Production of Monoclonal Antibodies from Hybridomas in a Hollow Fiber Bioreactor

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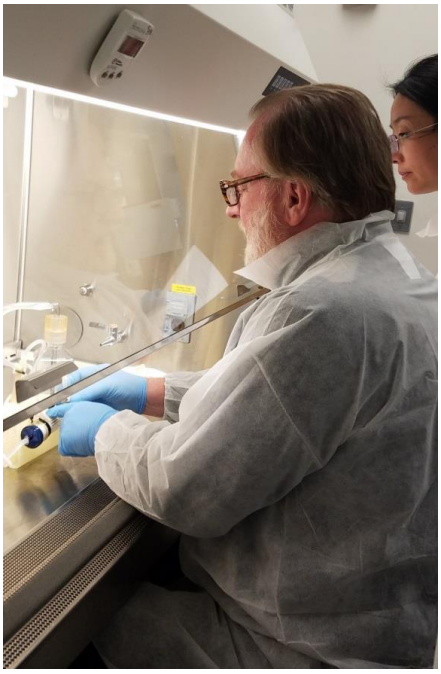
César Milstein, Ph.D. was awarded the 1984 Nobel Prize in Physiology or Medicine jointly with his former postdoctoral fellow Georges J. F. Köhler and theoretician Niels Jerne. Milstein and Köhler won the prize for developing the hybridoma method of producing monoclonal antibodies. Today, monoclonal antibodies continue to be at the cutting edge of medical research, as scientists continue to discover uses for them in both diagnostic and therapeutic applications. Nowhere has the impact been greater than in the field of oncology, where researchers are developing monoclonal antibodies that bind specifically to tumors and to target oncolytic drug delivery. At the time of their invention there was not much recognition of the vast impact that hybridoma technology would have on both research and therapy. For a more complete look at this story, which shows the human side of research click here: www.whatisbiotechnology.org/exhibitions/milstein/introduction



Prior to that the “one cell, one antibody” theory was becoming well accepted i.e. that a single B cell could be stimulated to produce a single antibody in response to a specific antigen, and that many different B cells contribute to the polyclonal response observed in antigen challenge. Prior thinking held that antibody diversity was due to either mutational drift, or the fitting of antigens into a pre-determined genetic template created over time by evolution. The presence of an antigen would stimulate the specific resident cell to proliferate in response to the presence of the antigen. The story is told here: www.jimmunol.org/content/182/3/1229.full

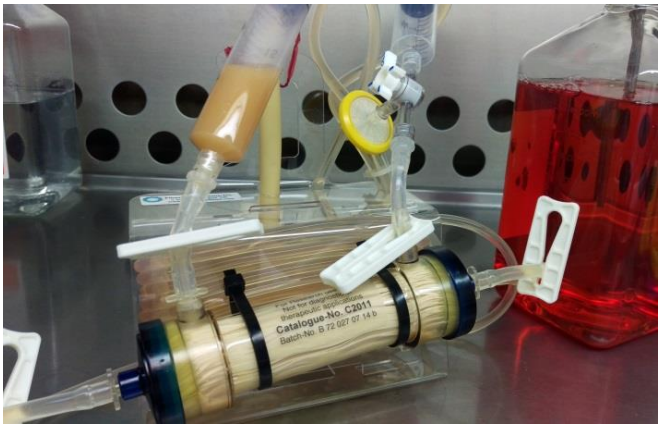
Milstein’s invention of hybridoma technology not only revolutionized the utility of antibodies by allowing the generation and production of a single antibody with a single specificity from a single clone, it also allowed monoclonal antibodies to be produced *in vitro*, instead of requiring an animal host. Interestingly, after more than 40 years of research and development, an intact *in vivo* immune system is still required to generate the specific B cell for hybridoma fusion.

