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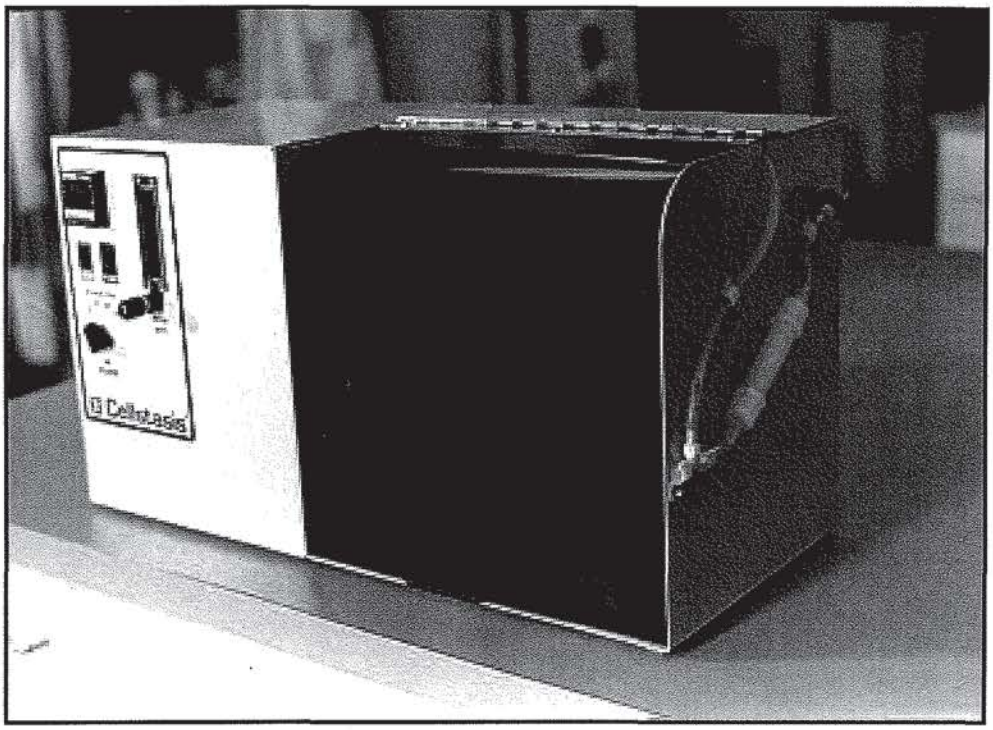
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Cellstasis®

3-D Cell Culture Technology



Operator's Manual

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Chapter 1
Introduction

Overview

The Cellstasis® Incubator[1] is a self-contained, bench-top instrument equipped with a highly efficient Cell Culture Chamber[2] that can be used for growing large quantities of cells required in transfection, drug screening, gene therapy research, stem cell research, *in vitro* modeling, tissue engineering and quantitative expression of novel genes by transfected plasmid DNA (i.e. proteomics). It significantly enhances cell growth by providing:

- Gas flow rate control for oxygen supply and maintenance of pH and CO_2 levels
- Programmed rotation of the Cell Culture Chamber for efficient, low shear mixing of cells
- Perfusion and recirculation of growth media that enables elimination of waste
- Precise temperature control

The Cellstasis® Incubator provides temperature control by means of pre-heated circulating HEPA-filtered air. Constant airflow under positive pressure reduces the possibility of contamination during long-term operation. These proprietary features combine to make the Cellstasis® Cell Culture Technology easy to install and operate for professionals with little or no prior cell culture experience.

Cellstasis® 3-D Culture Technology – A Novel Solution

The Cellstasis® 3-D Culture Technology consists of a patently unique Cell Culture Chamber and a customized Cellstasis® Incubator. This combination enables an operator without cell culture expertise and outside the typical clean room environment to grow cells for various applications. The Cell Culture Chamber, while possessing some superficial resemblance to a hollow-fiber bioreactor, is distinct in size and unique in the way that it provides oxygen and pH control for the cells. The Cellstasis® Incubator provides a localized clean room environment around the Cell Culture Chamber, provides precise temperature control for optimal cell growth, and has the functionality to rotate the Cell Culture Chamber on its central axis to keep cells in suspension and enhance transfection efficiencies.

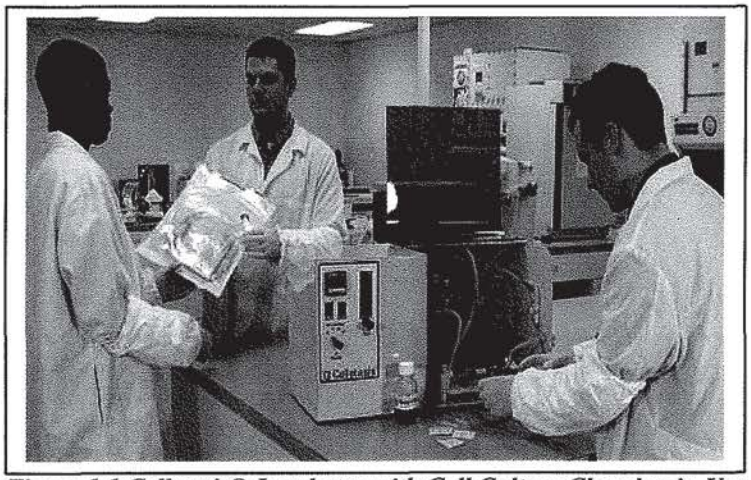


Figure 1.1 Cellstasis® Incubator with Cell Culture Chamber in Use

Footnote:

1. US Patent Number: 5,882,918
2. US Patent Number: 5,622,857

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A Comparison of a Conventional Hollow-Fiber Bioreactor and Cellstasis® 3-D Culture Chamber

Conventional Hollow-Fiber Bioreactor

The hollow-fiber bioreactor (Figure 1.2) is essentially a kidney dialyzer with varying packing densities of fiber membranes (up to a maximum of ~70% packing density). This device receives oxygenated media optimally from an oxygenator device in the media flow path. This media is perfused across the porous hollow-fiber membrane to feed cells grown on the outer surface of the membranes. Such bioreactor devices have been used with reasonable success in high-density perfusion culture, primarily for antibody production. Ironically, this cell immobilization technology has been most successful in culturing hybridomas, which are in fact non-adherent (or rather suspension cells).

Despite the success in hybridoma culture, hollow-fiber bioreactors have major limitations, which include:

- Low cell viability
- Difficulty in harvesting cells
- Oxygen limitation for productivity
- Heterogeneity of conditions, leading to problems with cGMP and 3-D cultures for tissue engineering

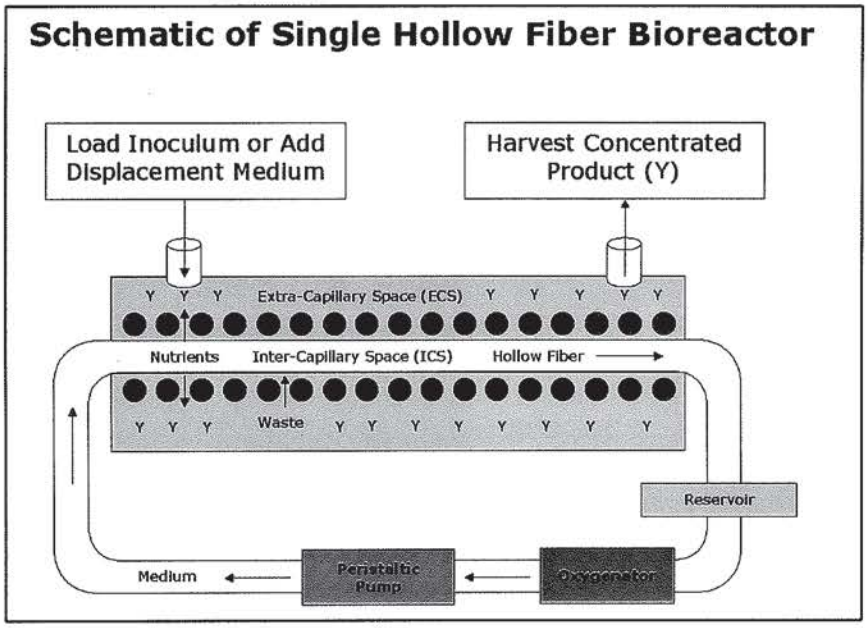


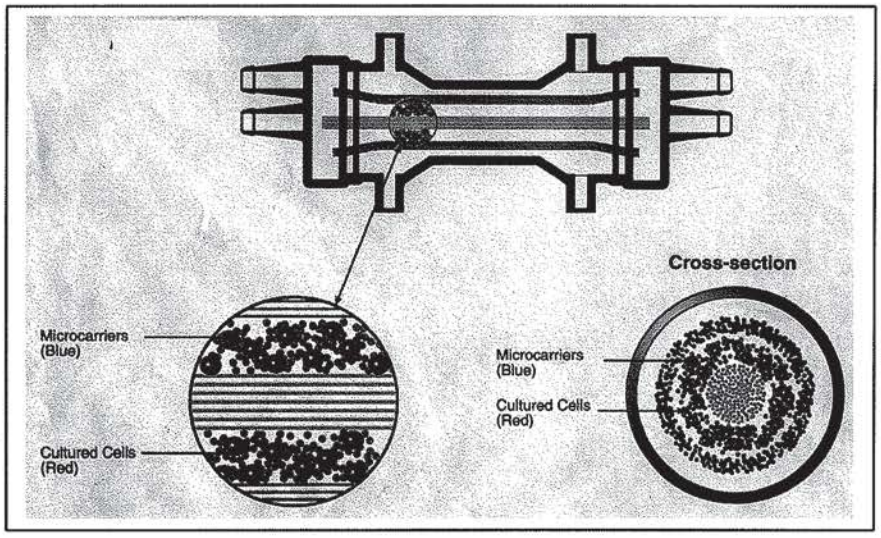
Figure 1.2 Hollow-Fiber Bioreactor

Cellstasis® 3-D Culture Chamber

The innovative approach represented by the Cellstasis® Culture Chamber (Figure 3) can be summarized as follows:

- Internal cell containment volume (17 mL) provides a chamber for 3-D culture of adherent cells. Cultivation of suspension cells (e.g. hybridoma) can also be accommodated.
- Cells are not encouraged to grow on the membrane, so that long-term high performance can be achieved as membrane pores do not become rapidly covered by cells or debris.
- Microcarriers are used as substrata for adherent cells.
- Two separate types of membranes are provided in adequate amounts to provide the necessary perfusion of nutrient media and oxygenated gases (for pO_2 and pH regulation of the microenvironment).
- Cells are maintained and harvested at high viability using standard procedures (e.g. trypsinization).

