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Frances Kirkland
Abstract for Senior Project
11-19-01

The Vascular Research Laboratory Experience

In the summer of 2001, I worked in a Vascular Research Laboratory (VRL). This senior project attempts to give a holistic approach to the experience by revealing three areas: 1) An overview of the VRL at The University of Tennessee Hospital in Knoxville 2) A summary of basic laboratory techniques learned 3) A paper on the research and experiments I performed this summer.

The VRL works in direct contact with the Division of Vascular Surgery to study new techniques and solutions to problems encountered during surgery. The laboratory also exists as part of the curriculum for fellows, residents, and graduate students, as well as an educational laboratory for undergraduate students interested in research and/or medicine.

Mastering basic laboratory techniques creates the foundation of any researcher. The VRL emphasizes cell and tissue culture, cell morphology, gel electrophoresis, protein assay, immunohistochemistry, protein isolation, aseptic technique, densitometry, and buffer formulation. An attempt is made in the paper to acquaint a researching neophyte with these techniques to ease the transition and shorten the time introducing these procedures when needed.

The actual research and experiments accomplished this summer comprises the last segment. An emphasis is placed on isolating smooth muscle cells from other cell types in the artery, especially from the fibroblasts, using fluorescent techniques. This project hypothesizes that fluorescent labeling might provide a faster and less expensive way to collect pure human aortic smooth muscle cells at low passages. An overview of a heat shock protein (HSP-70) project studying HSP-70 expression in canine arterial endothelial cells at different localized fluid shear stress conditions is also included.

The Purpose of the Vascular Research Laboratory

at the

University of Tennessee Graduate School of Medicine

The Vascular Research Laboratory at the University of Tennessee Graduate School of Medicine is supported through the Surgery Department at the University of Tennessee Hospital. The premise is to work in collaboration with the vascular surgeons at the hospital on ideas and problems associated with vascular surgery. The laboratory's efforts are based around "rigorous, basic and translational research in the biology of the vascular system" (Mission Statement). The laboratory focuses on the necessary understanding of cellular interactions and mechanisms in the peripheral vessels to better treat vascular disease medically and/or surgically. The Vascular Research Lab is dedicated to training talented surgeons in "experimental design, basic and advanced cellular techniques, pre-clinical biomedical device evaluation and scholarly presentation of experimental data" (Mission Statement). Inclusion of outside investigators, graduate students and undergraduate students is considered a strength of the program as it introduces non-surgical perspectives which encourage creative solutions and challenge assumptions to solve complicated problems.

The main functions of the lab are as follows: 1) research and training of select surgical residents and vascular fellows, 2) support of clinical research and development projects, 3) basic support research and clinical projects requiring technical expertise in tissue and cell cryopreservation, cell isolation and cell culture, retroviral insertion of genes into endothelial cells, fluorescence activated cell sorting (FACS) and analysis, protein isolation and Western blotting, RNA isolation and Northern blotting, PCR, immunohistochemistry, histologic interpretation and lab animal pathology.

Learned Techniques

As a Student Assistant in the Vascular Research Lab at UT Hospital in Knoxville, Tennessee, I learned many basic techniques that create the foundation of any researcher. An emphasis was placed on cell and tissue culture, cell morphology, gel electrophoresis, protein assay, immunohistochemistry, protein isolation, aseptic technique, densitometry, and buffer formulation. Several of these topics were covered in this section in order to acquaint a researching neophyte with these techniques. The desire was to ease the transition and shorten the time introducing these procedures when needed.

CELL CULTURE

Introduction:

Cell culture using sterile technique is the process of freezing, unfreezing, growing, and splitting specific cells in media compatible for the cells. Sterile work with the cells always occurs in a laminar flow hood where the airflow, temperature, and equipment are contained. Cell containers should not be opened outside of the hood. A glass window is also used to insure that sterility is not comprimised.

