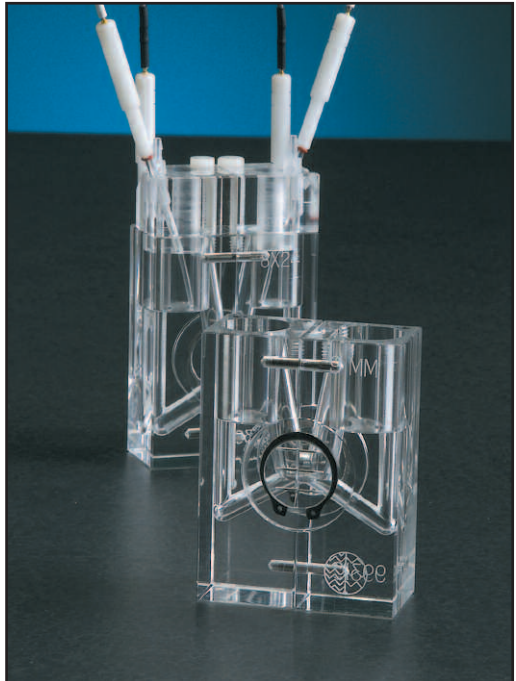


Diffusion

Chamber System

User's Manual

*Vertical Ussing & Horizontal
Diffusion Chamber Systems MA1 66-00XX*



HARVARD

A P P A R A T U S

Publication 5403-007-REV-D

WEEE/RoHS Compliance Statement

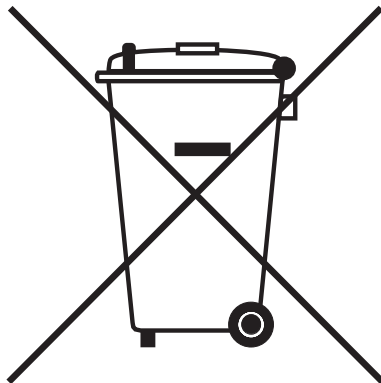
EU Directives WEEE and RoHS

To Our Valued Customers:

We are committed to being a good corporate citizen. As part of that commitment, we strive to maintain an environmentally conscious manufacturing operation. The European Union (EU) has enacted two Directives, the first on product recycling (Waste Electrical and Electronic Equipment, WEEE) and the second limiting the use of certain substances (Restriction on the use of Hazardous Substances, RoHS). Over time, these Directives will be implemented in the national laws of each EU Member State.

Once the final national regulations have been put into place, recycling will be offered for our products which are within the scope of the WEEE Directive. Products falling under the scope of the WEEE Directive available for sale after August 13, 2005 will be identified with a "wheelie bin" symbol.

Two Categories of products covered by the WEEE Directive are currently exempt from the RoHS Directive - Category 8, medical devices (with the exception of implanted or infected products) and Category 9, monitoring and control instruments. Most of our products fall into either Category 8 or 9 and are currently exempt from the RoHS Directive. We will continue to monitor the application of the RoHS Directive to its products and will comply with any changes as they apply.



- **Do Not Dispose Product with Municipal Waste**
 - **Special Collection/Disposal Required**

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Navocyte Diffusion Chamber System

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Using This Guide



WARNING: NEVER USE ORGANIC SOLVENTS AND CLEANERS WITH THE DIFFUSION CHAMBERS AND THE FRONT PANEL!

1. Using This Guide

This User's Guide is divided into five sections and one appendix.

Section 1

This section describes the general organization of this User's Guide.

Section 2

This section is the introduction which provides descriptions and illustrations of the Costar® Snapwell™ Cell Culture Insert, the Horizontal and Vertical Diffusion Chamber Systems, and the separately-provided electrodes for the chamber systems. This section also includes general information about diffusion chamber and electrode maintenance.

Section 3

This section describes preparations for using the diffusion chambers for a model epithelial transport experiment with Caco-2 cells. Specific information includes instructions for buffer preparation, collagen coating of Snapwell™ filters, cell seeding, Caco-2 cell culture, culture expansion, cell freezing and storage.

Section 4

This section describes the experimental set up for using diffusion chambers with Snapwell™ inserts or with tissues. Specific information includes the experimental set up, use of the Heat Block with the chambers, mounting Snapwell™ inserts or tissues, and electrode use.

Section 5

This section lists selected references for specific experimental applications using tissue and cell culture diffusion chamber systems.

Appendix A

Includes information on minimal diffusion chamber volumes and permeability calculations.

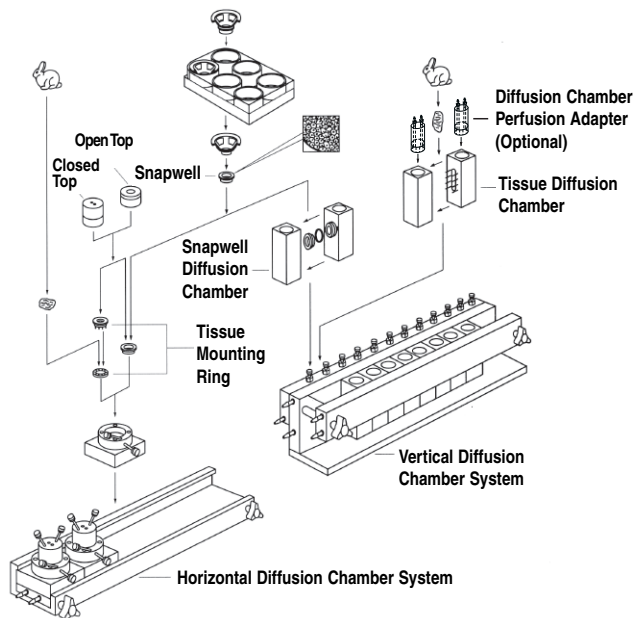
2. INTRODUCTION

Studies of fundamental cellular properties such as uptake and transport of materials across membranes and membrane electrical resistance and conductivity have been greatly facilitated by the availability of cell culture systems permitting access to basolateral and apical cell surfaces. Costar Snapwell™ cell culture devices and diffusion chamber systems permit convenient permeability measurements using cultured cells or tissues as models for drug absorption and transport studies. Use of diffusion chamber electrodes allows assessment of cell monolayer or tissue resistance and electrophysiological properties including short circuit current measurements.