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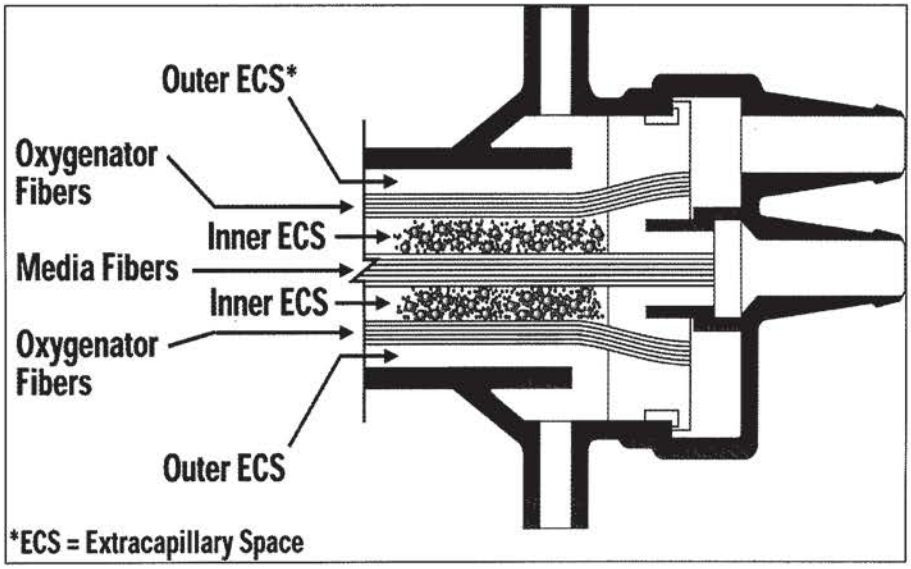


Figure 1.3 Schema of Cellstasis® Cell Culture Chamber

Due to the novel design of the Cellstasis® Culture Chamber, a wide variety of applications are possible under GMP with a totally scalable device. Scaling can be achieved in both directions (both larger and smaller). The design also makes it possible to employ a range of hollow-fiber or porous tubes as the perfusion and oxygenation means.

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Cellstasis® Incubator

Finally, the 3-D Culture Chamber is operated by an equally novel bench-top customized incubator, which offers many advantages over conventional hollow-fiber bioreactors (Figure 4). The Cellstasis® Incubator is simple to operate; it does not have the complexity of other bioreactors. A single technician can operate many Cellstasis® Incubators easily due to the minimal daily maintenance required.

Cellstasis® is designed specifically for the Cell Culture Chamber. It has the functionality to maintain uniform media flow through a closed-loop system, it regulates temperature of the media and chamber, provides gas-flow control of pre-mixed gases, and allows the Cell Culture Chamber to axially rotate back and forth, providing the mixing preferred by cells growing in suspension. This mixing feature overcomes any possible mass transfer limitation in 3-D culture.

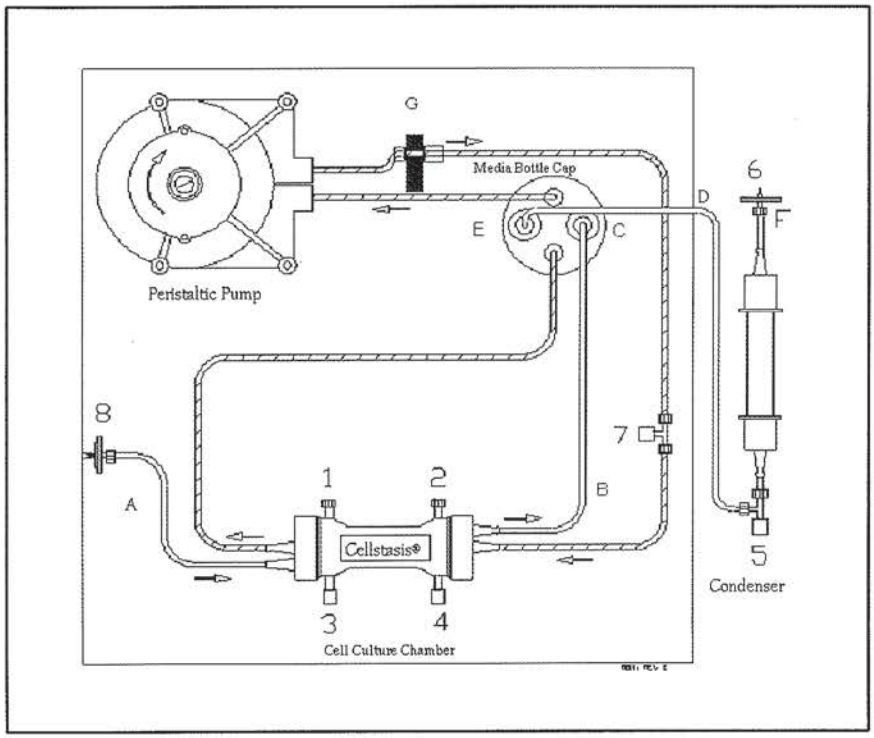


Figure 1.4 Schematic Diagram of Cellstasis® Incubator

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Operating Environment

For best operation, set the Cellstasis® Incubator on a level surface and in a well-ventilated area. Use grounded outlet only.

Gas source (User supplied):
5%-10% (v/v) CO_2 in air or user defined gas mixture CO_2:O_2:N_2. Regulator for gas cylinder should limit pressure to no more than 25 psi.

Operating Tips and Precautions

The Cellstasis® Incubator is a very rugged and dependable system; however, a few precautions are necessary:

1. When working with sterile components, always work in a sterile environment such as a Biological Safety Hood. The system is designed such that sterile connections and procedures requiring opening the "closed cell culture system" are made in a sterile Biological Safety Hood. When connections or procedures have been completed, the entire closed system of Cell Culture Chamber, tubing, media bottle, and condenser are then transported to the Cellstasis® Incubator. It is important that the two 0.2 µm filters connected to the tubing set remain attached to the tubing.

2. Prior to inserting needles into Sample Ports, wipe septum with alcohol to sterilize injection area.

3. The Cell Culture Chamber has media and gas fibers running along its length. Care must be taken not to damage the fibers. When injecting media or inoculating cells into the Cell Culture Chamber make sure to attach a receiving syringe to the opposite Sample Port to allow venting and prevent pressure build-up which could damage the fibers.

4. Always "break" the seal on any syringe that will be used for injection, inoculation, or venting.

5. Turn off the media pump before sampling, inoculating, harvesting, or changing media. Restart the pump after completing each operation.

6. Turn off the rotator and heater control switches before opening the Incubator compartment door. Turn control switches back to "on" position after closing compartment door.

7. If leaks are detected turn off media pump and secure any loose tubing connections. Contact Genespan Technical Service for assistance.

8. Securely tighten Luer and Heparin Locks, however, take care not to overtighten as this may cause Cell Culture Chamber or port caps to crack.

9. When closing the Incubator compartment door, be sure all tubing are cleared to avoid being pinched.

10. The gas inlet connection on the back of the instrument should be tight to avoid leaks resulting in high gas consumption.

11. During assembly and installation of the Cell Culture Chamber and the tubing components, if media bottle contains liquid, take care not to tip the bottle over. If media bottle is accidentally tipped over, media may flow into the gas tubing and impede proper gas flow. See Chapter 7 Troubleshooting Guide for directions in removing media in gas tubing.

12. When door is to be left in the "open" position for a prolonged period of time, turn off heater. Otherwise, the heater will over compensate for the drop in temperature and result in overheating the incubator when door is in the "close" position.

13. Always drain condenser of any fluid before moving Culture Chamber and tubing set from Cellstasis ® Incubator to Biological Safety Hood. Keep condenser in upright position so as to prevent any remaining fluid from plugging the sterile filter. A plugged filter will prevent proper gas flow. Replace sterile filter if needed.

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Chapter 2
Cellstasis® Incubator Set-up

Unpacking Cellstasis® Incubator

On each side of the carton-style packaging are two white plastic "locking devices" (four total, located near the bottom). Pinch the top of the tabs together to release each clip. Pull the tabs outward and remove the locking devices from the carton. Lift the top of the carton from the base, and remove the top foam pieces. Remove the Start Up Kit and lift the Cellstasis® Incubator out of the carton base.

Save all packaging materials in the event Cellstasis® Incubator needs to be returned to Genespan for repair or upon termination of lease or rental specified in agreement.

The cartons can be collapsed by carefully cutting the tape holding the joints together. Fold the cartons flat. To reuse, expand the cartons and tape the joints.

Repackaging Cellstasis® Incubator

If the unit is to be returned, contact Genespan Corporation for return authorization prior to shipment. The unit must be packaged in its original carton for shipment. For a nominal fee, Genespan can provide packing material. Reassemble and tape the carton if disassembled previously. Place two of the cushioning plastic pieces in the base of carton. Gently place the Incubator onto the cushion pieces, put the remaining two cushions on top of the Incubator. Slip the top of the carton over the Incubator. Insert the four white plastic clips into each of the slot; push the center section until it locks into place. No additional taping should be required. Attach a shipping label and ship to:

Genespan Corporation
19310 North Creek Parkway, Suite 115
Bothell, WA 98011-8006, USA
Telephone: (425) 482-3003

List of Components Included with the System

1. Cellstasis® Incubator with power cord
2. Operator's Manual
3. Start up kit (for practice):
    - 1- Non-sterile Cell Culture Chamber with attached tubing set and filters
    - 2 - Non-sterile 500 mL media bottle
    - 1- Non-sterile bottle cap with fittings
    - Sterilization instructions for media bottle and cap
    - HEPA filter
    - Tubing key, for pump tubing installation

Note: Sterile Cell Culture Chambers with Tubing set are purchased separately. Refer to the Appendix for a list of Cell Culture Chamber models.