HYBRIDOMA CELLS INHIBIT THEIR OWN PROLIFERATION

Milstein recognized at the time that when you fuse a B cell with a cancer cell to generate a hybridoma, you create a cell that inhibits its own growth. One of the ways that cancer cells locally immune suppress is by secreting a factor called TGF-Beta. TGF-Beta has a molecular weight of 27 kd and functions to inhibit the growth and activity of B cells. The molecular weight of an IgG is 147 kd. If you can remove the TGF-Beta while retaining the IgG, you can greatly enhance the production of monoclonal antibodies in cell culture.

The invention of hybridoma technology created the perfect platform for the application of hollow fiber bioreactors: allow TGF-Beta to diffuse away from the cells, while concentrating the antibody to high levels inside the hollow fiber bioreactor itself. Based upon this principle hollow fiber bioreactors are the ideal system for the production of monoclonal antibodies from hybridoma cell lines.

RECAPITULATING *IN VIVO* CONDITIONS FOR HIGH FIDELITY PROTEIN PRODUCTION *IN VITRO*



The production of secreted products from mammalian cells such as recombinant proteins and monoclonal antibodies is generally performed in the laboratory by standard flask, roller, or spinner culture. Only recently has it been recognized how culture conditions can dramatically affect protein quality. In conventional culture systems, cells that originally grew attached to a porous matrix at high densities with little variability in per-cell nutrient and oxygen supply (i.e. homeostatic *in vivo* conditions) are adapted to low-density plastic-bound 2-D conditions or suspension culture. While well-understood, robust, and convenient, classical batch-style 2-D cultures are not very biologically relevant systems. Cell culture conditions affect the quality and purity of the antibody or protein produced.

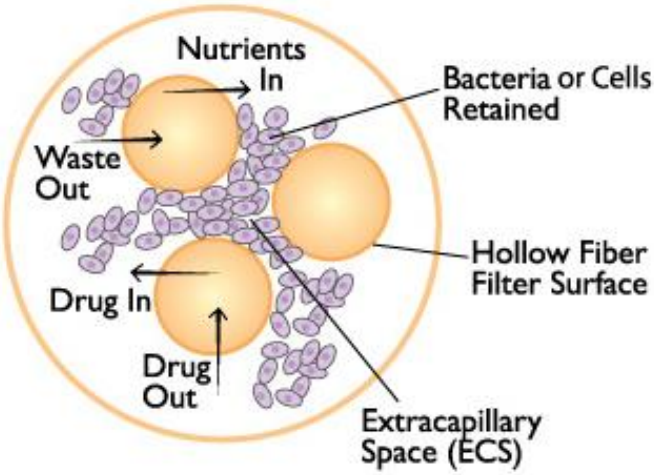
In order to more closely approximate *in vivo* cell growth conditions, Richard Knazek developed the hollow fiber bioreactor (HFBR) in 1972. The HFBR is a high-density, continuous perfusion culture system (see Figure 1). It consists of thousands of semi-permeable hollow fibers in a parallel array within 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 both ways 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 cm^2 to 200 cm^2 per mL.

Cells are bound to a porous support much as they are *in vivo*. It is not necessary to split cells and passage number is irrelevant. Cells in a HFBR maintain viability in a post-confluent manner for extended periods of time–months or longer. For example, one hybridoma was reported to maintain productivity for over one year 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 result in significantly reduced apoptosis. The majority of cells become necrotic and do not release cytoplasmic 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. Desired products can be retained to significantly higher concentrations, and the effects of cytokines can also be controlled. This is especially important for hybridoma culture, in which the inhibitory cytokine transforming growth factor (TGF) beta can be selectively removed from the culture while the secreted antibody is retained. The FiberCell C2011 and C5011 cartridges with a 20 kd MWCO (50%) are ideal for hybridoma culture.



There is an extremely high surface area-to-volume ratio. The small diameter of the fibers (200 um O.D.) creates a surface-area-to-volume ratio of 150-200 cm^2/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 10^8 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 (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.