The following User's Guide details the use of the Costar Snapwell™ cell culture device, in conjunction with diffusion chamber systems, to perform transport and electrical studies, with specific application to Caco-2 cells as a model system.

2.1 Product Definitions and Descriptions

This section describes Snapwell™ cell culture inserts, the Vertical and Horizontal Diffusion Chamber Systems, and Micro-Reference Electrodes.



2.1.1 Snapwell™ Cell Culture Insert

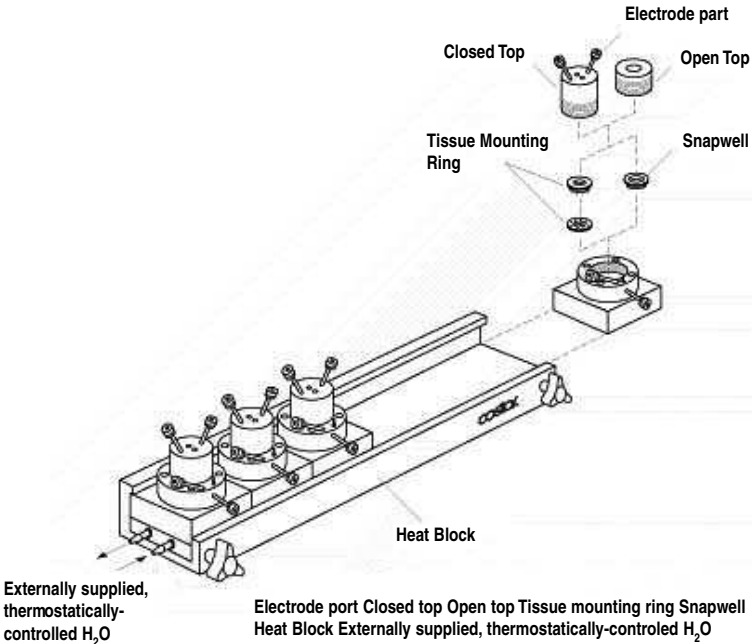
Snapwell™ devices are two-piece Transwells™, which allow convenient polarized cell culture on a permeable support. Snapwell™ cell culture devices consist of 12 mm diameter, 0.4 micron pore-sized polycarbonate or polyester membranes housed in detachable rings. These two-part assemblies are used together with Costar Diffusion Chamber Systems for performing a variety of physiological studies. Once cell monolayer formation is complete, the bottom section of the Snapwell™ is easily detached from its upper assembly for placement in the diffusion chamber system, without cell monolayer disruption.

Introduction

2.1.2 Vertical Diffusion Chamber

The Vertical Diffusion Chamber System can be used for transport studies on both filter-grown cell monolayers and surgically excised tissue sections. The Vertical Diffusion Chamber System includes six diffusion chambers, a 12-channel Gas Manifold, and a Heat Block. Two general types of vertical chambers are currently available; one for use with cells grown on Snapwell™ membrane supports and the other for use with tissues. Tissue diffusion chambers are available with both circular and oblong openings, depending upon the tissue type under study. For example, for intestinal tissue studies, oblong openings can increase the effective surface area of the sample. A low volume tissue chamber that reduces the amount of compound required to conduct permeability studies is also available. Up to six chambers of either type can be placed in the Heat Block at one time, allowing convenient execution of six simultaneous transport studies under precise experimental control.

Both the vertical and horizontal (described in 2.1.3) chambers permit the use of electrodes (not included with these systems, but which can be purchased separately) for measurement of parameters including voltage potentials and transmembrane electrical resistance.



2.1.3 Horizontal Diffusion Chamber System.

The Horizontal Diffusion Chamber System is designed for transport and toxicology studies using cells and tissues which are exposed to an air interface in their normal in vivo environment such as nasal, pulmonary, corneal or dermal cells. The chambers create an environment in which the apical surface of the tissue or cell monolayer is exposed to liquids, semi-solid compounds or gases, while the basolateral surface is perfused with medium. The Horizontal Diffusion Chamber consists of six horizontal chambers, into which either Snapwell™ devices or Tissue Mounting Rings may be fitted on a horizontal plane, and a Heat Block.

Either a peristaltic or a syringe pump (not included with the system) can be used to perfuse the lower surface of the Snapwell™ insert, usually the basolateral surface.

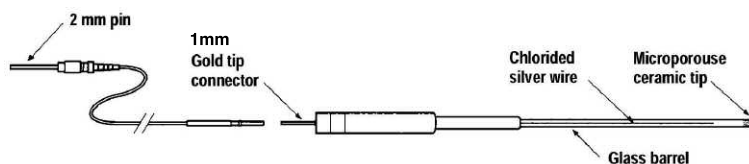
The apical (upper) surface of the diffusion chamber can be used in either open or closed configuration, the cell surfaces being more accessible for drug transport or cytotoxicity testing of liquids and semi-solid materials in the open configuration.

In the closed configuration, cells can be exposed to solutions, perfused with gas, or gas pressure can be applied. The closed system also accepts diffusion chamber electrodes for resistance measurements and electrophysiological studies. Both open and closed tops are included with the Horizontal Diffusion Chamber System; electrodes can be purchased separately.

Chambers can be sterilized with ethylene oxide, allowing sterile cell culture under air interface conditions to be examined.

Introduction

It should be noted that due to the horizontal orientation of the cells or tissue within the horizontal device, a hydrostatic pressure gradient will occur and be maintained across the cells or tissues. There are methods which can minimize these pressure differences, but for experiments in which such pressure may be undesirable, the Vertical Diffusion Chamber System is recommended. Please refer to Section 4.8 of this Users Guide for hydrostatic pressure gradient minimization techniques.



