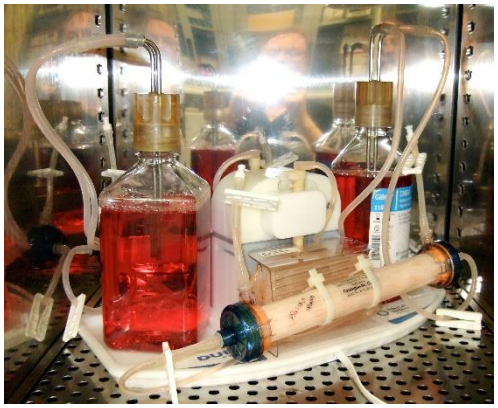
Hybridoma Troubleshooting Guide



FiberCell Systems®
A BETTER WAY TO GROW CELLS

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Be sure to follow the instructions in the Quick Start Guide. Perform each step of the pre-culture protocol as described.
www.fibercellsystems.com/quickstartguide

Also, watch the FiberCell Systems Instructional Videos on YouTube.
https://www.youtube.com/user/fibercellsystems

"I seeded 1 x 10^8 hybridoma cells into the C2011 cartridge. I am using DMEM/10% FBS for my medium. The reservoir bottle volume is 125 mL and the flow rate is set to 25. After two days my cells have not consumed any glucose. Normal consumption of glucose should be between 100-300 mg/day for the first day or two."

- Ensure that you are using medium based upon the FiberCell Systems guidelines. Medium should be DMEM or other high glucose mediums. Nothing less than 3.5 g/L should be used.
- Ensure that there are no additives such as insulin, transferrin, or other supplements not specifically recommended. These are not required.
- Use a good grade of serum, bad lots of serum can affect growth. "Good" serum is triple 100nm filtered, full 9CFR and mycoplasma tested and source traceable.
- Ensure that end port slide clamps are open, side port slide clamps are closed.

At this point it is a good idea to pull out a small sample of cells and check for viability using Trypan Blue. Another good viability check is to place a sample of cells from the ECS into a T25 flask with serum containing medium. High viability coupled with no or low glucose consumption is an indication that the cells are in lag phase. This means the volume of the medium is too large for the number of cells and the conditioning factors, specifically IL6 are too dilute to support cell growth. What to do?

- Reduce the volume of the medium in the reservoir bottle to 75 mL or less.
- Seed more cells, an additional 1 x 10^8 at the very least.
- Increase serum to 20%

RESERVOIR CAP

FiberCell Systems offers three different sizes of reservoir caps. 33 mm to fit onto glass media bottles with the black plastic cap, 38 mm to fit onto most standard Nalgene media bottles, and 45 mm to fit onto the wide mouth Gibco media bottles. The most common is the 38 mm reservoir cap. The length of the stainless-steel tubing can be adjusted after running the cap under some water. When you first get the reservoir cap adjust the length so that the tubes reach the bottom of a Nalgene 250 mL media bottle. This will allow you to use a 250 mL bottle to initiate the culture with 125 mL of medium and permit the next step of 250 mL. It will also allow you to use 500 mL in a 1-liter bottle. The 45mm cap reaches the bottom of a 500 mL Gibco bottle. The size of the reservoir bottle is most important for hybridoma culture.

RESERVOIR SIZE

Hybridoma culture in the hollow fiber bioreactor presents a balancing act between two cytokines 1) TGF-Beta which is inhibitory and 2) IL6 which is stimulatory. Different hybridoma clones have differing sensitivities to these two cytokines. In some cases when changing the medium and increasing the volume in the reservoir bottle during culture initiation it is useful to add fresh medium on top of the conditioned medium rather than removing and replacing 100% of the conditioned medium in the reservoir bottle. For example, after the first 125 mL of medium has its glucose depleted by 50% add 125 mL of fresh medium to the existing 125 mL for a final volume of 250 mL.

HOLLOW FIBER BIOREACTORS AND HYBRIDOMA TYPE

Hollow fiber bioreactors can support the production of monoclonal antibodies from any species or fusion partners. There are a few things to keep in mind. There is tremendous variability in the sensitivities of different clones to IL6 stimulation and TGF-beta inhibition. Some species, such as rabbit, are very poor producers and production using recombinant CHO or 293 expression systems should be considered. Some clones, in particular NSO, are cholesterol dependent. In this case CDM-HD should be supplemented with 2% FBS in the circulating medium only.

CELL NUMBER, GLUCOSE UPTAKE RATE AND ANAEROBIC METABOLISM

A common beginner's mistake when performing hybridoma culture in a hollow fiber bioreactor is allowing the cell mass to get too high inside the bioreactor. The rate limiting factor in any bioreactor is the ability to deliver oxygen and remove carbon dioxide. Hybridoma cells, freed from the inhibition of TGF-Beta through the action of the fiber filters, will grow quite rapidly and without the user taking action the cell number can easily exceed the ability of the system to deliver oxygen. This is to be avoided since otherwise the cells can be pushed into anaerobic metabolism. Once they go into anaerobic metabolism it can be very difficult to get the cells back into purely aerobic metabolism. Anaerobic metabolism is characterized by high glucose consumption and very poor antibody production. It is important to be aware of the possibility of cell overgrowth early in the culture. Switching from serum containing medium to CDM-HD should be performed just as soon as the glucose uptake rate reaches 1 gram per day. CDM-HD makes it easier to control the cell mass as protein-free medium renders the cells less adherent to each other.