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Controls and Hardware Features

- Power inlet ***(Figure 2.1)***
- Gas inlet ***(Figure 2.1)***
- Power switch ***(Figure 2.1)***
- HEPA filter enclosure ***(Figures 2.1 & 2. 4)***
- Power switches for heater control and peristaltic pump ***(Figure 2.2)***
- Gas flow control valve and flow indicator ***(Figure 2.2)***
- Adjustable digital temperature read out and controller, pre-set for 37°C ***(Figure 2.2)***
- Rotator speed controller switch for regulating low shear mixing of the Cell Culture Chamber ***(Figure 2.2)***
- Rotator platform ***(Figure 2.3)***
- Hangers for media and gas tubing ***(Figure 2.5)***
- Connector for the incoming gas system ***(Figure 2.5)***
- Peristaltic pump for circulating media ***(Figure 2.5)***

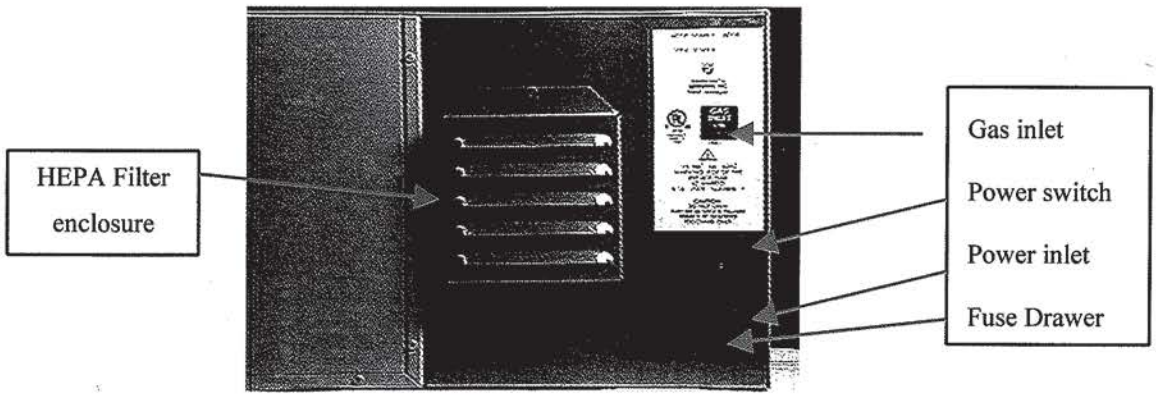


Figure 2.1 Back Panel

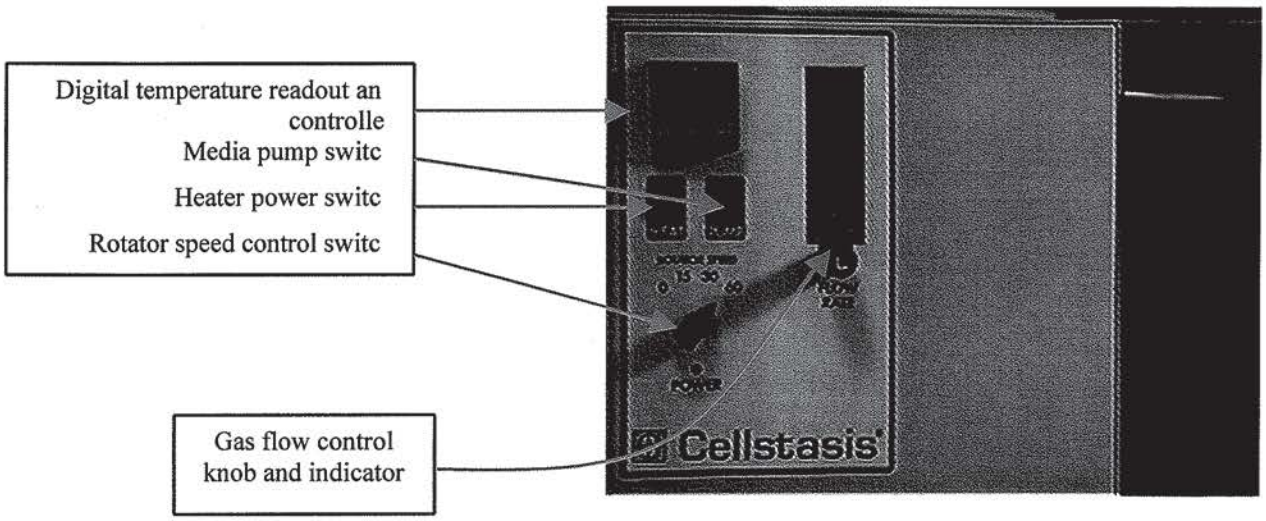
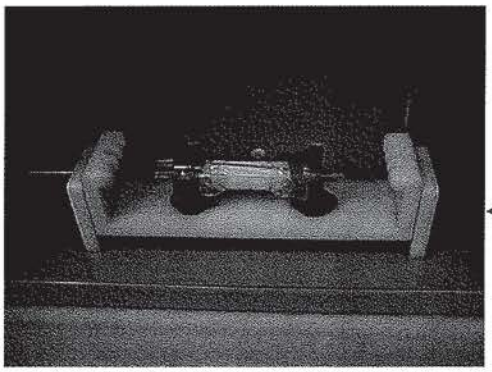


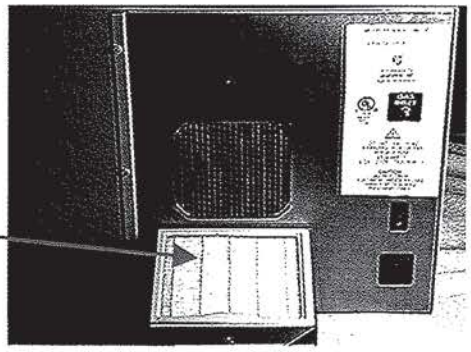
Figure 2.2 Controls

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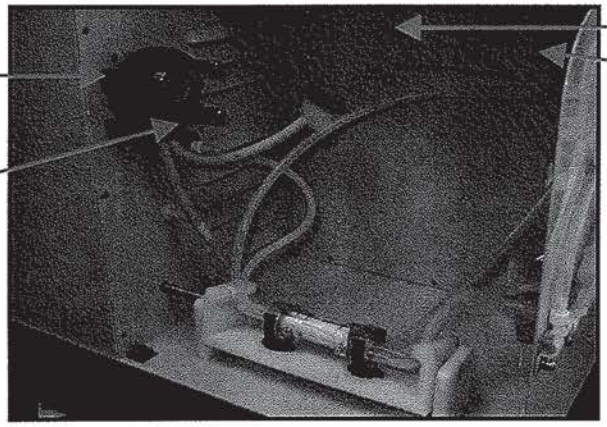
Cell Culture Chamber, without attached tubing, installed on rotator platform.

Figure 2.3 Cell Culture Chamber



HEPA Filter door in open position

Figure 2.4 HEPA Filter



Peristaltic Pump

Incoming Gas Connector

Media Tubing Hangers

Figure 2.5 Peristaltic Pump

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Initial Set-up

Connecting Power Source

Assure that the Incubator is positioned such that the power cord (6-7 ft in length) will reach an electrical outlet. Attach power cord to power inlet located at the back of instrument (Figure 2.1).

Connecting Gas Source

Attach a sterile hydrophobic filter to the gas inlet fitting located at the back of the instrument (Figure 2.1). Connect the gas source line to the sterile hydrophobic filter. Gas source is typically 5% to 10% (v/v) CO_2 in air depending on cell line, or a defined gas mixture CO_2:O_2:N_2.

Installing HEPA Filter

Place the HEPA filter into the filter holder (Figure 2.4). Close filter holder, making sure foam strip on HEPA Filter is in contact with the back of the instrument. This ensures a good seal for proper air filtering. Tighten bolt securely with fingers.

Adjusting Gas Flow

- With the gas source valve off, completely open the Cellstasis® Incubator gas flow valve by gently turning the knob counterclockwise.
- Open the gas source valve and slowly open the fine control regulator until the ball in the Incubator's flow control is at maximum flow (50 mL/min).
- Adjust the gas flow valve on the Cellstasis® Incubator so that the flow indicator ball is between 23 to 27 mL/min.

Resetting Temperature

The Temperature/Process controller is pre-set to 37°C. If a different temperature setting is required, the temperature can be reset as follows:

- Push the "INDEX" button.
- Verify 'SP1' is displayed on lower display.
- Push "UP" arrow to raise temperature or "DOWN" arrow to decrease the temperature setting. The upper panel digital readout will indicate temperature setting.
- After the desired setting is reached, push the "ENTER" button.
- Push "INDEX" to complete the process.
- With heater and pump turned on, allow 30 minutes for the Cellstasis® Incubator to reach equilibrium.

Rotator Platform Check

The rotator is controlled by a four-position knob: 0, 15, 30, and 60 cycles per minute ***(Figure 2.2)***. One cycle is defined as a complete ±120° to-and-fro motion around the longitudinal axis of the Cell Culture Chamber. Check the operating range of the rotator platform (without the Cell Culture Chamber and tubing set installed) by switching speed control knob to "15" and allowing the platform to rotate uninterrupted for one minute. Repeat the test at the "30" and "60" cycle positions. Contact Customer Service if rotator platform is not functioning properly.

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Chapter 3
Cellstasis® Culture Chamber Set-up

Schematic Diagram of Media and Gas Tubing Connections

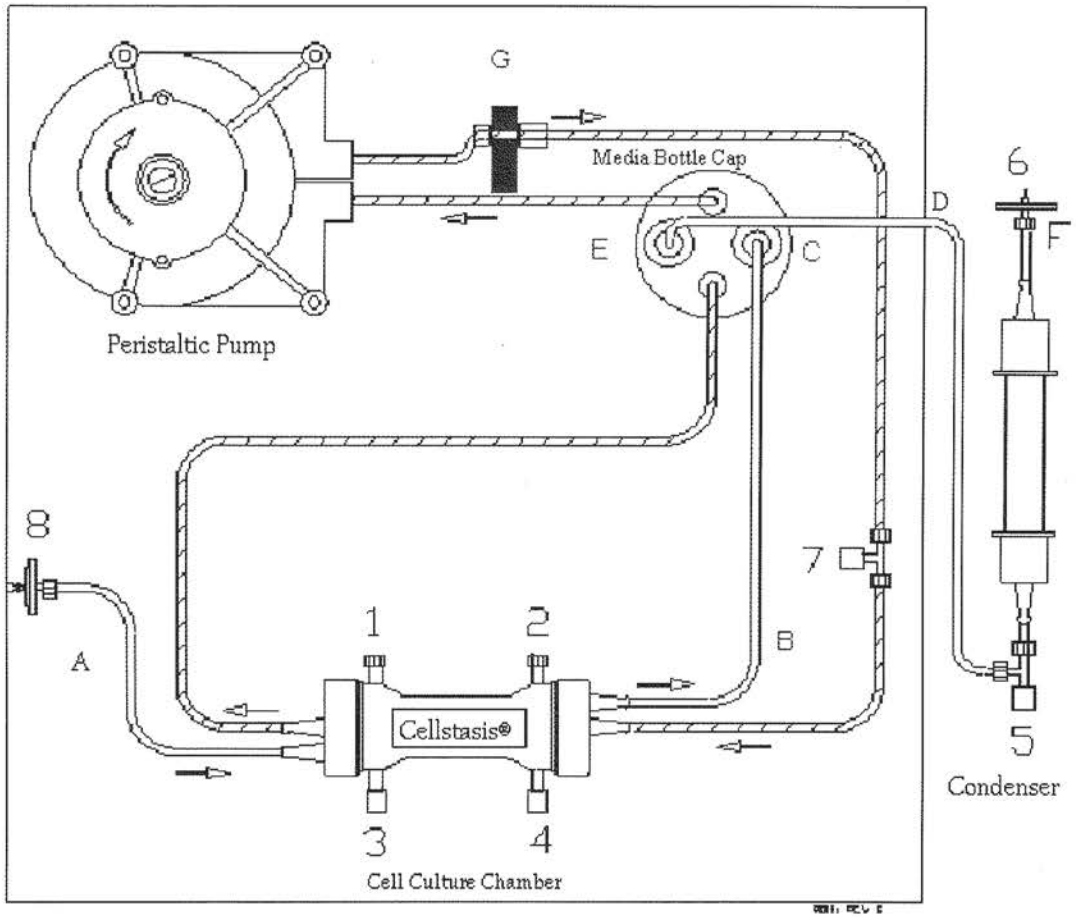


Figure 3.1 Diagram of Tubing Set-up.
(Cross-hatched tubing is media flow; clear tubing is gas flow.)