2.1.4 Micro-Reference Electrodes

Harvard/Navicyte Micro-Reference Electrodes fit both Vertical and Horizontal Chamber Systems without modification. These electrodes allow measurement of the electrical parameters of tissues and cell monolayers. The electrodes have a shaft diameter of 2.5 mm and a shaft length of 5.0 cm. The silver/silver chloride electrode uses a refillable glass barrel that can contain any suitable electrolyte solution. Usually, the filling solution is the buffer used for the external medium, or silver chloride (AgCl)-saturated 3 M potassium chloride (KCl). The glass barrel surrounds a chlorided silver wire and terminates with a ceramic tip. The microporous ceramic tip limits the flux of ions into and out of the electrode, and in most applications, serves as a suitable replacement for bridge systems.

The electrode connects to a wire one meter in length, which terminates in a standard 1mm pin plug. Snapwell™ Diffusion Chambers, 4mm Round, 9mm Round, 4x8mm oblong, 6x9mm oblong, 8x24mm oblong, and 5x24mm oblong require electrode caps to position the electrodes correctly. The Low Volume and Horizontal Chambers do not require these caps. Micro-Electrodes are not included with either the Vertical or Horizontal Diffusion Chamber Systems, and must be ordered separately.

2.2 Diffusion Chamber System Care and Maintenance

This section describes the general care, cleaning, maintenance, and storage conditions for Diffusion Chambers, Heat Block, Air Manifold and Micro-Reference Electrodes.



WARNING: NEVER USE ORGANIC SOLVENTS AND CLEANERS WITH THE DIFFUSION CHAMBERS! DIFFUSION CHAMBERS SHOULD NOT BE WASHED IN ALCOHOL OR AUTOCLAVED!

2.2.1 Diffusion Chamber Cleaning

New diffusion chamber systems require an initial washing to remove all traces of machining lubricants. Ultrasonic cleaning with a detergent is also recommended after each use.

Diffusion chambers can be disinfected with 50% bleach, or sterilized with Wescodyne.

Introduction

Cleaning agents such as Terg-A-Zyme made by Alconox® can be used. This is a concentrated, anionic detergent with protease enzyme for manual and ultrasonic cleaning. Excellent for removal of proteinaceous soils, tissue, blood, and body fluids from glassware, metals, plastic, ceramic, porcelain, rubber and fiberglass with no interfering residues. Ideal as a cleaning agent in Reverse Osmosis and Ultra-Filtration Systems. USDA authorized. Dilute 1:100. pH 9.5

2.3 Electrode Cleaning and Storage

Following each use of an electrode in protein-containing solutions, the electrode should be soaked for 10-15 seconds in an enzyme-cleaning solution or a chromic/sulfuric acid glass cleaning solution to remove the protein from the reference barrel and the reference junction. This soaking prolongs the useful life of the electrode. Always clean an electrode prior to storage. **Do not allow salt solutions to crystallize in the ceramic junction.**

For long term storage (longer than four weeks):

1. Remove the glass barrel containing the electrolyte, then store the barrel in a stoppered test tube filled with reference electrolyte or distilled water.
2. Rinse the silver wire and electrode cap to remove salt solution, then dry using an absorbent towel.
3. Store the electrode in a closed container to prevent dust accumulation.

For short-term storage place the tip of the electrode in a test tube or beaker containing reference electrolyte.

2.4 Heat Block and Air Manifold Cleaning

The Heat Block and Air Manifold can be rinsed with ethanol or washed with detergent.

Do not wash the Heat Block's acrylic front panel with ethanol or other organic solvents.

Preparation and Storage

3. Preparation of Buffers, Culture and Storage of Cells for an Experiment

3.1 Buffer(s) Preparation

The following section details the preparation of Krebs Bicarbonate Buffer, bicarbonate buffer stock solution, and buffers for transport studies, Mucosal (apical) and Serosal (basolateral) Buffers.

3.1.1 Krebs Bicarbonate Buffer

Krebs Buffer is a typical buffer to which test substances can be added, and in which this experiment will be described. Krebs Buffer should be sterile filtered for long-term storage. Shown below are recipes for stock solutions, and instructions for proportioning them to prepare the final Krebs Buffer.

3.1.2 Bicarbonate Buffer Stock Solution

To prepare each stock solution:

1. Weigh salts
2. Add salts to a volumetric flask.
3. Dissolve and dilute to the appropriate volume with reagent-grade water.
4. Store in refrigerator until use. (Stock solutions may be stored, refrigerated, for one week.)

Sample Krebs Buffer Stock Solutions are given in Table 1.

3.1.3 Preparation of Krebs Buffer From Stock Solutions

1. Add 50 mL of each solution in the numbered order as depicted in Table 1 above to a small volume of reagent grade water (about 200 mL). Mix in each solution prior to adding the next to prevent calcium salt precipitation.
2. Dilute the mixture with reagent grade water to a final volume of 1 liter. Final salt concentrations of salts in Krebs Buffer are shown below in Table 2.

Table 1.

Krebs Buffer Stock Solutions			
Solution	Salt	Grams per	
		500 mL	200 mL
Solution 1	MgCl ₂ /6H ₂ O	2.235	0.894
	CaCl ₂ /6H ₂ O	1.835	0.734
Solution 2	NaCl	66.60	26.640
	KCl	3.750	1.50
Solution 3	Na ₂ HPO ₄	4.420	1.768
	NaH ₂ PO ₄	0.415	0.166
Solution 4	NaHCO ₃	21.00	8.40

Table 2.

Krebs Bicarbonate Buffer, pH 7.4	
Salt	Final Concentration (mM)
MgCl ₂	1.10
CaCl ₂	2.15
NaCl	114.00
KCl	5.00
Na ₂ HCO ₃	25.00
Na ₂ HPO ₄	1.65
NaH ₂ PO ₄	0.30

Preparation and Storage

3.2 Transport Study Buffers

The following buffer preparations are typically used in transport studies, but may not necessarily be suitable for all applications. For information regarding specific applications, refer to the references in Section 5.