Freezing Cells Back:

When freezing cells back, media is aspirated off the cells and buffer is added and aspirated to wash the remaining media from the cells and the container. Trypsin is added to strip the attached

cells from the flask or well, taking about 5 minutes. Gentle tapping or incubating may quicken the releasing of the cells from the flasks. Once the cells have lifted off of the flask, media with serum is added to stop the enzyme trypsin. The cell solution is transferred to a tube and centrifuged in a balanced centrifuge for 5 minutes at 1500 rpm. The supernatant is aspirated from the tube and the pellet is re-suspended in 0.9mls fetal bovine serum (FBS) and 0.1ml of dripped DMSO and the solution is put in a cryovial. The cryovial is placed in a step-down cryo-preservation container which is then placed in a –80 degree Celsius freezer. The following day, the cryovial is removed from the freezer and placed in liquid nitrogen. The location of the cryovial in the liquid nitrogen must be logged. The hood should be sprayed with 70% ethanol.

Unfreezing Cells:

When unfreezing cells, the specified cryovial is taken out the nitrogen tank, and put in a warm water bath. The cryovial must remain standing, and water must not cover the top of the vial because the cap is porous with the potential to compromise sterility if contacted by water. Once unfrozen, the vial should be sprayed with 70% ethanol excluding the cap. The cells are then diluted in the desired amount of media and put into the appropriate flask(s). The media must be changed the following day. The hood should be sprayed with 70% ethanol.

Growing Cells:

Growing cells are contained in flasks or wells, depending on the desired amount and usage. They are kept in incubators that maintain a temperature of 37 degrees Celsius and a pressure of 5.0 atm Nitrogen, which optimizes growth. Cells must be kept in media that is best for the type of cell growing. Typical media includes IMDM (Iscores Modified Dulbecco's Media, Sigma

Inc, St. Louis, MO), SMGM2 (Smooth Muscle Growth Media, Clonetics), DMEM (Dulbecco's Modified Eagle's Media), and EGM (Endothelial Growth Media), and all include 10 % FBS.

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Splitting Cells:

When splitting cells, the cells are typically confluent from growth in a flask. Media is aspirated off the cells, and CMF is added and aspirated to wash the remaining media from the cells and the container. Trypsin, using half of the amount of media used to grow the cells, is added to strip the attached cells from the flask or well, taking about 5 minutes. Gentle tapping or incubating may quicken the stripping. Once the cells have lifted off of the flask, media with serum is added in the same amount as the trypsin. The cell solution is transferred to a tube and centrifuged in a balanced centrifuge for 5 minutes at 1500 rpm, (Notice, this is the same procedure as that for freezing cells back thus far). The supernatant is aspirated from the tube and the pellet is re-suspended in a volume or media divisible by the split. For instance, if splitting the cells into 3 different flasks, the pellet may be re-suspended in 3 mls of media. The media should be spread all over the side growing the cells to insure equal growth throughout the flask. The flasks should be labeled appropriately with a change of passage number and placed in the incubator. The hood should be sprayed with 70% ethanol.

IMMUNOHISTOCHEMISTRY

Introduction:

Immunohistochemistry is not considered a sterile technique.

Technique:

The tissue is embedded in paraffin. The embedded tissue is secured in a Jung histocut. The tissue is angled in the holder so that the embedded block is perpendicular and parallel to the razor to minimize wasteful cutting and to insure the entire tissue is cut in each slice. Sharp razors should be used at all times during the cut to ensure clean cuts, so safety is extremely important. A safeguard should be placed on the razor at all times the histocut is not being used, or the razor should be removed. Anytime the embedded tissue is not being cut, especially when positioning the tissue, the wheel should be in a locked position. Most tissue cuts have a thickness between 4 and 6 micrometers. The tissue is cut in bands with a manually-controlled wheel. If the tissue is not cutting well, the tissue and paraffin can be frozen with an aerosol freezer and/or hydrated with water. Once several cuts have been made insuring that the entire section is included, the bands are transferred to a water bath of 40 degrees Celsius. Fixation solution can be put in the water to aid in mounting on the slides. Each slide is angled in the water bath to pick up a slice of the embedded tissue. The slide is allowed to dry. The slide is placed on a hot plate to melt the paraffin. The slide(s) are placed in slide books to await the desired staining. Typical stains include H and E (Hematoxylin and Eosin) to stain the cytoplasm and nucleus, Smooth Muscle Alpha Actin to stain smooth muscle cells, antibody additions for adhesion, and quadrochrome to stain smooth muscle cells, collagen, endothelial cells and elastin, (mostly for vessels).