Cell mass is controlled by harvesting cells from the ECS, by using the high glucose rate harvest. Please refer to the video instruction manual. It is not unusual to harvest anywhere from a 2 to 5 mL cell pellet. Viability of the harvest can range from 5% to 95%. This is not indicative of the average viability inside the cartridge because non-viable cells are preferentially harvested as they are not adherent to the fibers. Do not be concerned about removing too many cells from the ECS, it is very difficult to remove too many cells. We know that a glucose uptake rate of 1 gram per day equals about 1×10^9 cells in the cartridge. You should strive to keep the glucose uptake rate below 2 grams per day, when using the C2011 cartridge. As long as the glucose uptake rate is below 2 grams per day there should be no anaerobic metabolism.

The C5011 cartridge has 4 times the oxygenation capacity and can support glucose uptake rates of up to 6 grams per day. The C5011 should be used for maximizing hybridoma antibody production and with this cartridge we suggest using 2 x 1L bottles in series as your media reservoir (https://www.fibercellsystems.com/technical-resources/tech-tips/). CDM-HD

also facilitates higher cell densities by reducing cell-to-cell adhesion and adding buffering capacity to the medium. The hollow fiber bioreactor is a continuous process, and the potential for anaerobic metabolism is always there if the cells overgrow the cartridge. Controlling the cell mass through harvesting and monitoring glucose uptake rate is critical to avoiding anaerobic metabolism and maximizing antibody yield.

HARVESTING

- Do not start harvesting until you have a glucose uptake rate of 1 gram per day and have consumed the first liter of medium.
- It can take 3-5 flushes of the ECS to remove most of the serum proteins after switching to CDM-HD.
- Gauge the number of cells to remove, and the type of harvest(s) to perform (low glucose rate, high glucose rate or both) based upon the glucose uptake rate. With the C2011 do not allow the glucose uptake rate to get above 2 grams per day, with the C5011 do not allow the glucose uptake rate to get above 4 grams per day.
- Harvest every other day to allow antibody to concentrate in the ECS.
- One of the most common mistakes people make is to not harvest and remove enough cells when the glucose uptake rate is too high. It is normal to have a cell pellet harvest of 2-4 mL.

PURIFICATION

- Be sure to spin down the harvests immediately after collection to remove cells from the supernatant. This will prevent antibody degradation and contamination with host cell proteins.
- When using CDM-HD you may be able to simplify purification protocols as the antibody of interest will be in the range of .5 mg/mL to 5 mg/mL and be the primary protein present.
- When using CDM-HD avoid phosphate buffers. CDM-HD contains a significant amount of free iron (there is no transferrin in CDM-HD) that can interact with phosphate buffers. If you are planning on using Protein A or Protein G for antibody purification, please check the CDM-HD usage instructions for alternate buffers. https://www.fibercellsystems.com/wp-content/uploads/2018/12/4-5-CDM-HDUsageInstructions-1.pdf

ADDITIONAL QUESTIONS

What medium is best? How do I adapt to a serum-free medium?

For hybridoma culture at high density the simpler the medium used, the better. Cells grown at high density, i.e., 1 x 10^8/mL, can create their own optimum micro-environment. One important factor in choosing a medium is glucose concentration, a starting glucose concentration of at least 3.5 grams/L (Ham's F12) or 4.5 grams/L (high glucose DMEM) should be used. Glucose supplementation of RPMI (1 gram/L) does not provide a robust medium for hollow fiber bioreactor culture. Some clones, i.e. NSO, can be cholesterol dependent. In this case artificial cholesterol in serum free mediums is bound up with cyclodextrin which will bind to the silicone tubing. With cholesterol dependent cell lines, it is recommended to culture using DMEM/10% CDM-HD with .5-1% FBS in the circulating medium only. The cholesterol and other nutrients can cross the fiber, but bovine IgG and other large components cannot cross the fibers.

- The medium that the cells have been growing in works best for the initial medium, provided it is high glucose.
- CDM-HD is the ideal medium for most hollow fiber bioreactor applications and requires no adaptation, after the cells have reached high density in the bioreactor.
- If the cells are already adapted to a specific serum free medium you can continue to use that medium in the hollow bioreactor with the understanding that there may be some high molecular weight components that may not cross the fiber.
- Adaptation to a different medium or other cell culture conditions are best made after the cells have reached high density in the hollow fiber bioreactor module. This is defined by a glucose uptake rate of 1 gram per day or higher. At that time one can change the medium in the reservoir bottle in the following manner:
    - First medium change: 75% old medium, 25% new medium
    - Second medium change: 50% old medium, 50% new medium
    - Third medium change: 25% old medium, 75% new medium
    - Change the medium in the ECS (during harvesting) with the new formulation of medium as well.
- Monitor the glucose rate to ensure that the cells are not adversely affected.
- Continue this transition until the cells have adapted to the new medium.