1 – ECS Port 1
2 – ECS Port 2
3 – Sample Port 1
4 – Sample Port 2
5 – Condenser Port
6 – Condenser Filter
7 – ICS Port
8 – Gas Port
A – Gas Inflow Tubing
B – Gas Outflow Tubing
C – Gas Outflow Connection
D – Condenser Tubing
E – Condenser Tubing Connection
F – Condenser Vent
G – Pall Filter

ECS Port 1& 2 – For Draining contents of Cell Culture Chamber and sampling media in Extra Capillary Space (ECS)
Sample Port 1 & 2 – For Injecting/Aspirating media or cells
Condenser Port – For Draining accumulated condensate
ICS Port – For sampling media in Inner Capillary Space (ICS) or injecting reagents/compounds

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Note: The following procedures must be done in a sterile Biological Safety Hood.

Filling Media Bottle

- Aseptically fill a sterilized media bottle with appropriate growth media recommended for cell line. Avoid overfilling media bottle.
- Carefully insert sterilized media tubings connected to bottle cap into media bottle. Securely tighten media bottle cap. Media bottle cap and attached tubing must be sterilized according to recommended instructions (See Appendix).

Setting-up Cell-Culture Chamber

- Remove the Cell Culture Chamber and tubing set from the package and lay out the tubing set so that the Cell Culture Chamber label is in a readable orientation (left to right), ***Figures 3.2 and 3.3***. Set the condenser aside for the moment.
- Insert the two white Luer caps into the two ECS ports of the Cell Culture Chamber(***Figure 3.1,* Positions 1 & 2**). Securely tighten with fingers.
- Insert the two sterile heparin lock into the two Sample ports of the Cell Culture Chamber(***Figure 3.1,* Positions 3 & 4**). Securely tighten with fingers.

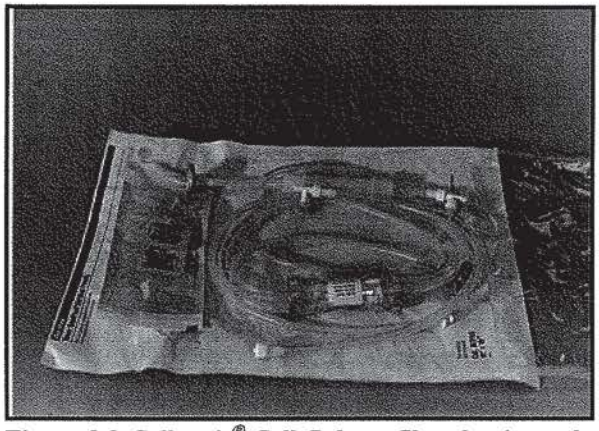


Figure 3.2 ***Cellstasis® Cell Culture Chamber in packaging***

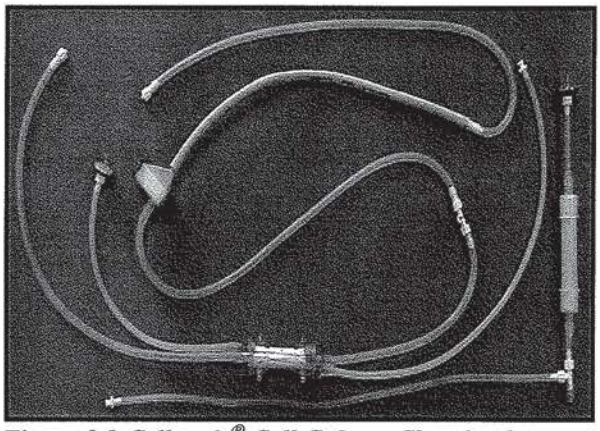


Figure 3.3 ***Cellstasis® Cell Culture Chamber layout.***

Connecting Media Tubing

- Connect the media inlet tubing (with the Pall filter) to the media bottle cap (Luer connected to long piece of tubing). Securely tighten with fingers.
- Connect the media outlet tubing to the media bottle cap (Luer connected to short piece of tubing). Securely tighten with fingers.

Connecting Gas Tubing

- Connect the gas outflow tubing (***Figure 3.1*, B**) on the right side of the Cell Culture Chamber to the media bottle cap fitting (***Figure 3.1*, C**). Securely tighten with fingers.
- Connect the Luer fitting on the condenser tubing assembly(***Figure 3.1*, D**) to the media bottle cap fitting (***Figure 3.1*, E**).

At this point all connections should be complete. Check all connections, including filters and ICS "T" junction to ensure all connections are secure. The tubing, Cell Culture Chamber, and media bottle are effectively isolated from any outside contaminants. The entire assembly can now be installed in the Cellstasis® Incubator.

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Installing Cell Culture Chamber on Rotator Platform

- Snap the Cell Culture Chamber into place on the rotator platform using the holding clip. Note: this may require some effort to overcome the tension of the holding clips ***(Figure 3.4)***. Make sure there is enough slack in tubing length to allow the rotator platform to rotate properly.

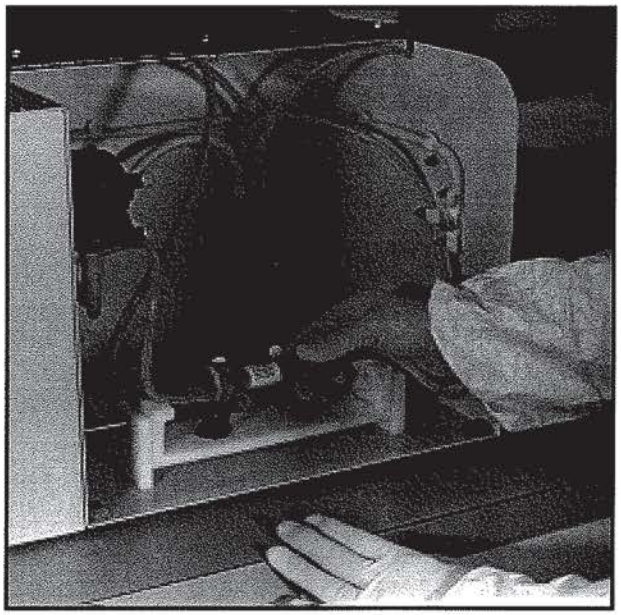


Figure 3.4 Installing Cell Culture Chamber in holding clips.

Installing media tubing through pump housing

- Detach the peristaltic pump from the side of the Incubator by loosening the four bolts. Remove one half of the peristaltic pump device. Install the rotor hub into the bottom half of the pump housing. Place the media pump tubing (rubber section) between the rollers and the inside of the pump housing ***(Figure 3.5)***.
- Hold both ends of the tubing and pull gently to stretch the tubing. The tubing key may be used to push the tubing onto the rollers as shown in ***(Figure 3.6)***. Insert the slot of the tubing key on the rotor so that the bottom edge of the tubing key is pressing the tubing into the pump cavity. Push down and turn the tubing key counterclockwise to guide the tubing deep into the cavity. Hold the tubing and rotor in the pump head and remove the tubing key carefully.

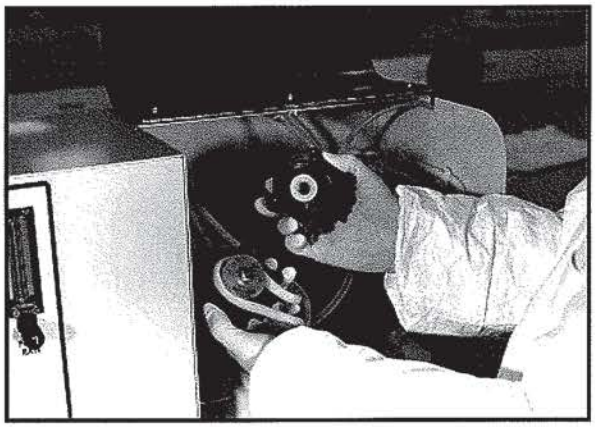


Figure 3.5 Installing tubing in the pump housing.

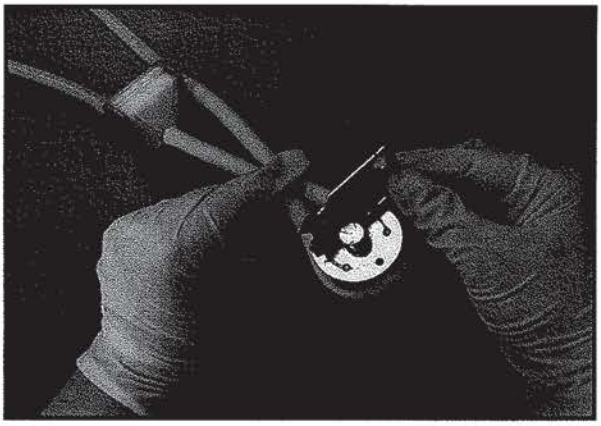


Figure 3.6 Use of the pump tubing key

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- Place the other half of the pump on the rotor shaft. Squeeze the two halves together, being careful not to pinch the tubing ***(Figure 3.7)***. Leave approximately 8 to 10 cm of tubing length on the intake side of the pump. If the tubing is installed correctly, it should slide through the pump housing with minimal resistance. If the tubing does not easily slide through the pump housing but requires heavy pulling, the pump tubing is either pinched or kinked at the rollers. Test this by pulling on one side of the tubing. Make the necessary adjustments before proceeding. *Note: Failure to make the necessary adjustments will cause damage to tubing and pump.* Cellstasis® Incubators that have been in use for some time may require a coat of silicon spray applied to the rollers to minimize friction.
- Align the rotator hub on the back of the pump with the shaft in the Cellstasis®Incubator. Reattach the pump using the four bolts ***(Figure 3.8)***. Make sure the intake and outtake sections are facing down.

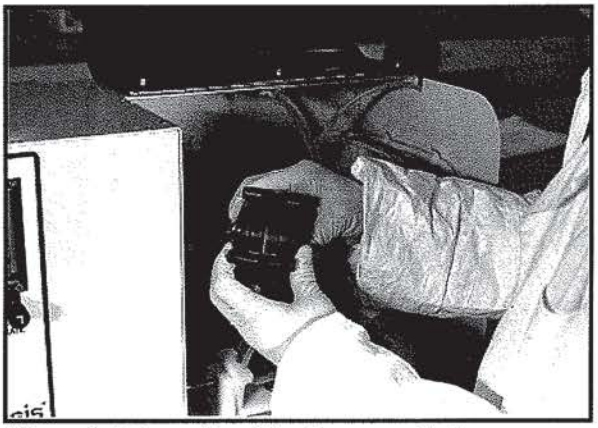


Figure 3.7 Assembly of the two pump halves.