PROTEIN MEASUREMENTS

Introduction:

Most protein measurements do not have to be sterile. The protein measurements include protein isolation or extraction, protein assay, and Western blotting using gel electrophoresis.

Protein Isolation:

Using a nuclear and cytoplasmic extraction kit, non-denatured proteins can be purified in less than two hours. Protease inhibitors as well as Cytoplasmic and Nuclear extraction Reagents (CER and NER) (Pierce Co., Rockford, Il) are made in the specified concentrations. CER I is a hypotonic solution so that cells swell. CER II is a detergent that makes holes in the cells so that the intracellular components excluding the nucleus exit the cell membrane. NER is added causing the cell to shrink and nucleus to exit the cell membrane. NER is only needed if nuclear proteins are desired.

Homogenizing tissue:

1 ml of PBS is added to each specimen. 2 tubes are filled with 50 ml of isopropyl alcohol and 2 tubes are filled with de-ionized water. The homogenizing blade is washed in a tube of de-ionized water. The tissue is then homogenized, which is simply grounding the tissue so that it is well mixed. The blade is then washed with water, alcohol, and then PBS. The steps can be repeated as needed for each specimen. Once all of the specimens are homogenized, they are centrifuged at 1,000 G's for 5 minutes. 1.5 ml microfuge tubes are labeled appropriately. 100 ul of CER I plus some extra is obtained for each vial. Protein inhibitors are added to the CER I in the correct concentration, as noted in the extraction kit. The new CER I solution is kept on ice. Before adding the CER I to the specimens, the supernatant is taken off of the centrifuged tubes. The pellet is resuspended in 0.5 ml of PBS and transferred to smaller vials. The new vials are

centrifuged at 500 G's for 2 minutes. The supernatant is aspirated, and 100 ul of the chilled CER I is added to each pellet. The pellet is re-suspended via vortex at the highest setting for 15 seconds. The new solution is incubated on ice for 10 minutes. After incubation, 5.5 ul CER II is added to each vial. The solution is vortexed again for 5 seconds on the highest setting. It is then centrifuged for five minutes at the highest setting. The tubes are then put on ice. The supernatant, which is the cytosolic protein, is extracted and stored quickly. Care is taken not to get too close to the pellet.

Protein Assay:

Using a protein assay kit and the Lowry procedure, protein specimens can be measured against a standard. The goal is to generate a graph of absorbance versus concentration against the standards. Protein standard solutions are made using the given directions in the protein assay kit and vials are labeled appropriately. The concentrations are typically the following:

Standard #	Protein concentration (ug/ul)	Protein Standard Solution (ul)	Water (ul)
1	0	0	1000
2	5	125	987.5
3	10	25	975
4	25	62.5	937.5
5	50	125	875
6	100	250	750
7	200	500	500

Mixtures are made with 10 uls of each sample and 990 uls of water. Starting with the least amount of water concentration needed, the water is added for the standards and then the correct amount of water is added to the samples. Next, 1 ml of Lowry's Reagant is added to every tube including the standards and the samples. Each tube is vortexed at minimum speed and then sit for 20 minutes. While vortexing for quick and immediate mixing, 0.5 ml of folin is added. The solutions sit for 30 minutes and the spectrophotometer is turned on. All the solutions are then taken to the spectrophotometer. On the spectrophotometer, the quantitative route is chosen, and parameters are changed to print the seven standards, and concentrations are entered. Then, 1 ml of water is added to each cuvet, and auto-zero is pressed. The cuvets should be placed such that the clear sides face sideways and the rippled sides face the front and back. Care should be taken not to touch the cuvet on the flat, clear sides so that the results are not skewed. The front cuvet is discarded and 1 ml of each solution is added, going through the standards first, using a new cuvet each time. The results are printed with a graph of the standard to insure there is a small deviation from the expected results, and a table reporting the unknown concentrations of the protein samples. The desired mass is typically around 30 ug. In order to obtain the desired amount of mass from each sample, the desired mass should be divided by the concentration, and the quotient is the volume needed for each sample to have that mass. Sometimes, samples must be re-concentrated because the amount is too small or large for the desired masses. Microconcentrators with filters are used. Frozen vials are thawed and 90 uls of each sample is used and centrifuged.