SERUM-FREE AND PROTEIN-FREE MEDIUM READILY SUPPORTS HYBRIDOMAS 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. CDM-HD is designed to take advantage of the unique, high density cell culture conditions found within a hollow fiber bioreactor. It contains specific micronutrients, amino acids, free iron, additional buffering capacity, and no surfactants as part of its proprietary formulation. It is supplied as a dry powder to make one liter, and is used at 10% with standard basal mediums such as DMEM. It can be used as a serum replacement for most cell types, except those that are cholesterol dependent. The cell culture conditions inside a hollow fiber bioreactor are different enough from standard cultures that a cell culture medium can be specifically designed to take advantage of these conditions. CDM-HD does not work well in flask or spinner culture as 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 perhaps 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.

CONCENTRATED PRODUCT FOR HIGH YIELDS AND EASIER PURIFICATIONS

The above features of hollow fiber cell culture result in protein and antibody concentrations that can be 100 x higher than those found in flask or spinner culture, with almost no contaminating proteins from either the cell culture medium or the cells themselves. The more *in vivo*-like cell culture conditions result in improved protein folding and more uniform and complete glycosylation patterns 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 20-40 mL and antibody concentration can range from .5 mg/mL to 5 mg/mL. The C2011 cartridge will typically consume one liter of medium every two days and produce 5-50 mg of antibody every two days, while the C5011 will consume a liter of medium per day and produce between 10-100 mg every two days. Since no animal host is required (such as mice or nude mice), chimeric (mouse/human) and non-murine antibodies such as rabbit, rat and hamster can be easily produced.

A hollow fiber bioreactor from FiberCell Systems is the ideal method for the *in vitro* production of 25 mg on up to gram quantities of a monoclonal antibody.

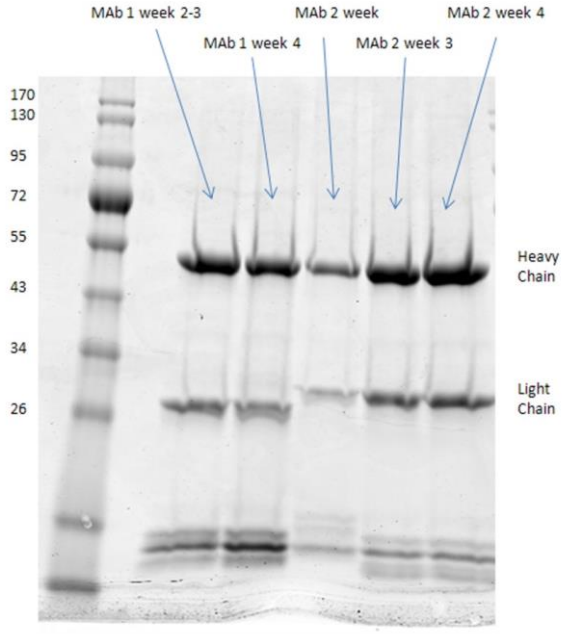


Figure 4: Hybridoma cell line using FiberCell Systems CDM-HD

- MAb 1–2.6 mg/mL week 2 & 3 harvest: (40 mL)
- MAb 1–3.2 mg/mL week 4 harvest: (20 mL)
- MAb 2–0.8 mg/mL week 2 harvest: (25 mL)
- MAb 2–3.2 mg/mL week 3 harvest: (20 mL)
- MAb 2–3.0 mg/mL week 4 harvest: (25 mL)

Data courtesy of Dr. Erin Bromage, University of Massachusetts, Amherst.