3.2.1 Mucosal (Apical) Buffer Preparation

For mucosal buffer preparation, add drug or test buffer substance. When glucose is used in serosal buffers (3.2.2) add an equivalent mM amount of unlabeled mannitol to the mucosal buffer. (The mannitol equalizes the osmotic load between the mucosal and apical buffers).

If the drug is radiolabeled:

1. Vacuum distill the drug to remove the organic solvents.
2. Distill tritiated compounds prior to use to remove any free tritium.
3. Dissolve the drug in the mucosal buffer solution.

If the drug is unlabeled:

1. Add the drug at a concentration that will dissolve completely in the buffer.
2. Adjust the mannitol concentration so that the drug plus the mannitol concentration totals 40 mM.

For both radiolabeled and unlabeled drugs:

1. Sample the mucosal buffer at the beginning of the experiment to determine the initial drug concentration.
2. Sample the mucosal buffer at the end of the experiment to determine the decrease in donor drug concentration, if any, during the experiment.

3.2.2 Preparation of Serosal (Basolateral) Buffer

For Serosal Buffer preparation, add 40 mM unlabeled D-glucose to Krebs Buffer (pH. 7.4). D-Glucose helps maintain tissue or monolayer viability.

3.3 Culturing Cells on Snapwell™ Filters

3.3.1 Coating Filters for Optimal Cell Adhesion and Culture

While a variety of cell types can be successfully grown and maintained on Snapwell™ filters without a collagen coating, many cell types adhere to the filter surface more efficiently with collagen. Some cell types may require substrates other than collagen for adherence and growth. For specific application information, please refer to the references in Section 5. For cell types requiring collagen coatings, a filter coating procedure is described below in section 3.3.2.

3.3.2 A Typical Filter Coating Procedure

1. Prepare a collagen solution by mixing one part rat tail collagen with three parts 60% ethanol (EtOH).
2. Add 100 µl collagen solution to each Snapwell™ filter insert, making sure to cover the entire filter surface.
3. Completely evaporate ethanol by drying the filters for 4 hours in a laminar flow hood with cluster plate lids slightly opened.

Preparation and Storage

3.3.3 Seeding Cells Onto Filters

1. Prewet coated or uncoated filters with phosphate buffer, 15 minutes outside and 15 minutes inside.
2. Trypsinize cells from stock, centrifuge, and resuspend pellet.
3. Count cells and calculate concentration to seed at appropriate growth density. Seeding density may depend upon cell type. Typical seeding densities for Caco-2 cells are 63,000 cells/cm²; for example, if plating 0.5 mL into each Snapwell™ insert at 63,000 cells/0.5 mL, adjust the concentration to 126,000 cells/mL.
4. Centrifuge and suspend cell pellet in the volume of medium required to obtain the desired cell concentration.
5. Seed cells onto filters and add additional medium to cluster plate (0.5 mL inside; 2 mL outside of each well).
6. Maintain the cells at culture conditions and change medium as required, usually every other day.

3.4 Caco-2 Cell Culture:

General Instructions

The following describes procedures for proper cell culture and maintenance. General instructions for proper culture and maintenance include the following:

1. Perform all procedures in a sterile environment.
2. Keep the laminar flow hood clean by wiping with 70% ethanol after use.
3. Keep the UV light on in the hood when hood is not in use.
4. Have all supplies at hand in the hood to minimize movement in and out of the hood when the cells are out of the incubator.
5. Properly dispose of all pipettes, flasks, vials, etc. that have been used to handle the cells.
6. Treat spent medium with bleach, and flush down the sink if appropriate.
7. Following medium preparation, thawing and resuspension of cells (see below for specific instructions) cells should be incubated at 37°C, with 90% humidity in 5% CO₂ and 95% O₂.

3.4.1 Cell Culture Medium Preparation

To Prepare the Caco-2 cell culture medium, add the following to Dulbecco's modified Eagle's Medium (DMEM/high) containing 25 mM HEPES:

- 10% fetal bovine serum
- 1% non-essential amino acids
- 1% L-glutamate
- 100 units/mL penicillin
- 100 g streptomycin

Medium components can be divided into aliquots in individual vials and frozen to facilitate complete medium preparation.

Preparation and Storage

3.4.2 Thawing Cells

Prior to removing cells from liquid nitrogen, prepare an ice bath, a 50 mL conical centrifuge tube, and have on hand cold complete medium.

To thaw cells:

1. Remove the cells from the liquid nitrogen storage tank, placing them on ice while transferring to the laboratory.
2. Warm cells immediately to 37°C in a water bath, shaking occasionally.
3. Thaw cells until no ice crystals remain.
4. Transfer cells gently to the 50 mL centrifuge tube.
5. Mix cell suspension with about 10 mL of cold complete medium.
6. Mix the medium with the cell suspension by performing 1:1 dilutions until a complete volume of medium has been added. For example, add 1 mL of medium to 1 mL of cell suspension and mix, add 2 mL of medium and mix, add 4 mL and mix, etc. Dilution should occur over a 2-minute time period.

3.4.3 Dimethyl Sulfoxide Removal

The dimethyl sulfoxide (DMSO) used in freezing cells must be removed from the cells carefully since it is toxic to cells at higher temperatures. To remove DMSO:

1. Centrifuge cells at 300 x g for 5 minutes.
2. Decant supernatant and gently resuspend cells in 10 mL of complete medium.
3. Transfer cells to a T-75 flask and incubate.

3.4.4 Culturing Caco-2 Cells in Plastic Flasks

Following thawing and/or trypsinization, cells require a few days to recover and resume normal growth. Speed of cell division and growth depends upon cell density, as well as culture conditions such as pH and timely medium replacement. Monolayer formation should occur in about 7-8 days under normal conditions. For culture in plastic flasks following thawing:

1. Maintain the cells in about 15 mL of medium in a T-75 flask or 30 mL of medium in a T-150 flask.
2. Change the culture medium every other day by removing spent medium from the flask with a sterile pipette and replacing it with fresh medium.
3. Note the color of the culture medium. Color changes as pH changes. While a slight color change is normal, drastic color changes indicate abnormal conditions. (A change toward yellow indicates acidity; toward red, more basicity).