Project: Isolating Aortic Smooth Muscle Cells via Fluorescent Markers

Background:

Current techniques to sort aortic smooth muscle cells from arterial tissue are both expensive and inefficient. The current method available physically sorts the smooth muscle cells, and the only one available for the University of Tennessee at Knoxville is at the Veterinary School.

This experiment attempts to test a possible alternative to current methods of obtaining pure lines of smooth muscle cells by chemically labeling smooth muscle cells and fibroblasts. Using Levamisole and Vector Red (Vector Lab Inc, Burlingame, CA), an attempt will be made to use these fluorescent markers as flags to sort the smooth muscle cells from the artery. If successful, this method would be useful in identifying and then obtaining the smooth muscle cells separated from the rest of the artery. Not only would it be cheaper, but sorting could be accomplished easily in any lab.

Experiment:

Canine Fibroblasts (K9 Fibro) and Human Aortic Smooth Muscle Cells (HuAOSMC) were pulled from the storage freezer and grown separately in T-25 and T-75 flasks in Dulbecco's Modified Eagle's Media (DMEM) and Iscores Modified Dulbeccos's Media (IMDM), respectively. Once confluent, (and the fibroblasts were confluent much sooner), the cell lines were each plated in wells at varying concentrations (see table below). These solutions were then each tested with Vector Red and Levamisole to determine the best concentration for viability and fluorescence of the cells with the markers.

Vector Red was made using one drop of the A, B, and C alkaline phosphotase droppers for every 2.5 mL of 100mM Tris-HCL. 1 mL of this new solution was mixed with every 9 mLs of PBS. The final solution was then filter sterilized using a syringe and a .02 micron filter with acrodiscs. The Vector Red should be added first in the dark, and incubated immediately because Vector Red reacts with light and is less sensitive the longer the final solution sits.

The Levamisole solution was made using 2 drops of Levamisole per 2.5 mLs of 100 mM Tris-HCL.

When checking for toxicity and flow, the amounts used with the fibroblasts were as follows:

Cells Treated with Vector Red

Well	Concentration	Amount Vector Red	Amount Media
1	100	2 ml	0 ml
2	1:10	200 ul	1.8 ml
3	1:100	20 ul	1.98 ml
4	1:500	4 ul	1.996 ml
5	1:1000	2 ul	1.998 ml
6	Control (0)	0 ml	2 ml

Cells Treated with Levamisole

Well	Concentration	Amount Levamisole	Amount Media
1	1:25	80 ul	2 ml
2	1:50	40 ul	2 ml
3	1:100	20 ul	2 ml
4	1:200	10 ul	2 ml
5	1:400	5 ul	2 ml
6	2:25	160 ul	2 ml

Note: The control used in the wells treated with Vector Red also served as the control for the wells treated with Levamisole.

After 16 hours of incubation with the mixed solutions, the wells were observed under microscopes to get approximations of dead cells which look rounded and/or floating versus viable which are flat and attached to the plate. The following observations were made:

Cells Treated with Vector Red

Well	Concentration of solution	Approximate Percent dead (floating)
1	100 %	90%
2	1:10	10%
3	1:100	<5%

4	1:500	<1%
5	1:1000	<1%
6	Control (0 %)	<1%

It should also be noted that Wells 5 and 6 appeared to have more dead cells because of over-confluency.

Well	Concentration of solution	Approximate Percent dead (floating)
1	1:25	10%
2	1:50	5%
3	1:100	<5%
4	1:200	<5%
5	1:400	<1%
6	2:25	90%

It should be noted that wells 1-5 follow a decreasing concentration pattern, while well 6 had the highest concentration of Levamisole.