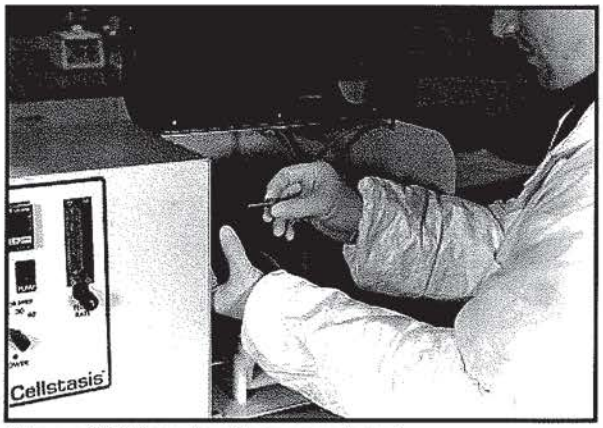


Figure 3.8 Securing the pump head.

Routing Media and Gas Tubing

- Route the media tubing from the right side of pump behind the media bottle and down to the right side of the Cellstasis® Incubator using the wall-mounted hangers. Note the placement of the Pall filter **(Figure 3.9)**. Note placement of ICS port **(Figure 3.10)**.
- Route the media tubing on the left side of pump to the ceiling of the Incubator using the mounted hanger.
- Route the gas tubing to the right of the Cell Culture Chamber down the right side of the Incubator using the mounted hanger ***(Figure 3.10)***.
- Press the sterile gas filter into the gas port **(Position 8, *Figure 3.1*)**.

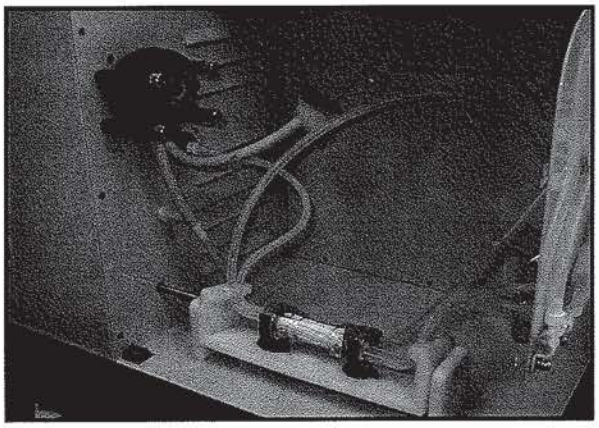


Figure 3.9 Filter placement.

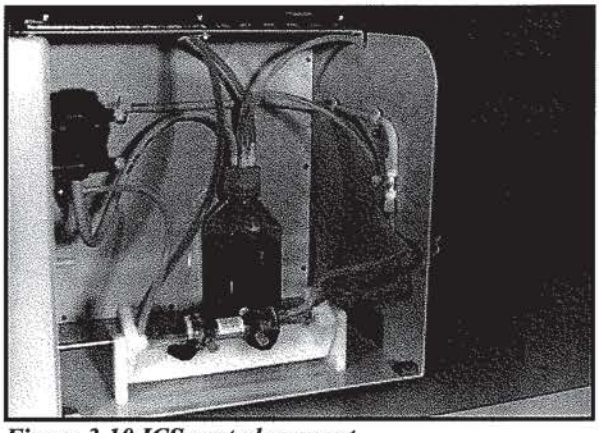


Figure 3.10 ICS port placement

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Routing Condenser

Route the condenser to the outside of the Cellstasis® Incubator using the hangers provided ***(Figure 3.11 and 3.12)***.

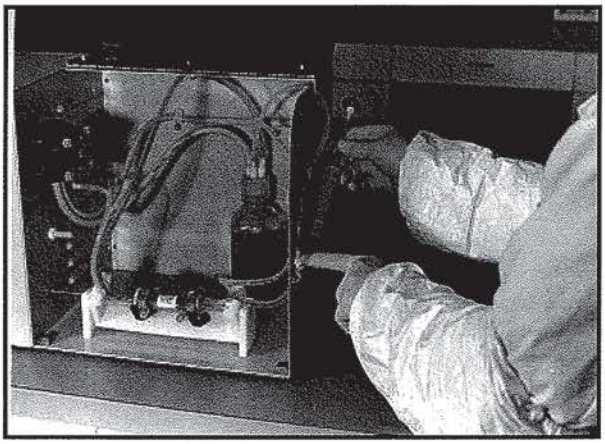


Figure 3.11 Routing of condenser and tubing.

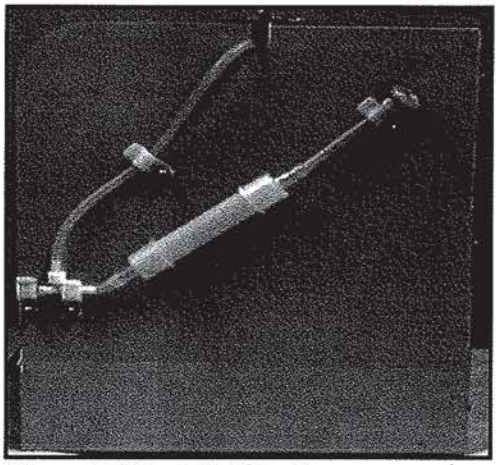


Figure 3.12 Installed Condenser.

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Chapter 4
Operation

Preventing Cell Culture Contamination

When working with sterile components, always work in a sterile environment such as a Biological Safety Hood. The system is designed such that sterile connections and procedures requiring opening the "closed cell culture system" are made in a sterile hood. When connections or procedures have been completed, the entire closed system of Cell Culture Chamber, tubing, media bottle, and condenser are then transported to the Cellstasis® Incubator. It is important that the two 0.2 µm filters connected to the tubing set remain attached to the tubing.

Operational Procedure

A. Hydrating Media Fibers

PBS/Antibiotic Flush

Note: It is recommended that the Cell Culture Chamber and tubing set be flushed overnight or longer with Phosphate Buffered Saline (PBS) containing antibiotic such as Gentamicin, prior to culturing cells. The overnight flush is necessary to allow full hydration of the media fibers, flushing of extractables, and to ensure that the system is operating smoothly without any leaks. Cell Culture Chambers designed for protein production will require longer exposure to the PBS/Antibiotic solution because of the slower diffusion rate of the media fibers used for this application. Sterile PBS may be used in lieu of PBS/Antibiotic solution if necessary.

- Set up a sterile Cell Culture Chamber and tubing set, and a sterilized media bottle and cap, in a sterile Biological Safety Hood as described Chapter 3. Fill media bottle with PBS/antibiotic solution. Do not overfill media bottle.
- Fill one 20 mL Syringe with 17 mL of PBS/antibiotic Solution. Prepare a second 20 mL syringe for venting. Wipe Sample Ports 1 & 2 with 70% (v/v) alcohol. Remove the heparin locks from both ports. Insert the PBS/antibiotic filled syringe into Sample Port 2. Insert the venting syringe into Sample Port 1. Gradually inject the PBS/antibiotic solution. Air will vent into the left syringe. The Extra Capillary Space should now be filled with the PBS/antibiotic solution. Alternatively, the Cell Culture Chamber can be filled by diffusion once the solution is allowed to circulate using the peristaltic pump.
- Install the Cell Culture Chamber, tubing set and media bottle and cap in the Cellstasis®Incubator as described in Chapter 3. Turn on the gas supply as described in the Initial Set Up Instructions and adjust the flow rate to 23- 27 mL/min. The backpressure at the gas regulator should be no greater than ~1.5 psi. Turn the media pump switch to the "ON" position to start circulating the PBS/antibiotic solution. Immediately check to make sure that bubbles are not coming out of the long suction tube located inside the media bottle. If bubbles are coming out of the long tube, check that all connections on the media bottle cap are correctly installed (***Figure 3.1***). If connections are incorrect, remove the entire Cell Culture tubing set to a biological hood and correctly install the media and gas tubing. Reinstall the Cell Culture Chamber and continue.
- Run the pump overnight with the heat switch on and gas flowing through the Cell Culture Chamber; longer runs are acceptable.

Removing PBS/Antibiotic Flush or Media from Media Tubing

- Move the entire Cell Culture Chamber to a Biological Safety Hood. Clamp media tubing below the ICS port (***Figure 3.1***). Fill a 60 mL syringe with 50 mL of sterile air. Holding the ICS port no lower than the media bottle cap, uncap ICS port, insert syringe and inject the air to force the PBS/antibiotic solution into the media bottle. Bubbles will be evident in the short media inlet tubing. Remove clamp and place above the ICS port. Purge the remaining PBS/antibiotic solution into the media bottle by injecting sterile air using the 60 mL syringe.

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Removing PBS/Antibiotic Flush or Media from Cell Culture Chamber

- Uncap ECS Port 2 and Sample Port 1. Allow contents of Cell Culture Chamber to drain completely into a suitable container. Alternatively, a sterile 20 cc syringe filled with air can be attached to ECS Port 2 and a bolus of air pushed through the chamber to expel the contents of the Cell Culture Chamber through Sample Port 1.
- Recap ECS Port 2 and fill Cell Culture Chamber using a 20 cc syringe with 15 mL media. Inject media through Sample Port 1; recap Sample Port 1. Aseptically switch the media bottle containing PBS/Antibiotic with a new media bottle containing culture media.

Changing Media Bottles

- Remove cap from used media bottle and attach a new bottle taking care not to touch any of the cap tubing on the outside of bottle. Securely tighten media bottle cap. The entire set-up is now ready to be re-installed to the Cellstasis® Incubator. When switching media bottles, always move Cell Culture Chamber into a sterile Biological Safety Hood.

B. Microcarrier Preparation for Adherent Cells

Preparing Microcarriers

Refer to Appendix to determine type of microcarrier best suited for particular adherent cell line to culture. Microcarrier slurry may be prepared ahead of time and stored at 4°C.

Calculating Amount of Prepared Microcarriers Needed for Cell Culture

Sample calculation: Cytodex® 3 Microcarrier

Assumption: For 100 cells per microcarrier, if expanding cells to 1×10^8, need 1×10^6 microcarriers.

Swollen microcarriers = 14 mL/g dry weight.
Dry microcarriers = 4.0×10^6 microcarriers/g dry weight.
4.0×10^6 microcarriers/14 mL = 2.86×10^5 microcarriers per mL of packed, swollen microcarriers.
1×10^6 microcarriers required/2.86×10^5 microcarriers per mL of packed microcarriers = 3.5 mL packed microcarriers. Pack microcarriers obtained by centrifugation at 75g/room temperature/10 minutes.

- The dry Cytodex microcarriers are added to a suitably siliconized glass bottle and are swollen in Ca^{2+}, Mg^{2+}- free PBS (50-100 mL/g Cytodex) for at least 3 hr at room temperature with occasional gentle agitation. The hydration process can be accelerated by using a higher temperature e.g. 37°C. The supernatant is decanted and the microcarriers are washed once with gentle agitation for a few minutes in fresh Ca^{2+}, Mg^{2+}- free PBS (30-50 mL/g Cytodex). The PBS is discarded and replaced with fresh Ca^{2+}, Mg^{2+} - free PBS (30-50 mL/g Cytodex) and the microcarriers are sterilized by autoclaving with steam from purified water (115°C, 15 min, 15 psi).*Note: It is not recommended that conditions for autoclaving exceed 120°C, 20 min, 20 psi. When hydrating Cytodex 3 initial surface tension may occasionally prevent wetting and sedimentation of the microcarriers. Should this occur, Tween 80 can be added to the PBS used in the first hydration rinse (2-3 drops Tween 80/100 mL PBS).* The microcarrier slurry may be stored at 4°C or used immediately.