Preparation and Storage

3.4.5 Caco-2 Cell Subculture

To expand Caco-2 cell colonies:

1. Decant medium from flask and rinse 3 times with phosphate buffered saline (PBS).
2. Add trypsin solution (0.25% trypsin with 0.2% EDTA) to the flask, distributing it gently over the cell monolayer.
3. Maintain the culture at 37°C for about 5 minutes. (Overexposure of cells to trypsin can damage cell membranes, and can either kill the cells or retard their recovery. Care should be taken to minimize exposure to the least amount of time required to release the cells from the flask.)
4. Terminate the trypsinization process by adding about 10 mL of culture medium to the flask.
5. Resuspend the cells by gently shaking the flask, or by scraping them with a rubber policeman if necessary, then shaking.
6. Centrifuge the cell suspension for 5 minutes.
7. Decant the supernatant and resuspend the cell pellet in 10 mL of fresh medium.
8. Divide the suspension among three new flasks, adding additional medium to achieve the appropriate volume.

3.4.6 Freezing Cells For Storage

(For additional information, refer to the Corning guide for Cryogenically Storing Animal Cells)

To minimize mutations or viral contamination, cells should be frozen relatively early in their growth cycle. Prior to freezing cells, have on hand a 10% DMSO solution cooled on ice, as well as labeled and cooled 1.2 mL freezing vials. (DMSO prevents crystal formation, and thus aids in maintaining cell membrane structure during freezing.) To freeze cells for storage:

1. Trypsinize the cells and centrifuge the suspension.
2. Resuspend the cell pellet in the ice cold 10% DMSO solution.
3. Dispense 1 mL aliquots of the cell suspension into the cooled freezing vials and seal tightly.
4. Place vials in the center of a 10 cm² freezer box.
5. Place the freezer box in a -80°C freezer for at least two hours. The temperature of the cells will decrease approximately 1°C per minute. (Vials can be left in the freezer overnight.)
6. Transfer the frozen cells to the vapor or liquid phase of a liquid nitrogen storage tank. Keep the vials on dry ice during the transfer process.

Setting Up the Experiment

4. Setting Up the Experiment

In any experiment, the minimal usable volume of tissue culture medium (that volume allowing fluid circulation across the bottom of the open reservoir) should be used in the diffusion chambers. See Appendix A, for usable volumes.

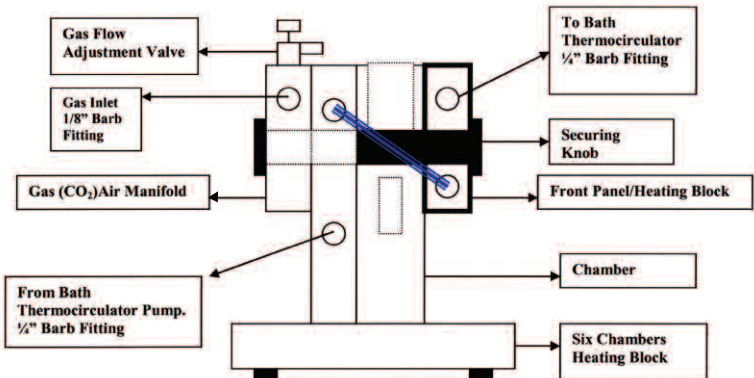
The following steps should be followed for all diffusion chamber set-ups.

1. The Heat Block should be connected to a circulating water bath.
2. The Snapwell™ inserts, or Tissue Mounting Rings (Horizontal Diffusion Chamber System only) should be mounted.
3. The Air Manifold should be attached to the chamber (Vertical Diffusion Chamber System only).

These steps are described in detail below.

A 25% antifreeze in distilled water solution should be circulated through the external heating unit and aluminum block to prevent corrosion and to retard mold growth in the tubing. Prior to use, Diffusion Chambers and Heat Blocks should be preheated (or cooled) for about 30-40 minutes. Since adding cold solutions to the tissue or cell monolayer may cause damage, the temperature of buffer solutions should also be appropriately adjusted.

For Snapwell™ insert and diffusion chamber surfaces and volumes, as well as permeability calculations, See Appendix A



Setting Up the Experiment

4.1 Connecting the Diffusion Chamber Heat Block

Connect the Heat Block to a circulating water bath to permit temperature control. The acrylic stand provided with the units should be used in conjunction with the Heat Block to prevent radiated heat loss from the back and bottom of the Heat Block. Use of a high-throughput rate, large heating capacity pump ensures the best temperature control and allows multiple heat blocks to run from one unit. Since the Heat Block operates with high efficiency however, almost any circulating water bath can adequately maintain one complete chamber system.

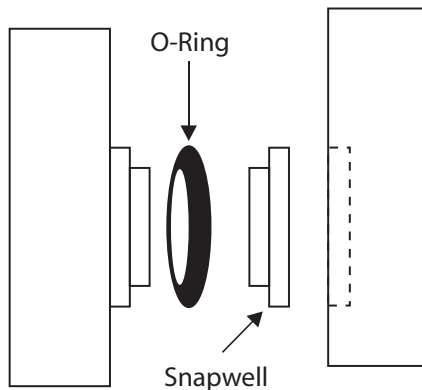
For Vertical Diffusion Chamber Systems:

1. Connect the outflow from the thermocirculator pump to the lower fitting on the back of the Heat Block.
2. Connect the upper fitting on the back of the block with an 8-10 inch section of tubing to the lower fitting on the front panel of the Heat Block.
3. Connect the upper fitting on the front panel back to the pump.