What is the optimum serum concentration?

- Most cells should be started with 10% serum in the medium.
- After the cells have reached ~150 – 250 mg/L glucose/day the serum can be reduced to 7.5%. Monitor the glucose rate.
- At the next change of medium, reduce the serum to 5%. Monitor the glucose rate.
- Most cell lines will be able to adapt to 2 – 3% serum. Serum levels below this amount may support cell growth but protein secretion may be inhibited. It is not recommended to use serum levels below 2%.
- Provided the cells can be cultured at high density CDM-HD is a protein-free replacement for serum in most cultures.

If the culture dies, can I re-use or re-inoculate a cartridge?

- If the culture dies a second inoculation can be performed provided the cartridge is not contaminated.
- Increase the inoculum size by 2 – 3-fold to provide a sufficient cell mass to adapt to the bioreactor if the likely cause was insufficient cell numbers inoculated. Reduced medium volume in the reservoir may also be helpful.
- Always flush the ECS of dead cells and cell debris prior to re-inoculation.

How long will my bioreactor cartridge last?

- The all-time record is 560 days for a glioma-based culture. A hybridoma culture has been maintained for almost a year with no change in antibody affinity or specificity.
- Tips for long life:
    - Always wipe the Reservoir bottle cap and luer fittings with an ethanol swab during feeding and harvesting.
    - Perform a reverse ultra-filtration harvest (high glucose rate harvest described in the video manual) at least once a week. This helps to keep the pores of the fibers open.
    - Use new luer caps each time you access the ECS via the sideports.
    - Use one medium bottle per culture.
    - Never open a cold medium bottle – the negative pressure inside the bottle may draw in water, condensation and microorganisms.
    - Keep the hood clean and uncluttered.
    - Don't rush your bioreactor work!

SYSTEM PROBLEMS

Flowpath won't prime.

- Check that the slide clamps on the inlet and outlet are open.

- Check that there are no kinks in the oxygenation loop of tubing.
- Ensure the reservoir bottle inlet tube is submerged in medium.

Pump doesn't work.

- Check all connections.
- Is the transformer plugged in?
- Check the current in the wall outlet.
- Contact FiberCell Systems

Air bubbles in the flowpath tubing.

- Pump the system by hand making sure that the Reservoir Bottle outlet tube is submerged and not pulling in bubbles.
- Purge the system and cartridge by hand pumping.
- Open the Reservoir Cap in the hood to equilibrate the system.
- Always use prewarmed medium.

Air bubbles at the cartridge inlet.

- Tilt the outlet side of the cartridge up and hand pump the system.
- Always use prewarmed medium.

Air bubbles in the ECS.

- This is common early in preculture. Continue to preculture and check after a few hours.
- In the laminar flow hood flush the bubbles into a syringe with medium and remove.
- Elevate the medium reservoir bottle so that the level of medium is above the level of the ECS in the cartridge.

MEDIUM COLOR

Medium is bright orange or colorless.

- Lactate concentration is too high. Replace the medium in the Reservoir Bottle.
- Change medium when glucose is 50% depleted.

Medium is bright red.

- CO_2 level is too low. Check the CO_2 tanks for pressure. Check the lines from the tank to the incubator.
- Check the CO_2 concentration in the incubator with a Fyrite at least once a week.

ECS is yellow but the flowpath medium is red.

- Cells, cell debris, cell derived extracellular matrix proteins may have compromised diffusion across the fibers. Flush the cartridge to remove excess cells. Reverse ultra filtration, pulling medium from the flowpath into the ECS, may also help.
- Bacterial or yeast contamination in the ECS. Remove an aliquot and examine microscopically.

CONTAMINATION

Contamination of the cartridge with yeast, fungus or bacteria is a terminal event and the cartridge must be disposed of. There is no reliable way to clean and sterilize a hollow fiber bioreactor cartridge.

Medium in the reservoir bottle or ECS is cloudy and yellow or pink.

- The system has a bacterial (yellow) or yeast (pink and cloudy) contamination. Check microscopically. If contaminated dispose of immediately. Rinse bottle and cap assembly in a bleach solution. Clean the laminar flow hood thoroughly afterwards. Dispose of all medium used for this bioreactor.

Inlet side of cartridge is occluded with a plug of white material.

- Fungal contamination. Mycelia in the perfusing medium have collected at the inlet of the cartridge. If contaminated dispose of immediately. Rinse bottle and cap assembly in a bleach solution. Clean the laminar flow hood thoroughly afterwards. Dispose of all medium used for this bioreactor.

Remember, as long as you don't contaminate the cartridge, we can fix anything!

Feel free to contact FiberCell Systems with any technical questions at info@fibercellsystems.com.

Tips for good sterile technique in cell culture: https://www.bionique.com/mycoplasma-resources/technical-articles/better-aseptic-technique.html