- Remove an amount of microcarrier slurry greater than double the packed volume of microcarriers required and transfer to a centrifuge tube. Centrifuge at 75g for 5 min at room temperature to pellet the slurry.
- Decant the PBS and unpacked microcarriers. Add sufficient culture media to resuspend microcarriers; mix microcarriers. Centrifuge tube at 75g for 5 min at room temperature to repack the slurry.
- Decant supernatant and add media sufficient to resuspend microcarriers. Attach an 18gauge needle to a 20 cc syringe and aseptically fill with the microcarrier slurry. If microcarriers are not pulled into syringe add more media (up to a total volume of 13 mL).

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Injecting Microcarriers into Cell Culture Chamber

- Wipe Sample Ports 1 & 2 with 70% alcohol. Attach a 22gauge needle to a 20 cc syringe; insert into Sample Port 1. Insert needle attached to syringe containing microcarrier slurry into Sample Port 2 and gradually inject microcarriers while venting media into receiving syringe. Take care not to inject the microcarriers too quickly such that microcarriers travel to the opposite end of the Cell Culture Chamber and into the receiving syringe. After injection, gently agitate the Cell Culture Chamber to evenly distribute the microcarriers.
- Prior to inoculating adherent cells, allow the microcarriers to settle for ½ hour. Position the Cell Culture Chamber with the Sample Ports facing up.

C. Inoculating Cells (Adherent or Non-Adherent)

- Harvest cells from culture plates or flask using the appropriate enzyme or dissociation solution. Count cells and determine number of cells to inject. Transfer the desired number of cells to inject into a 50 mL tube containing culture media. Centrifuge cells at appropriate time and condition for cell line to produce a pellet. Remove supernatant and resuspend cells in culture media (~5 mL).

- Attach a sterile needle (18G) to a 20 cc syringe and fill with the resuspended cells. Wipe Sample Ports 1 & 2 with 70% alcohol. Attach a sterile needle (18G) to a 20 cc syringe; insert into Sample Port 1. Slowly inject cells into Sample Port 2 while venting media into the receiving syringe. Take special care not to lose any microcarriers during this process. The microcarriers should have settled during the ½ hour wait. If cells are injected too quickly the microcarriers might migrate into receiving syringe.

- Remove needles and syringes from both Sample Ports. Disengage Cell Culture Chamber from clamps on rotator platform. Gently mix cells and microcarriers (if using adherent cells) with a swirling motion and gentle end-to-end and side-to-side rocking. Reinstall the Cell Culture Chamber on the rotator platform and close door. Turn switches on for gas, heat, and pump. Wait a minimum of four hours before turning rotator platform on. This will allow cells to attach to microcarriers. Occasionally rotate the Cell Culture Chamber manually during this wait period to resuspend microcarriers and cells.

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Chapter 5
Cell Culture Maintenance

Sampling Cells

It may be necessary to sample cells from time to time to determine cell number, viability, and other parameters. Follow the instructions below when a homogenous sample is needed.

- Prepare two 10 cc syringes in sterile hood; attach a sterile 18gauge needle to each syringe. Fill one syringe with 3 mL growth media.
- Turn off heat, pump and rotator platform. Move Cell Culture Chamber with tubing and media bottle to sterile Biological Safety Hood. Manually mix cells in Cell Culture Chamber by gently rotating the Cell Culture Chamber and tipping from side-to-side.
- Wipe Sample Ports 1 & 2 with 70% alcohol. Insert the empty syringe into Sample Port 1. Insert syringe filled with media into Sample Port 2. Slowly inject media into Cell Culture Chamber. Media, cells and/or microcarriers should be filling into other syringe. If the empty syringe is not filling, stop and ensure that the seal of empty syringe is broken, then continue with injection. If empty syringe is still not filling, gently pull on its plunger while gently pushing on the media syringe's plunger.

Note: It may be necessary to pump the plungers of both syringes back and forth to obtain a well-mixed sample.

- Analyze the cell sample as desired.

Sampling Media from Inter-Capillary Space (ICS)

- Prepare a 5cc syringe in a sterile hood; attach a sterile 18gauge needle. Turn off heat, pump, and rotator platform. Wipe ICS port (***Figure 3.1*, Position 7**) with 70% (v/v) isopropyl alcohol. Insert needle into ICS Port and aspirate desired volume.

Sampling Media from Extra-Capillary Space (ECS)

- Turn off heat, pump, and rotator platform. Orient the Sample Ports upward. Let cells and/ or microcarriers settle for 30 minutes.
- Prepare two syringes in sterile hood; attach a sterile 18gauge needle to each syringe. Fill one syringe with a volume of feeding media equal to the volume desired for sampling.
- Wipe Sample Ports 1 & 2 with a 70% (v/v) isopropyl alcohol. Insert syringe-containing media into Sample Port 2 (***Figure 3.1*, Position 4**); insert other syringe into Sample Port 1 (***Figure 3.1*, Position 3**).
- Very slowly inject the growth media. Make sure that no cells/microcarriers are being pushed into receiving syringe.
- After sampling, manually rotate Cell Culture Chamber to resuspend contents. Turn rotator platform, heat, and pump on.

Feeding Cells

- Depending on the particular cell line's growth rate, the schedule for changing the media will vary. Fast growing cells may need their media changed daily or every other day. Slow growing cells may require changing less frequently. It is recommended that the pH be monitored daily by sampling of the media. If pH quickly becomes too acidic or basic, media will need to be replaced more frequently.

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Chapter 6
Harvesting Contents of Cell Culture Chamber

Removing Tubing Set

- Shut down the Cellstasis® Incubator.
- Unhook the tubing from the hangers, remove the pump tubing and move the entire set to a sterile Biological Safety Hood.
- Aseptically disconnect the media and gas tubing from the ends of the Cell Culture Chamber. A sterile razor blade may be used for this procedure.

Draining Cell Culture Chamber

- Gently shake the Cell Culture Chamber to loosen cells and micro-carriers (for adherent cells) from media and gas fibers.
- Remove cap from Sample Port 2 **(*Figure 3.1*, Position 4)** and set cap aside. Aseptically attach an air-filled 20cc syringe to Sample Port 2.
- Remove cap from ECS Port 1**(*Figure 3.1*, Position 1)** and set cap aside. Position ECS Port 1 over an uncapped 50 mL centrifuge tube. With the air-filled 20cc syringe, inject air into the culture chamber to force contents out into the uncapped centrifuge tube. This tube may be kept separate from other collection tubes if further analysis of ECS media is desired.

  Note: Alternatively, contents may be drained by removing caps from Sample Port 2 and ECS Port 1. Contents are then allowed to drain into a sterile centrifuge tube. Contents may also be removed by uncapping ECS Port 2 and attaching a syringe to Sample Port 1, then aspirating contents.

Flushing Cell Culture Chamber

- Fill the 20cc syringe from Sample Port 2 with 15 mL serum-free media and attach to Sample Port 2. Carefully inject the serum-free media into the Cell Culture Chamber.
- Remove syringe from Sample Port 2. Re-cap Sample Port 2 and ECS Port 1. Gently mix the Cell Culture Chamber to dislodge remaining cells or micro-carriers trapped between fibers.
- Refill the 20cc syringe with 15 mL serum-free media and attach syringe to Sample Port 2. Attach another 20 cc syringe to Sample Port 1. Pump media through Cell Culture Chamber by alternating pushing the plungers of both syringes.
- Remove syringe filled with media and transfer contents to a sterile centrifuge tube. Aspirate the remaining contents of Cell Culture Chamber with the other syringe. It may be necessary to tilt the Cell Culture Chamber to one side to recover remaining contents. Remove filled syringe and transfer contents to a centrifuge tube.
- Repeat chamber flushing several times as needed to ensure complete removal of cells. Note: Very dense cell cultures may require more than four flushes. For non-adherent cells, proceed to "Washing Cells". For adherent cells, proceed to "Final Flush for Adherent Cells".

Final Flush for Adherent Cells

- Inject 15 mL of trypsin-EDTA solution or Cell Dissociation Solution through Sample Port 2.
- Gently rotate the Cell Culture Chamber to thoroughly distribute the solution throughout the chamber. Incubate the Cell Culture Chamber at 37°C for 10 minutes to allow the enzyme to further dislodge cells.
- Drain the Cell Culture Chamber into another centrifuge tube as described above, and proceed to "Washing Cells". *Note: This step may need repeating to completely recover microcarriers and cells.*

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Washing Cells

- Wash cells by centrifuging tubes at 225g for 5 minutes. Remove and discard the supernatant.
- Add 2 mL of serum-free media to each tube and mix contents thoroughly. Combine contents from each tube into a single tube, and add sufficient serum-free media to ensure thorough washing.
- Wash cells again by centrifuging the tube at 225g for 5 minutes. Remove and discard the supernatant. **For non-adherent cells**, add appropriate volume of media to obtain a sample dilution needed for counting cell number. Count the number of cells and determine percent cell viability.
- **For adherent cells**, add sufficient volume of enzyme or cell dissociation solution to the tube. Incubate tube for 10 to 15 minutes. Examine that cells have completely detached from micro-carriers by pipeting a small amount of sample onto a slide and examining under a microscope. If cells are still attached, incubate the tube for a few more minutes and re-examine a sample under a microscope. After incubation, if trypsin is used, deactivate the enzyme by adding sufficient media containing 10% FBS. Separate the cells from the micro-carriers using a 100 µm nylon screen (e.g. Falcon #2360) or stainless steel sieve No. 140. Count the number of cells and determine percent cell viability.

Disposing Cell Culture Chamber

- Inject 17 mL bleach (1:10) and let sit for 10 minutes. Drain bleach into an appropriate container and properly discard. Dispose Cell Culture Chamber into biohazard container. **DO NOT REUSE.**

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Chapter 7
Trouble Shooting

The Cellstasis® Incubator has been found to be very reliable under a wide variety of conditions; however, a number of situations can arise to cause the system to deviate from the usual performance. The cause of a problem should be identified and corrected as soon as possible to avoid losing cells and/or protein product. Table 1 is a quick guide to possible problems users may encounter.

