressed as a properly folded hexamer. This is due to the superior cell culture.
conditions inside the hollow fiber cartridge. Medium used was DMEM with 2% FBS. 478 mg of purified protein was produced in FiberCell Systems catalog number C2018 in a period of 8 weeks in a collected volume of less than 5 liters.

**CHO Expression**

246 mg of purified recombinant IgG1 from a CHO (DG44) cell line was harvested from the C2011 20 kd MWCO cartridge. Medium was a DMEM 10% CDM-HD. Each harvest was 19 mL in volume; total harvest volume was 304 mL for an average concentration of over 800 micrograms per day per milliliter. The cartridge consumed 1 liter of medium every two days and the culture was maintained for a total of 35 days. Even though cell viability in the harvest was fairly low the expressed protein was very clean as demonstrated by the gel of the unpurified harvest shown below. Contamination with cellular proteins and DNA was quite low.

**293T Expression**

Recombinant immunoglobulin produced by 293t cells in a C2011 cartridge. This gel nicely demonstrates the reduction in contaminating serum proteins after switching to CDM-HD. In this case the researcher harvested every two days rather than every day, so the elimination of serum proteins takes a little longer. Note that the IgG is the dominant protein in the unpurified supernatant after the switch to CDM-HD is complete.

**BHK Cells Cultured with FiberCell Systems CDM-HD**

BHK cells had initial popularity as serum free media were developed early on to support their culture in suspension for scale-up. However, there were issues with productivity under serum free conditions in some cases. FiberCell Laboratories cultured BHK cells expressing PEDF (pigment epithelium-derived factor, MW approximately 50KD) using DMEM 10% FBS followed by a switch to DMEM 10% CDM-HD on day 7 with no adaptation. Data demonstrates stable glucose
uptake rate and no loss of PEDF production when using CDM-HD. BHK cells can be cultured in hollow fiber bioreactors using protein-free medium with no loss of production.

<table>
<thead>
<tr>
<th>Cartridge used</th>
<th>Cat #C2008 5 KD MWCO PS cartridge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells inoculated</td>
<td>3 X 10⁸ cells</td>
</tr>
<tr>
<td>Medium Consumed</td>
<td>14 L</td>
</tr>
<tr>
<td>Total protein produced</td>
<td>99.8 mg</td>
</tr>
<tr>
<td>Total volume of harvest</td>
<td>164 mL</td>
</tr>
<tr>
<td>Average concentration</td>
<td>0.6 mg/mL</td>
</tr>
</tbody>
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Above: Culture was terminated at 22 days as the target of 100 mg of protein produced was reached.

Mammalian Overexpression of IL-12

A stable, high producing cell clone 293-H/78 was cultured in a hollow fiber bioreactor in serum-free medium. SDS-PAGE of culture supernatants showed two prominent closely moving bands corresponding to the p40 (Mw ~40 kDa) and p35 (Mw ~ 35 kDa) subunits of IL-12. The average concentration of IL-12, as assessed by ELISA, in culture supernatants was 364 ± 62 µg/mL. In addition, the total amount of protein in culture supernatants was measured using the Bradford protein assay. Results indicated that IL-12 accounts for ~25% of total proteins in culture supernatants.

![Figure 3: Purified recombinant IL-12 is well-folded and biologically active.](image)

![Figure 4: Amino acid sequence and three-dimensional structure of hIL-12.](image)
Production of IL-15 Receptor Complex

IL15 receptor complex is perhaps the ultimate difficult-to-express protein. It consists of two subunits, held together by hydrophobic interaction and is 45% glycosylated.

The IL15 heterodimer demonstrates superior pharmacokinetics and in vivo bioactivity to the single chain IL15 expressed in E.coli. Expression in standard cell culture systems is problematic. Efficient production of this noncovalently linked but stable heterodimer in clonal human HEK293 cells is demonstrated in a 5 kd MWCO hollow fiber bioreactor. Cell supernatants (20 mL) were harvested daily for up to 5 months and assayed for IL-15 levels by ELISA (R&D Systems).

IL-15 is an important cytokine with potential clinical applications as a lymphocyte growth and activation factor. Recombinant human IL-15 generated in E.coli has been produced as a non-glycosylated monomer of 12 kDa. Although monomeric E.coli-produced IL-15 is in the initial stages of clinical testing, this form of the molecule poses multiple challenges for clinical use due to its instability and rapid plasma clearance. HEK293 human cells produce correctly folded, processed, and glycosylated human IL-15 sIL-15R heterodimeric cytokine.