"Thanks for the great product! I am actually more impressed with the Bioreactor now using the CDM-HD than I was using FBS. CDM-HD is cheaper than FBS, no purification required (Protein A or Melon gel), and the yields are about the same."
– Dr. Erin Bromage

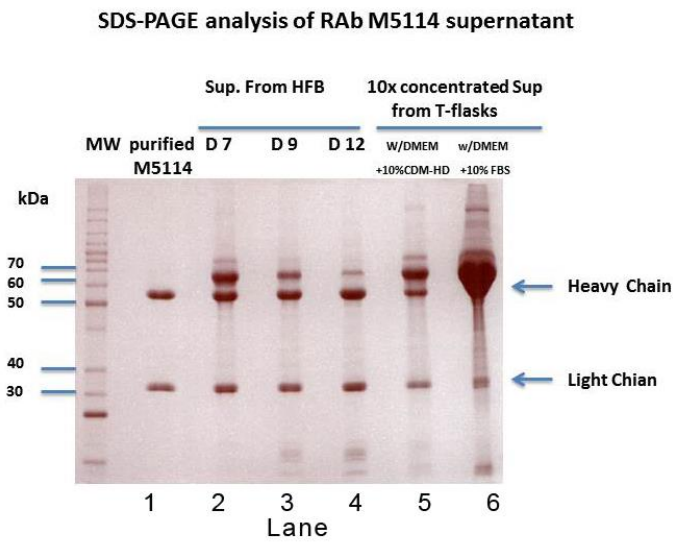


Figure 5: RAb M5114 Production results from the Fibercell Systems hollow fiber bioreactor.

Cartridge: C2011 20 kd MWCO polysulfone fiber
Medium: DMEM with 10% CDM-HD
Protocol: per the FiberCell Systems manual

In the gel shown, purified M5114 was a positive control purified from Protein G column, lane 1. Lanes 2, 3, and 4 are harvests from the cartridge on day 7, 9 and 12. No purification has been performed. Note how clean the harvests are and free from extraneous proteins. Lane 5 is 10 X concentrated supernatant from a T-flask using DMEM and 10% CDM-HD. Lane 6 is from a T flask using DMEM and 10% serum. 3 µg of control was loaded, and the supernatant from HFB was loaded with 2 µL of each. The supernatant from T-flasks were 10 X concentrated and 2 µL of each were loaded. Based on the gel, it is estimated that the concentration of supernatant from HFB was about 2.5 mg/mL. 140 mL of supernatant was harvested to date for a total about 350 mg of non-purified antibody. Both SDS-PAGE gel and gel filtration analysis showed that the antibody from the hollow fiber bioreactor was very clean and properly folded. The cartridge was allowed to run for several weeks after this initial data was collected, producing additional antibody. *Data courtesy of Liying Lu, University of Massachusetts, Worcester, Worcester, Massachusetts.*

COMPARISON TO A WAVE BAG

To demonstrate the capacity of the C2011 HFBR cartridge and the utility of CDM-HD, a direct comparison was performed between monoclonal antibody production from the same standard murine SP2 clone in a 20 L wave bag containing 10 L of medium, T175 tissue culture flasks (1.2 L consumed, data normalized to 10 L for comparison) and a FiberCell Systems HFBR Flask data and Wave bag data were generated using a commercially available serum free hybridoma medium (SFM), while FiberCell Systems data was generated using DMEM medium with 10% CDM-HD chemically defined serum replacement. Total protein was measured before and after Protein A purification and antibody quantitation performed on column eluent.

The FiberCell Systems cartridge produced 5 X the amount of antibody as the Wave bag produced, at 50 X higher concentration with no exogenous protein. The FiberCell Systems cartridge produced slightly more antibody than the T175 flasks but at a concentration 50 X higher with no detectable exogenous protein. CDM-HD is optimized for higher density cell culture, and therefore the media employed in this comparison were not identical. The comparison demonstrates the power of the

combination of hollow fiber bioreactors and a medium designed to take advantage of this higher cell density. It should also be noted that the Wave bag is a 1-2 week batch mode process, while the FiberCell Systems cartridge can be maintained producing antibody for more than 6 months if so desired, further increasing amount of antibody produced per reactor cycle or annum.