Trouble Shooting Guide Table

Problem	Possible Causes	Remedy
Unresponsive when electrical switches are turned on	1. Lack of power to the outlet 2. Instrument not plugged in 3. Fuse burned out	1. Check if in need of repair. 2. Attach power cord securely and plug into outlet. 3. Replace with 6 Amp fuse.
Gas flow is not detected	1. Empty gas cylinder 2. Regulator not turned on 3. Leak at connection 4.Clogged gas filter 5. Clogged condenser	1. Replace gas source. 2. Turn on and adjust flow as described in Chapter 2. 3. Check connection; retighten if necessary. 4. Aseptically replace gas filter. 5. Aseptically replace drain condenser.
High backpressure in gas lines at regulator pressure indicator	1. Regulator set incorrectly 2. Media contains precipitate or debris	1. Reset as described in Chapter 2. 2. Halt experiment and determine cause.
Drop of gas flow at the flow meter	1. Clogged gas filter 2. Clogged condenser	1. Aseptically replace filter. 2. Drain condenser.
Improper flow of gas or media	Incorrect tubing routing	Reroute tubing as directed in Chapter 3.
Leaks in the tubing or Cell Culture Chamber	1. Leak in tubing connections 2. Luer or Heparin Lock stripped 3. Cell Culture Chamber leaks	1. Check connection is tight. 2. Move entire set to sterile hood and install new lock caps. 3. Halt experiment, replace entire disposable set; contact Genespan Technical Support.
Cells or microcarriers detected in the media reservoir	Media fiber broken	Move set up to sterile environment and harvest cells from chamber. Recover cells from media reservoir.
Gas bubble growing and Extra Capillary Space (ECS) drying out	Gas pressure at regulator too high	Readjust as outlined in Chapter 2.

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Problem	Possible Causes	Remedy
Fluid build up in Cell Culture Chamber end cap(s) or in gas line(s)	1. Gas pressure at regulator too low 2. Broken gas fiber 3. Media bottle tipped over	1. Readjust as outlined in Chapter 2. 2. Halt experiment, replace entire disposable set; contact Genespan Technical Support. 3. With the gas flow turned on, remove the Cell Culture Chamber from the holding clips and tip the media back into the gas line. The airflow will carry the media back to the reservoir. Alternatively the gas flow rate can be increased to 50 mL/min to aid this process.
Moisture in condenser	Accumulation of condensate	Drain every 1 – 2 days.
Incubator temperature is too high or low as measured by laboratory thermometer	1. Verify calibration of thermometer 2. Thermocouple may be malfunctioning inside instrument.	1. Adjust temperature controller as described. 2. Contact Genespan Technical Support.
Rotator Platform not rotating properly	1. Not enough tubing length 2. Motor or control board malfunctioning	1. Adjust tubing length to allow platform to rotate properly. 2. Contact Genespan Technical Support.

Unresponsive electrical components

Make sure the electrical outlet has power; check circuit breaker and reset if necessary. Make sure the power cord is firmly inserted into the power inlet slot and plugged into an electrical outlet. Check the fuse in the back of the incubator and replace if necessary with a 6 Amp fuse. The fuse drawer is located just below the power chord inlet (***Figure 2.1***). To replace the 6 Amp fuse, pull the power cord from behind the instrument. Pull on both top and bottom tabs of the fuse drawer, pull out old fuse and replace with new fuse.

Gas flow is not detected

Check gas source and replace cylinder if empty. Make sure the main and fine regulating valves are open and adjusted as per the Initial Set Up instructions. Verify that the hydrophobic filter is not clogged. Replace if necessary. Do not use a hydrophilic filter.

High backpressure

High backpressure could develop in the gas or media lines. This is evident if swelling is observed in the media or gas lines. If high backpressure is in the gas line, reset the gas pressure at the tank using the gas regulator as per Initial Set Up instructions. Should a high backpressure develop in the recirculating media, check for precipitates. Halt experiment and determine cause of precipitation.

Whenever high backpressure is observed, turn off the flow at the source immediately.

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Drop of gas flow at the flow meter

If the flow meter shows a drop in gas flow, the gas filter may be clogged. Move the entire Cell Culture Chamber and tubing set to a sterile Biological Safety Hood and replace filter. If condenser is full, drain the condenser as described in Chapter 5.

Improper flow of gas or media

If the tubing set is installed incorrectly, the media and/or gas will not flow properly. Refer to Chapter 3,***Figure 3.1*** for the correct installation and routing of the media and gas tubing.

Leaks

If the leak is coming from the pump tubing, check that it is installed correctly as outlined in Chapter 3,***Figure 3.1*** and make the necessary adjustments. If a leak develops at a connection, aseptically adjust the connection so that it is tight and secure. If leak is due to a stripped Luer or Heparin lock, move the entire tubing set to a sterile environment and aseptically install a new sterile Luer of Heparin lock. If the leak is detected on the Cell Culture Chamber, halt the experiment and contact Genespan Technical Support.

Cells or microcarriers are detected in the media reservoir

This is usually caused by a broken media fiber that allows cells to be circulated to the media reservoir. If this happens the experiment can be saved by shutting down the system and moving the entire tubing set to a sterile environment, then harvesting the cells. The media bottle can be sampled through port 7 to determine the number of cells present. If a significant number of cells are present they can be centrifuged and collected.

Extra Capillary Space (ECS) drying out

If the ECS starts to dry out, or a large gas bubble forms, the gas pressure at the tank regulator is too high. Check that the flow rate is 23-27 mL/min. If flow rate is high, make the necessary adjustments. Also, check to verify that the gas pressure is correctly set at the tank regulator. Formation of a substantial gas bubble in the ECS should be avoided.

Fluid build up in the end cap(s)

A small amount of condensation is normal in the Cell Culture Chamber end caps, particularly where the gas is exiting from the Cell Culture Chamber. When substantial fluid builds up, this situation indicates that the gas flow rate may be low or the gas pressure may be low at the tank regulator. Refer to Chapter 2 of the Operator's Manual for adjusting gas flow rate. Also check to verify that the temperature in the Cellstasis Incubator is at 37°C. The fluid can be removed from the end caps by taking the Cell Culture Chamber out of the rotator platform and tilting the Cell Culture Chamber to allow the fluid to flow out of the end caps into the tubing. Another possible cause would be a broken gas fiber. Contact Genespan Technical Service if liquid and bubbles can be seen coming from gas fibers into end caps.

Moisture in the condenser

It is important to drain the condenser before too much fluid collects. Typically, the condenser should be drained daily or every other day, depending upon laboratory conditions. Shut off gas flow. Tap the condenser to move liquid to the bottom of the condenser. Use a sterile 22gauge needle to drain the condenser at Condenser Port (***Figure 3.1,*** **Position 5***)*. Sterilize heparin lock before draining condenser.

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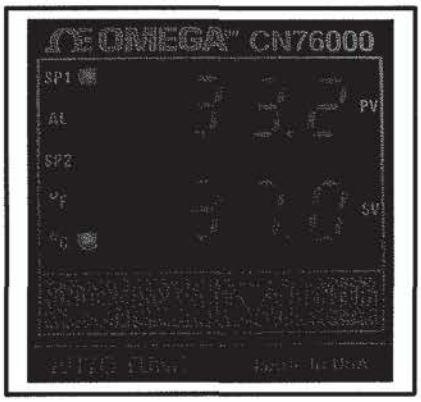
Incubator temperature is too high or low as measured by laboratory thermometer

Cellstasis® Incubators are calibrated to provide accurate temperature readings when operated at 37°C in an environment at STP (standard temperature and pressure) and 50% RH (relative humidity). When outside of these parameters it is recommended that the operating temperature at the Cell Culture Chamber be confirmed.

1. Monitor the temperature at the Cell Culture Chamber with a calibrated thermometer.
    a. With heater and peristaltic pump on, allow at least 30 minutes for the Cellstasis® Incubator to equilibrate.
    b. Record the temperature reading at the Cell Culture Chamber.
2. If the temperature at the Cell Culture Chamber differs from the Cellstasis® Incubator readout, a simple adjustment can be made at the digital temperature controller.

    a. Calculate the Input Correction needed according to the following formula:
        Input Correction = $T_{CCC} - T_{SV}$ where
        T_{CCC} = Recorded temperature at Cell Culture Chamber
        T_{SV} = Cellstasis® Incubator Temperature Set Variable
    b. Push the "UP" arrow and "ENTER" buttons simultaneously to display the settings menu.
    c. Push the "INDEX" button to advance the lower display to "InPC".
    d. Push the "UP" or "DOWN" arrow buttons to correct the "InPC" value displayed. **Warning: It is important that other parameters are not altered. If a parameter other than the "InPC" is accidentally altered, refer to Appendix for proper Omega Controller settings.**
        Note: The "InPC" value displayed and the required correction may both be negative numbers.
    e. Push the "ENTER" button to save the value.
    f. Push the "INDEX" button to advance back to the temperature readout.

    Example:



SV (Set Variable) digital reading (T_{SV}) = 37.0

Thermometer reading (T_{CCC}) = 40.0
"InPC" value displayed = 2.0

(T_{CCC}) – (T_{SV}) = Input Correction
40.0 – 37.0 = 3.0

Therefore, push "UP" arrow button to increment + 3.0 units until "InPC" value displayed is 5.0 (2.0 + 3.0).

3. Confirm the correction by allowing the Cellstasis® Incubator to equilibrate for at least 30 minutes with heater and pump on and rechecking the temperature at the Cell Culture Chamber.

If Incubator has difficulty maintaining temperature, the thermocouple may be malfunctioning, contact Genespan Technical Support.

Rotator Platform Malfunctioning

Adjust tubing length providing enough slack for platform to rotate properly. If platform continues to malfunction, test platform without the Cell Culture Chamber installed. If the problem persists, contact Technical Support.

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Specifications

	USA	Europe	Japan
Operating Voltage	120 VAC	250 VAC	100 VAC
Operating Frequency	60 Hz	50 Hz	50/60 Hz
Operating Current	6A Maximum	3A	6 A
Operating Temperature	10-30°C	Same	Same
Operating Humidity	10-90% Non Condensing	Same	Same
Installation Category	II		
Pollution Degree	2		
Chamber Temperature Setpoint	37°C	Same	Same
Setpoint Limits	20°C to 45°C	Same	Same
Rotator Speed	0, 15, 30, and 60 rotations per minute	Same	Same

Dimensions:

Width - 25 inches
Height – 13 inches
Depth – 11 inches

Warranty

Leased or Rented Equipment

Genespan retains the ownership of all leased Cellstasis® Incubators and is responsible for maintaining the instrument in good working order when used, handled and maintained in the manner detailed herein for all lease and rental agreements. The user assumes total responsibility for ensuring the proper and safe use of this product while in their possession. Furthermore, the user is responsible for any damage (e.g. from misuse and abuse) and theft of the product.

Purchased Equipment

Genespan warrants the instrument for a period of one (1) year from the original purchase date against defective materials or workmanship. This Warranty is limited to the original purchaser and cannot be transferred. All claims under this Limited Warranty must be made in writing to Genespan, Attention: Technical Support. Purchasers may return the instrument if it is found to be malfunctioning or defective anytime during the first year of ownership, provided that Genespan has issued a Return Authorization Number. Genespan will repair or replace the Cellstasis® Incubator free of charge. After a one-year warranty period, the instrument will be repaired for a nominal fee. Contact Genespan Technical Support for additional information (1-800-231-2215).