4.2 Mounting Snapwell™ Inserts

To Mount Snapwell™ Inserts:

1. Rinse medium from the filter with 1-2 mL of Krebs Buffer, pH 7.4.
2. Remove the lower filter ring from the Snapwell™ insert upper assembly.
3. Insert the filter as shown in figure at right with the O-ring in place on the chamber half-cell.
4. Join the matching half-cell and seal the chamber with the retaining rings.
5. Place the assembled chambers in the Heat Block and fill with the buffers.
6. Add buffers to each side of the chamber simultaneously, if possible. Fill the apical (mucosal) side first if only one buffer can be added at a time. See Appendix for appropriate half-cell buffer volumes.) Simultaneous buffer addition prevents hydrostatic pressure from forcing cells off the filter surface.



Setting Up the Experiment

4.3 Mounting Tissue Segments

To mount tissue segments in the diffusion chambers:

1. Mount the tissue segments on the pins of a Tissue Diffusion Chamber.
2. Trim the tissue if it protrudes beyond the outer chamber sides.
3. Place the assembled chambers in the Heat Block and fill with the buffers.
4. Again, as above, add buffers to each side of the chamber simultaneously, filling the apical (mucosal) side first if only one can be added at a time. Adding buffers simultaneously prevents hydrostatic pressure effects.

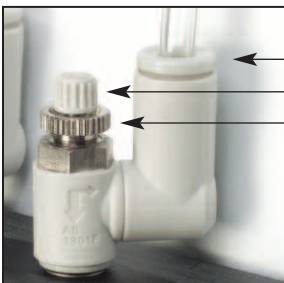
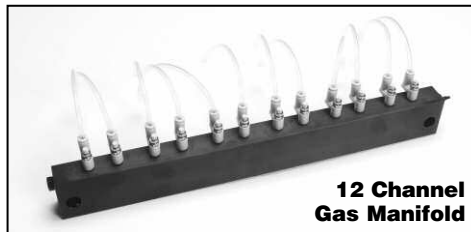
4.4 Attaching the Air Manifold

The Air Manifold for this diffusion chamber has been specially designed for use with mixed oxygen gases. Oxygen in the presence of hydrocarbons is combustible. Do not use this manifold to deliver gases which contain traces of hydrocarbons. Medical grade gases are recommended.

Other than to replace air lines, do not open the Air Manifold by removing fittings as this may introduce contaminants into the oxygen-cleaned environment.

To attach the Air Manifold:

1. Attach air lines to the chambers using the holes on the outside edge at the top of each half-cell.
2. Place a bubble trap between the air supply tank and the air manifold; then bubble the gas through water in this trap. Low humidity gas directly from the gas supply tank will cause significant fluid loss from the chamber if it is not humidified prior to delivery to the air manifold.
3. The inlet pressure to the Air Manifold should be 20-30 psi. Adjust the flow to provide adequate mixing and oxygenation, generally to 15-20 cc/mm in each half-cell, but this may vary according to application or experiment.



Press to Release
Output Hose

Locking
Ring

Flow
Adjustment

Input Pressure: 20-30ps.

Output Pressure: Generally 15-20cc/min in each half cell

NOTE: Output pressure may vary according to application and experiment.

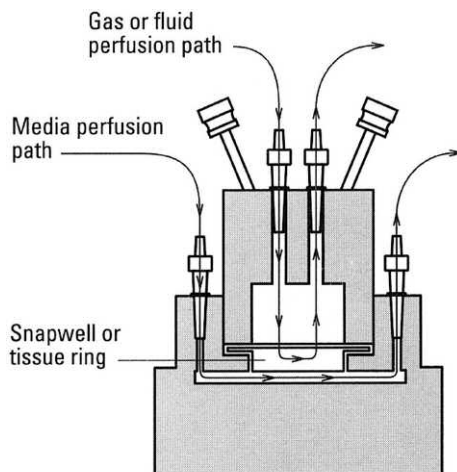
Setting Up the Experiment

4.5 Continuing the Experiment Once Snapwell™ Inserts or Tissue Segments are in Place and All Connections Appropriately Made:

1. Secure the front panel of the Heat Block to ensure a constant temperature.
2. Sample the receiver half-cell at regular intervals (every 15-30 minutes) for typical transport studies. A typical sample volume is 1 mL, but the volume can be varied depending upon the sensitivity of the assay used for analyte detection in the receiver solution. The volume removed should be replaced with fresh buffer to maintain constant volume in the receptor chamber and sink conditions.
3. Sample the donor side chamber at the end of the experiment.

4.6 Setting Up Horizontal Diffusion Chambers

For use of Horizontal Diffusion Chamber Systems, some special considerations apply. These systems are designed primarily as semi-perfused systems, in which buffer or medium is perfused through the receiver area.



**Horizontal Diffusion Chamber
Cross Section**

4.6.1 Pumps for Horizontal Diffusion Chambers

To adequately perfuse the cells or tissues in the Horizontal Diffusion Chamber System, syringe or peristaltic pumps are recommended. Syringe pumps deliver constant flow without pressure changes, but allow only a limited stock of donor volume, and do not permit access to the donor reservoir during the pumping process. Peristaltic pumps allow access to donor solution during pumping. Either a common medium or buffer reservoir may be used as a source for all channels, or individual reservoirs may be used. Medium or buffer should be oxygenated prior to pumping.

Setting Up the Experiment

4.6.2 Using the Heat Block with Horizontal Diffusion Chambers

Horizontal Diffusion Chambers are heated with a circulating water bath similarly to the Vertical Diffusion Chamber Systems. (See Sections 4, and 4.1 of this Users Guide.)

4.6.3 Cap Type Selection for Horizontal Chambers

Horizontal Diffusion Chamber Systems permit the use of either a closed or open configuration on the donor (upper) reservoir. Consider the following in choosing either configuration:

1. To prevent evaporation or to maintain a constant gas content or pressure, the closed cap should be used.
2. To use electrodes, the closed cap must be used.
3. For semi-solid materials or applications in which evaporation is not a concern, the open cap provides easier access to the upper chamber.