The cells from each well were then counted with a Hemocytometer using protocol VRL-SOP-607 (Vascular Research Lab-Standard Operating Procedure-607). Each of the 6 wells were trypsinized, i.e. sloughed from the plate to allow mobility of cells. They were then centrifuged

for five minutes at 1500 rpm's. They were each re-suspended in 1 ml of media (DMEM). Using Trypan Blue the cells were counted.

The remaining cells treated with Vector Red were then tested in flow conditions with a histogram by means of a FACS machine, (Fluorescent Activated Cell Sorting machine). First, they were re-centrifuged for five minutes at 1500 rpm's. The supernatant was aspirated and the pellet was re-suspended in 1 ml 1% PBS. The solutions were put through the FACS machine.

Human Aortic Smooth Muscle Cells were also grown in two 6-well plates in SMGM2 (Smooth Muscle Growth Media 2) and when confluent, where treated with Vector Red and Levamisole in the following concentrations:

Cells Treated with Vector Red

Well	Concentration	Amount Vector Red	Amount Media (SMGM2)
1	1:10	200 ul	1.8 ml
2	1:40	50 ul	1.95 ml
3	1:200	10 ul	1.99 ml
4	1:400	5 ul	1.995 ml
5	1:1000	2 ul	1.998 ml

6	Control (0)	0 ml	2 ml

Cells Treated with Levamisole

Well	Concentration	Amount Levamisole	Amount Media (SMGM2)
1	1:40	50 ul	1.95 ml
2	1:200	10 ul	1.99 ml
3	1:400	5 ul	1.995 ml
4	1:1000	2 ul	1.998 ml
5	1:1:100	10 ul Vector Red 10 ul Levamisole	1.98 ml
6	Control (0)	0 ml	2 ml

It should be noted that wells 5 and 6 were accidentally mixed.

After sixteen hours, the dead cells and viable cells were counted using Trypan Blue and a Hemocytometer using Standard Operating Procedure VRL-SOP-607 . Each well was trypsinized by aspirating the media, washing in CMF, aspirating the CMF, releasing with 1 ml of trypsin, and diluting in 1 ml SMGM2. 25 uls were taken of each sample in a 1.5 ml tube and 500 ul Trypan Blue and 475 ul CMF were added to the 25 uls of cell media. A cover slip was placed on top of a clean, dry hemocytometer. Approximately 10 ul of the suspension was added to each

side of the hemocytometer. The cells were then counted using a given formula. Only cells inside the lines counted, and blue ones reflect non-viability. The following results were found:

Cells Treated with Vector Red

Well	Concentration	Viable	Non-viable
1	1:10	0	30,000
2	1:40	20,000	0
3	1:200	10,000	0
4	1:400	10,000	0
5	1:1000	30,000	10,000
6	Control (0)	20,000	20,000

Cells Treated with Levamisole

Well	Concentration	Viable	Non-viable
1	1:40	10,000	10,000
2	1:200	20,000	30,000
3	1:400	10,000	0
4	1:1000	10,000	20,000
5	1:1:100	0	30,000

6	Control (0)	10,000	0

Note: These Smooth Muscle Cells did not grow very well. However, the cells were counted in 10,000 increments. When taken to FACS, the machine did count almost 5000 cells for each of the wells.

All of the remaining solution of cells, trypsin, and SMGM2 was re-centrifuged at 1500 rpm's for 5 minutes. The supernatant was aspirated, and the pellet was re-suspended in 1 ml of 1% PBS. The cell-suspended solution was taken to FACS.

The results using the FACS machine from the canine fibroblasts and the human aortic smooth muscle cells were compared. First it was noted that Levamisole and Vector Red fluoresced specific cells similarly. This showed that one fluorescent marker did not prove more favorable to either fibroblasts or smooth muscle cells. It was decided that Vector Red would be the fluorescent marker studied from this point.

The three fluorescent band pass filters, i.e. FL1, FL2, and FL3, fluoresced best at 530 nM for FITC/FL1, 585 nM for PI/PE or FL2, and greater than 650 for Red/FL3. It was also noted that the Vector Laboratories reported that Vector Red excitation is "broad and seems to be from 360 nm to 560 nm," while the emission spectrum seems to be above 560 nm.