The superior bioactivity of IL-15 in the heterodimeric formulation is mainly the result of the presence of IL-15R contributing to increased stability of the protein in vivo. These properties offer the potential to allow lower and less frequent dosing and simpler delivery methods, with increased convenience for both patients and caregivers.

ADVANTAGES OF PROTEIN EXPRESSION IN MAMMALIAN CELLS

In 1986 human tissue plasminogen activator (tPA, Activase; Genentech, S. San Francisco, Ca. USA) became the first therapeutic protein based upon recombinant expression in mammalian cells to be approved by the FDA. Expression in mammalian...
cells remains the primary methodology for therapeutic protein biopharmaceutical production of approved products, with hundreds more in corporate pipelines. Most of these proteins are expressed in Chinese Hamster Ovary (CHO) cells but other cell lines such as mouse myeloma (NSO), baby hamster kidney (BHK), and human embryonic kidney (HEK-293) cells.

This is a most interesting article from 1988, just as a side note: http://articles.latimes.com/1988-10-14/business/fi-3949_1_tpa-production

Different methods were used for therapeutic protein production in the early years, and at least one product was manufactured in a huge automated roller bottle facility. Production of recombinant proteins at the manufacturing scale has been refined over the years to follow a standard scheme, in part to facilitate regulatory compliance. The producing cells are adapted to suspension culture (or attached to microcarriers) to allow for large scale suspension culture in stainless steel stirred tank vessels, up to 10,000 or 20,000 liters in size. Cell culture medium formulations are endlessly tested and optimized, along with control parameters such as pH, dissolved oxygen, CO2 etc. Cells are seeded into the reactor, consume the medium and when it is exhausted the secreted product and cells are harvested from the reactor.

Production of mammalian expressed proteins in a hollow fiber bioreactor does not require adaptation to suspension culture, and medium optimization is not required in the initial stages, saving valuable time.

Protein research at the laboratory scale is the basis for the development of therapeutic products. It is critical that these be produced in a form that retains all of the characteristics of the final product so that results seen the research lab can be extended to the clinic.

Expression of proteins and especially difficult-to-express proteins in mammalian systems is efficient and cost effective in a hollow fiber bioreactor. There are many advantages to working with a protein that is correctly folded with tertiary structure intact.

1) Solubility is maintained.
2) Proper bioactivity.
3) Improper glycosylation from other methods can result in antigenicity and immunogenic reactions when infused into animals.
4) Half-life and pharmacokinetics can be shortened by improper folding and glycosylation.
HOLLOW FIBER AND DIFFICULT-TO-EXPRESS PROTEINS

Difficult-to-express proteins can be proteins that are expressed at low titers by mammalian expression systems. They are also defined as highly complex proteins; highly glycosylated, with high levels of post-translational modifications and in some cases consisting of several subunits. Case studies using HFBRs have demonstrated superior expression of these types of proteins such as the hexerimized IgG and the IL15 ligand-receptor complex. The next generation of therapeutic proteins include bi-specific and tri-specific antibodies, protein structures that are the creation of bio-engineers and not found in the templates of nature. These proteins require the in vivo-like cell culture conditions and concentration that HFBRs and mammalian expression systems provide.

A hollow fiber bioreactor system from FiberCell Systems allows any laboratory to take advantage of the superior folding, glycosylation and complete post-translational modifications that only expression in mammalian cells can provide. In vivo-like cell densities, constant provision of nutrients and removal of waste products result in complete and uniform post-translation modifications over long term cultures. The removal of ammonia specifically is a factor that contributes to proper and consistent glycosylation. Hollow fiber bioreactors are an effective method for producing milligram to gram quantities of recombinant proteins. The harvested product is concentrated and free of contaminating proteins, DNA, RNA, and proteases. Use of CDM-HD renders the medium used, economical and chemically defined/protein-free. Cultures can be maintained for long periods of time, meaning that scalability of the system is determined by length of culture, not new equipment. The protein-free medium and high-concentration product simplifies downstream processes, and can result in increased yields by reducing the steps required for purification. A hollow fiber bioreactor from FiberCell Systems is the ideal method for the production of 50 mg on up to gram quantities of recombinant proteins from mammalian cells, and is particularly useful for the production of difficult-to-express proteins.