4.6.4 Mounting Snapwell™ Inserts

To mount Snapwell™ inserts on horizontal chambers:

1. Unscrew and remove the top cap to gain access to the inside of the chamber.
2. Place the Snapwell™ insert with the membrane area toward the bottom of the chamber.
3. Replace the top cap on the chamber and tighten. Do not over tighten the top cap! Excessive pressure is not required to create a seal. If you encounter difficulty turning the cap, place a small amount of Vaseline on the threads.

4.6.5 Mounting Tissue in Horizontal Chambers

To mount tissues in horizontal chambers:

1. Unscrew and remove the top cap to gain access to the inside of the chamber.
2. Open the optional tissue insert and place the tissue across the pins. Trim the tissue so that it does not extend past the outer edge of the ring.
3. Place the lower portion of the insert back on the pins, then insert with the pins facing the chamber bottom.
4. Replace the top cap on the chamber and tighten as above, taking care not to over tighten.

4.7 Fittings for Horizontal Chamber Systems

Luer fittings supplied with the Horizontal Diffusion Chamber Systems are used to connect tubing for fluid and gas flow within these chambers. This flexibility permits the choice of larger tubing for higher flow rates or smaller tubing to minimize the overall volume of the receiver chamber. For lower and upper chamber connections:

1. The lower chamber fluid inlet can be found on the chamber front. Fluid exits the chamber from the back. If, following the initial setup, bubbles occur in the lower chamber, tilt the chamber toward the front to help clear the bubbles from the back. If the lower chamber is used as a stagnant reservoir, a Pasteur pipette can be used in the exit channel to add media and take samples.
2. The upper chamber fittings are not directional. To create a completely closed system, place a short loop of tubing between the two fittings. Additionally, a Pasteur pipette fits through the upper channels for sampling when the closed configuration is used.

Setting Up the Experiment

4.7.1 Screws for Horizontal Chambers

Four electrode, and four alternate screws without center holes are supplied with the Horizontal Diffusion Chamber System. While the use of the electrode screws will be described in a later section, the alternate screws, for applications in which electrodes are not required, prevent fluid leakage from the chamber.

4.8 Controlling Hydrostatic Pressure in Horizontal Diffusion Chamber Systems

Equalization of hydrostatic pressure across a membrane can be challenging in Horizontal Diffusion Chamber Systems. This is of particular concern for cell monolayers because a net pressure from the basolateral to the apical side can force the cells from the membrane. To minimize the potential for hydrostatic pressure problems, keep the perfusion tubing on both the inlet and outlet tubing as close to the benchtop as possible.

4.8.1 Equalizing Hydrostatic Pressure in Horizontal Diffusion Chamber Systems

1. Place a bare Snapwell™ insert into the chamber.
2. Leave the top reservoir empty of fluid.
3. Connect the tubing and pump to the lower chamber, and fix the tubing at the same height that will be used during the experiment.
4. Pump fluid through the lower chamber at the same rate as the rate to be used during the experiment. Fluid will begin to accumulate in the upper chamber as pressure forces it through the membrane.
5. Run the system until the rising fluid reaches a steady state level.
6. Mark the fluid level in the top chamber. This level is the fluid level that must be applied to the upper chamber to equalize the hydrostatic pressure. Alternatively, the pressure may be equalized by using the closed top configuration and applying a gas pressure through the luer fittings.

4.9 Using Electrodes with Various Chamber Types

Prior to using Harvard/Navicyte Micro-Reference Electrodes in any configuration, the reference barrel must be filled with internal reference solution. To fill the barrel:

1. Remove the glass barrel from the electrode cap by grasping each end of the cap and pulling until it comes apart.
Note: The top and bottom cap sections are matched sets and should not be interchanged with other electrode sections.
2. Fill the glass barrel with reference solution using the polyethylene tubing (filling fiber) provided with the electrode. A 26 gauge or 27 gauge syringe needle will fit inside the tubing.
3. Insert the silver wire into the glass barrel and reassemble the electrode cap.
4. Before use, place the tip of the electrode into a test tube or beaker containing the experimental buffer electrolyte for 1 hour or until there is no drift in measurements. If little or no response occurs:
 1. Visually inspect the reference electrode for broken or dissolving internal Ag-AgCl wire or for inadequate volume of reference electrolyte. The Reference electrolyte should cover at least the Ag-AgCl element.
 2. Soak the tip of the electrode in warm (not hot) distilled water for 5-10 minutes if the liquid junction is blocked or clogged. An ultrasonic bath may also be used.
 3. Soak overnight in distilled water if still clogged, or replace reference barrel with the extra barrel supplied.

Setting Up the Experiment

4.9.1 Using Electrodes with Snapwell™ Inserts, and Tissue Chambers

These chamber types, 4mm Round, 4x8mm oblong, 6x9mm oblong, and 5x24mm oblong require a specialized electrode mounting cap. There are two cap designs, one for the Snapwell™ device and the other for tissue chambers. The cap is fastened to the chamber by a screw that fits the threaded port at the top rear of the chambers. Electrodes can then be slid into the ports on the cap. Electrode set-up zeroing procedures depend upon the electronic device used with the electrodes. While Harvard/Navocyte electrodes are compatible with most commercially available voltage clamps, single and six channel voltage clamps from Precision Instrument Design and Physiologic Instruments are recommended, with the initialization procedures described in their respective manuals.



To zero electrodes for Snapwell™ inserts:

1. Mount a Snapwell™ insert in the diffusion chamber. This helps seal the system during the initialization process, allowing the system to be set with a bare membrane in place.)
2. Place the electrodes in the chamber.
3. Set offset and fluid resistances.

To zero electrodes for 9 mm Round and 8x24 mm Tissue Chambers:

1. Open the Tissue Diffusion Chamber.
2. Place a rubber band around the perimeter of the pins to seal the chamber during electrode initialization to prevent chamber leaking. (Alternatively, Parafilm can be placed over the pins and hole the same size as the chamber opening can be cut.)
3. Seal the chamber.
4. Place the electrodes in the chamber.
5. Set offset and fluid resistances.