This Warranty is VOID if the instrument has been damaged by accident or misuse, or has been damaged by abuse or negligence in the operation or maintenance of the instrument, including without limitation unsafe operation and failure to perform routine maintenance. This Warranty is VOID if the instrument has been repaired or altered by persons not authorized by Genespan. This Warranty is VOID if the instrument has not been connected, installed or adjusted strictly in accordance with written directions furnished by Genespan.

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Preventive Maintenance

Cleaning

The Cellstasis® Incubator does not require maintenance other than keeping the unit clean and contaminant free. The incubator may be cleaned with warm water and can be sanitized with 70% (v/v) isopropyl alcohol.**Make sure to unplug the unit prior to sanitizing with 70% (v/v) isopropyl alcohol.**

DO NOT USE STRONG AGENTS SUCH AS BLEACH OR OTHER FULL STRENGTH ORGANIC SOLVENTS.

Changing Filters

HEPA Filter

The HEPA filter will require replacement from time to time depending on the particulate level in each laboratory and the hours of use. Under normal conditions, the HEPA filter need not be replaced more than every other year. For optimal performance, inspect HEPA filter yearly for particulate build-up. If changing is required, contact Customer Service to order a replacement.

Gas Line Filter

The gas line filter located at the back of the instrument should be replaced every three months.

Draining Condenser

Typically, the condenser should be drained daily or every other day, depending upon laboratory conditions. Shut off gas flow. Tap the condenser to move liquid to the bottom of the condenser. At the Condenser Port, sterilize the heparin lock with alcohol wipe. Insert a sterile 22gauge needle and aspirate any formed condensate (***Figure 3.1,*** **Position 5)**.

Customer Service/Technical Support

We can be reached by telephone, fax, or e-mail for general or specific product and company information, to order products or just to discuss your experiments.

Telephone: **(425) 482-3003** or **(800) 231-2215**
Facsimile: **(425) 482-3005**
Email: **tech@genespan.com**

Mailing Address:

Genespan Corporation
19310 North Creek Parkway, Suite 100
Bothell, WA 98011-8006
USA

Web Site: **www.genespan.com**

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Omega Controller Settings

1	Auto	On	9	Prog	OFF
2	TunE	nor	10	StAt	OFF
3	ArUP	OFF	11	Irt	0.00
4	ArtE	OFF	12	ISt	0.00
5	PEA	35	13	PEnd	Hold
6	UAL	15	14	InPC	
7	Cy1	10	15	FiLt	2
8	Pct0	OFF	16	LPbr	OFF

Microcarrier Selection

Cytodex Microcarriers

	Cell Type[a]							
	Primary[b]				*Normal diploid cell strains*	*Established cell lines*		
Application Area[f]	Fibroblast	Epithelial	Mixed	Tumor			Fibroblast	Epithelial
Cultures for Production								
General purpose cell production								
Production of virus	1,2	1,3	1,3	1,3	1, 2[c]		1,2	1,3[d]
Production of cell products								
Cultures for other cell biology studies								
Differentiated culture systems[e]					Cytodex 3			
Microscopy	1,2	1,3	1,3	1,3	1,2		1,2	1,3
Uptake studies	2	3	3	3	2		2	3
Isolating cells with membranes intact					Cytodex 3			
Harvesting mitotic cells	1,2	1,3	1,3	1,3	1,2		1,2	1,3

[a] Definition of cell type is based on morphology in culture. Cells of epithelial, endothelial and mesothelial origin usually have an epithelial morphology in culture.

[b] Also includes secondary, tertiary etc. cultures, which have not become established.

[c] Cytodex 3 can be used for high yields of certain cell strains, e.g. FS-4.

[d] Cytodex 3 is more suitable than Cytodex 1 for cell lines with pronounced epithelial morphology, e.g. BSC-1.

[e] For example hepatocytes, hepatomas, muscle cells, endocrine cells, etc.

[f] Pharmacia Microcarrier Cell Culture Principles and Methods Handbook.

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Sterilizing Media Bottle and Cap

Media bottles - The media bottles may be sterilized by autoclaving or exposing to Ethylene Oxide (EtO) or sanitized by soaking in 70% filter-sterilized alcohol for 30 minutes at room temperature.

Media Bottle Cap- Sanitize the media bottle cap with fittings and tubings attached, in 70% filter-sterilized alcohol solution for not less than 30 minutes nor more than 4 hours at room temperature. Do not alcohol sanitize overnight. Allow the cap to dry in a sterile biological hood. Alternatively, the caps may be EtO sterilized. Do not autoclave the cap as this may damage the Luer fittings.

Tubing attached to the media bottle caps - The tubing can be removed from the bottle cap and autoclaved separately, however, it is not recommended, due to potential contamination during reconnecting to cap.

Cell Culture Chamber Models

The Cell Culture Chambers are available in several models depending on media fiber specifications. Each Cell Culture Chamber comes with gas and media tubing already attached.

- Model 60207 is constructed with 0.6 mm outer diameter, 0.2 µm pore size macroporous fibers.
- Model 60210 is constructed with 200 µm outer diameter, 10kD MWCO pore size dialyzer fibers.
- Model 60224 is constructed with 200 µm outer diameter, 30kD MWCO pore size dialyzer fibers.
- Model 60225 is constructed with 200 µm outer diameter, 70kD MWCO pore size dialyzer fibers.
- Model 60226 is constructed with 200 µm outer diameter, 100kD MWCO pore size dialyzer fibers.

Note: All Cell Culture Chambers use 0.2 µm pore size polypropylene oxygenator fibers unless otherwise noted.

Reordering

Replacement parts can be ordered from Genespan Corporation using the following part numbers or descriptions.

Caps with tubing (non-sterile) for 125 mL Media Bottle, Part No. 60195
Caps with tubing (non-sterile) for 250 mL Media Bottle, Part No. 60196
Caps with tubing (non-sterile) for 500 mL Media Bottle, Part No. 60197
Caps with tubing (non-sterile) for 1000 mL Media Bottle, Part No. 60198

125 mL Media Bottles with caps and tubing (non-sterile), Part No. 60199
250 mL Media Bottles with caps and tubing (non-sterile), Part No. 60200
500 mL Media Bottles with caps and tubing (non-sterile), Part No. 60201
1000 mL Media Bottles with caps and tubing (non-sterile), Part No. 60202

Sterilized single-use components are available as a custom order. Inquire with Genespan Customer Service.

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Procedure:

1. Set-up Cellstasis Cell Culture Chamber and Tubing Set (refer to Chapter 3).
2. Flush system with PBS/Gentamicin 24-48 hours prior to inoculating cells (refer to Chapter 4, p. 19).
3. After system flush, remove PBS/Gentamicin from system (refer to Chapter 4, p. 20). Aseptically replace PBS/Gentamicin bottle with a media bottle containing 250 mL GEM 1 (Chapter 4, p.20).
4. Inject 1 x 10^6 Cytodex 1 microcarrier beads into Cell Culture Chamber using either Sample Port 1 or 2 (Chapter 4, p. 21).
5. Inoculate the Cell Culture Chamber with 1 x 10^7 cells (Chapter 4, p. 21). Allow the cells to grow for 24 hours.
6. Drain GEM1 media from media tubings (Chapter 4, p. 19). Replace GEM1 media in Cell Culture Chamber with 12 mL Transfection media, taking care not to remove any cells or microcarriers (Chapter 4, p. 19). Aseptically replace the media bottle containing 250 mL GEM 1 with a media bottle containing 250 mL Transfection Media.
7. Add 20 µg plasmid DNA into 2.5 mL Transfection Media and mix thoroughly.
8. Add 80 µL lipid delivery reagent (GeneFectin® LT) to another tube containing 2.5 mL Transfection Media and mix thoroughly.
9. Combine plasmid DNA and lipid delivery solutions. Mix thoroughly and aspirate into a 5 cc syringe.
10. Carefully inject the DNA/lipid complex into the Cell Culture Chamber using either Sample Port 1 or 2. Make sure a receiving syringe is available to vent media that is displaced.
11. Reinstall Cell Culture Chamber and Tubing set into the Cellstasis® Incubator. Turn on heat, pump, and gas; set rotator platform setting to "15" cycles. Transfect cells for four hours.
12. Drain Transfection media from media tubings (Chapter 4, p. 19). Replace Transfection media in Cell Culture Chamber with 10 mL GEM 1 (Chapter 4, p. 20). Aseptically replace the media bottle containing 250 mL Transfection Media with a media bottle containing 500 mL GEM 1.
13. Turn on heat, pump, and gas.
14. Grow cells for 30 days; replace media bottle with fresh GEM 1 every other day. Sample ECS and ICS media daily to monitor metabolic conditions. Sample cells as needed to monitor viability and gene expression.

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Cultivating Hepatocytes

Freshly isolated porcine hepatocytes were cultivated in the Cell Culture Chamber for 5 weeks. Cells were isolated by a method based on procedures for rat liver reported in "Methods in Cell Biology, volume XIII", edited by D. M. Prescott, Academic Press, 1976.

Materials

All materials were obtained from either Sigma Chemical Co. or Life Technologies.

Attachment Media	Long-term Culture Media
CPMI 1640 (500 mL)	CPMI 1640 (500 mL)
50 mL (10%) FBS	No serum
4 mM L-glutamine	4 mM L-glutamine
1x Penicillin/streptomycin	1x Penicillin/streptomycin
50 µg/mL Gentamicin	50 µg/mL Gentamicin
15 mM HEPES	15 mM HEPES
10 mU/mL Insulin	10 mU/mL Insulin
6 µg/mL, Transferrin	6 µg/mL, Transferrin
6 µg/mL, Selenium	6 µg/mL, Selenium

The Cell Culture Chamber was flushed with PBS/Antibiotic for one day before attachment media was applied. Pre-swollen Cytodex 3 microcarriers (500 mg) were inoculated into the chamber. Viable porcine hepatocytes ($1x10^9$) were also inoculated into the chamber, and the device rocked and rotated by hand to achieve uniform mixing of cells and microcarriers. Assuming that the hepatocytes are between 15-20 µm in diameter, the cell-to-microcarrier inoculum ratio was about 500. The apparent viscosity of cells and micro carriers was observed to increase rapidly, suggesting that cell-to-microcarrier and possibly cell-to-cell attachments were proceeding rapidly. Within 2-3 minutes of this mixing, a discrete gel of cells and microcarriers was formed in the chamber.

Following an overnight incubation at 37°C in attachment media (no rotation), the media was changed to long-term culture media (2L). The hepatocytes were cultured for 5 weeks with fresh media applied to the system weekly. The metabolic function of the cells was monitored by testing samples daily.

After 5 weeks, >90% recovery of viable cells and microcarriers by the following procedure:

1. 0.1% collagenase in PBS mixed with 0.44 mL (0.23M) EDTA, was used to flush the ECS and the Cell Culture Chamber incubated at 37°C to 10 minutes.
2. The content of the ECS was expelled with sterile air from a syringe barrel.
3. This process was repeated with long-term culture media and the materials collected washed and separated.

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