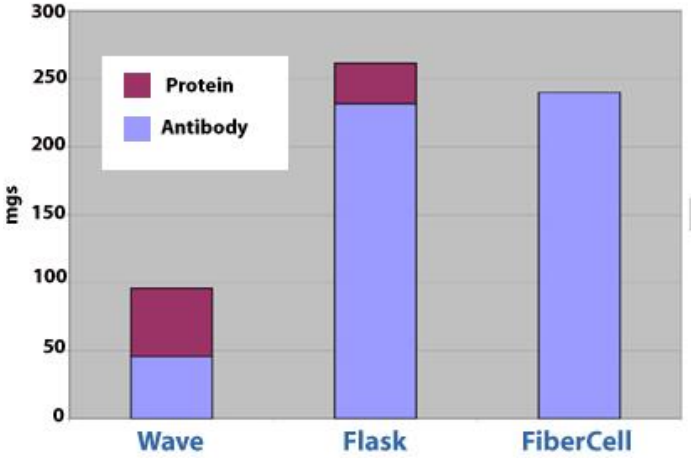


Figure 3: Purified antibody and ratio of antibody to total protein for the three systems. Flask culture yielded almost as much antibody as the FiberCell Systems cartridge, however the antibody in the FiberCell Systems harvests is about 50X more concentrated. Note the large amount of extra protein from the wave bag, probably due to cell lysis. Non-antibody protein in the flask is from the serum free media, which is not protein free. FiberCell Systems culture when used with CDM-HD is free from exogenous protein and cell lysis.

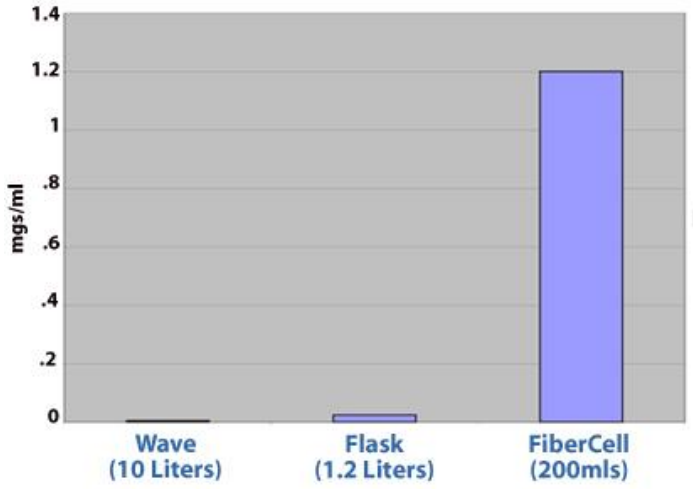


Figure 4: Antibody yield per 10 L of medium consumed, total harvested antibody concentration prior to purification. Note the extremely high concentration of antibody in the FiberCell Systems hollow fiber bioreactor cartridge. 200 mL of harvested supernatant was equivalent to 10 L of culture supernatant from the other systems again reflecting the 50 X higher concentration of antibody generated in the FiberCell Systems bioreactor cartridge.

SCALING UP

The scale-up of production in a HFBR is quite simple. The C5011 cartridge can be managed in the same manner as the C2011 but with 2 X greater productivity and twice the antibody concentration. The C5011 is the same cartridge as the C2011 but with more than twice the oxygenation capacity.

DATA SHARED BY A FIBERCELL SYSTEMS CUSTOMER

Cartridge	C5011
Cell Line	SP2 Hybridoma
Medium	H-SFM
Medium Consumed Per Day	1 L
Total Medium Consumed	66 L (11 weeks production)
Total Antibody Produced	1.7 grams after purification
Total Harvest Volume	450 mL
Average Concentration (after purification)	3.77 mg/mL

HFBRs are an effective method for producing milligram to gram quantities of monoclonal antibodies and recombinant proteins. The harvested product is concentrated and free of contaminating proteins, DNA, RNA, and proteases. Use of CDM-HD renders the medium used both economical and chemically defined/ protein-free. Cultures can be maintained for long periods of time, scalability of the system is determined by length of culture, not new equipment. HFBR from FiberCell Systems give the power of hollow fiber cell culture to any laboratory.

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