Setting Up the Experiment

4.9.2 Use of Electrodes with Horizontal Chambers and Low Volume (LV) Chambers

Horizontal Diffusion Chambers and Low Volume Tissue Diffusion Chambers do not require a cap system to position electrodes. Electrodes are held in position by a screw and O-ring assembly.

For mounting electrodes in Horizontal Diffusion Chambers:

1. Place the electrode through the screw and then the O-ring.
2. Insert the completed assembly into the electrode port and begin to tighten the screw.
3. Slide the electrode back and forth in the assembly once the O-ring begins to compress. (As the screw is tightened, the O-ring begins to compress around the electrode.)
4. Tighten the screw to hold the electrode in the appropriate position. (Do not over tighten the screw!)
5. Loosen the screw and slide the electrode out of the chamber without removing the O-ring or the screw at the conclusion of the experiment.
6. For use of Horizontal Diffusion Chambers without electrodes, replace the electrode assembly with the screw set without the center hole, with or without the O-ring.

For Mounting Electrodes in Low Volume Tissue Chambers:

1. Place voltage-passing electrodes through the two screw-mounting assemblies provided with the Low Volume Tissue Diffusion Chamber.
2. Place current-passing electrodes vertically into the tube where air bubbles rise at the outside of the chamber, to rest on the bottom of the tube.
3. Loosen, but do not remove the screw at the conclusion of the experiment and slide the electrode out of the chamber.
4. For use of Low Volume Tissue Diffusion Chambers without electrodes, a change of screw is not required.

4.10 Use of Perfusion Caps for Operation of Snapwell™ Inserts and Tissue Chambers (excluding LV Chambers) for Operation in the Perfusive Mode

Snapwell™ inserts and tissue chambers other than Low Volume chambers may be fitted with perfusion adapters. These allow continuous buffer perfusion of either or both sides of the chamber. For use of Perfusion Caps:

1. Fit a pumping system to the diffusion chamber similar to that described in Section 4.6.1.
2. Preheat and oxygenate medium before perfusing it through the chamber.
3. Do not use the Air Manifold for perfused chamber half-cells which are perfused.

5. Reference Literature for Tissue and Cell Culture Diffusion Chamber Systems

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Minimal Usable Volumes Allowing Fluid Circulation Across Open Reservoir Bottoms

Snapwell™ Inserts and Tissue Diffusion Chambers		
Chamber TYPE	Exposed SURFACE AREA (CM ²)	Half-Cell Volume (mL)
Snapwell™ Chamber	1.13	4.0 – 6.0
8 x 24 Tissue	1.78	5.0 – 7.0
9 mm Round Tissue	0.64	5.0 – 7.0
9 mm LV Tissue	0.64	1.0 – 2.0 (1.5ml)
5 mm LV Tissue	0.20	0.2 – 0.4
12mm LV Tissue	1.78	2.0 – 4.0

Calculation of Permeability

Permeability coefficients may be calculated from the following equation:

$$P = \frac{V \cdot \partial C / \partial t}{A \cdot C}$$

where:

- V = Volume of the receiver half-cell
- A = Exposed surface area (filter or isolated tissue)
- C = Initial donor concentraion
- $\partial C / \partial t$ = Change in receiver concentration over time.

Generally a plot is made of the concentrations determined in the receiver solution versus time. This is a flux curve ($\partial c / \partial t$). The permeability coefficient normalizes this curve for the starting concentraion and surface area so that comparisons can be made between dissimilar experiments.

Appendix: A

Calculation for Sampling with Replacement

When samples are removed from the receiver chamber and replaced with blank buffer, a correction must be made to account for the mass of drug the sampling procedure. This correction is additive for successive samples and can be calculated as follows:

M = mass of drug transported during the sample interval

S = Sample Volume removed

V = Total volume of the half-cell (receiver volume)

C = Concentration in the chamber at the time of sampling

C^* = Corrected Concentration (for no dilution)

$X = C/C^*$ = correction factor or, $C = C^* \cdot X$

For the initial sample:

Mass = M

$V = V$

$C = M/V$

$C^* = M/V$

Since there has been no previous dilution: $X = 1$ and $C^* = C$.

For the second sample,

$$M + \left[\frac{V-S}{V} \right] M = \frac{M}{V} (2V-S)$$

So that,

$$C = \frac{M}{V^2} (2V-S)$$

and,

$$C^2 = \frac{2M}{V}$$

Therefore,

$$X = \left[\frac{2V}{2V-S} \right] \text{ and } \frac{1}{X} = 1 - \frac{1}{2}$$

Appendix: A

Subsequent samples can be calculated in a similar manner and results in the following series for $1/X$:

Sample 1:

$$\frac{I}{X} = I$$

Sample 2:

$$\frac{I}{X} = I - \frac{I}{2} \left[\frac{S}{V} \right]$$

Sample 3:

$$\frac{I}{X} = I - \frac{2}{2} \left[\frac{S}{V} \right] + \frac{I}{3} \left[\frac{S}{V} \right]^2$$

Sample 4:

$$\frac{I}{X} = I - \frac{3}{2} \left[\frac{S}{V} \right] + \frac{3}{3} \left[\frac{S}{V} \right]^2 - \frac{I}{4} \left[\frac{S}{V} \right]^3$$

Sample 5:

$$\frac{I}{X} = I - \frac{4}{2} \left[\frac{S}{V} \right] + \frac{6}{3} \left[\frac{S}{V} \right]^2 - \frac{4}{4} \left[\frac{S}{V} \right]^3 + \frac{I}{5} \left[\frac{S}{V} \right]^4$$

The following patterns exist and make it possible to predict the correction factor for any sample number:

1. Each correction factor contains a (S/V) term raised to each power from 0 to $(n-1)$, where n is the sample number.
2. The denominator for each coefficient term is equal to the power of $(S/V) + 1$.
3. The numerator of each coefficient is equal to the corresponding number of Pascal's triangle.