For every concentration level, the canine fibroblasts fluoresced with one sharp peak just less than 10^1 and ending around 10^2. The human aortic smooth muscle cells, on the other hand, fluoresced with two peaks around 10^2 and 10^3. The most intriguing finding was that at a Vector Red concentration of 1:10 the smooth muscle cells fluoresced at curves beginning above 10^2 for

the FL2 and FL3 with peaks halfway between 10^2 and 10^3 as well as 10^3 and 10^4. These results are not only higher than the fibroblast emissions, but do not even overlap. Thus, the possibility of gating the cells at 10^2 or slightly above could result in a pure smooth muscle cell line.

Due to availability and unavailability of specific cell lines, cells were grown in 8 wells. The first two contained rabbit fibroblasts, the second two contained canine fibroblasts, the third two contained human smooth muscle cells T/G ATCC, and the fourth two contained human fibroblasts which was confirmed after a negative alpha actin staining test for smooth muscle cells. The rabbit fibroblasts and canine fibroblasts were grown in DMEM, the human smooth muscle cells were grown in SMGM2, and the human fibroblasts were grown in IMDM. One of each of the types of wells was considered a control, to which only media was added, and the other had 200 ul of Vector Red to 1.8 ml of media, a concentration of 1:10. The cells were incubated for sixteen hours. Upon retrieval they were centrifuged, the supernatant was aspirated, and the pellet was re-suspended in 1ml 1% PBS. The canine fibroblasts, wells 3 and 4, did not grow very well. It was assumed that they were grown for several days in media without 10% FBS, thus killing the cells.

The results showed the rabbit, canine, and human fibroblasts peaking between 10^1 and 10^2 and tapering quickly above 10^2. The fibroblast peak completely ends by 10^3. The human smooth muscle cells, on the other hand, peaked above 10^3, with an ascending slope sharpening halfway between 10^2 and 10^3, and remaining high until 10^4. A mild peak with the smooth muscle cells did occur around 10^2, however, the bulk of the cells remained in the peak to the right.

The goal is to efficiently and inexpensively isolate smooth muscle cells from arterioles so that the cost of future research using smooth muscle cells will be reduced, and the availability of smooth muscle cells will be increased.

Heat Shock Protein Project

Atherosclerosis is a progressive vascular disease causing plaque formation and an ensuing occlusion of the vessel lumen. The disease begins with endothelial lesions. It has been documented that endothelial lesions result from several local hemodynamic factors such as wall shear stress and turbulent flow. It has been reported that adhesive properties of endothelial cells are heightened by chronic shear stress. In response to high shear stresses in both in-vivo and in-vitro models, endothelial cells increasingly express heat shock proteins (HSPs). In direct correlation, studies have shown the enhanced adhesive properties would benefit from vascular graft implantation. Potentially, HSPs are targets of anti-HSP antibodies at primary endothelial lesion sites for decreasing auto-immunogenic activity. A transplant may be at risk of "arterio-venosclerosis" or post-implantation sclerosis if exposed to arterial hemodynamic conditions, which is relevant to the use of vein grafts.

The goal of this project was to study HSP-70 expression in canine arterial endothelial cells at different localized fluid shear stress conditions. Endothelial lesions at the beginning of atherosclerosis would be better understood if HSP-70 expression was upregulated with those stress conditions. Using native blood and artery of a canine, the expression of HSPs could be tested in an ex vivo model. An in vitro study occurred using media with the same viscosity as blood. This study included a flow apparatus allowing two vascular conduits to be exposed simultaneously at two different flow conditions. Further studies assessing the expression of other

molecules including adhesion molecules, could be done using this as a model. The ability to use native blood in the flow system makes the model as close to an in vivo model as possible while keeping other hemodynamic factors independent.

In the ex-vivo model, cells from the carotid arteries of dogs were exposed simultaneously to different flow conditions via a flow system. Each vessel was exposed to each condition and compared. The conditions included normal physiological stress at 15-30 dynes/cm2 and extra-physiological stress anywhere above 60 dynes/cm2. Blood was the fluid used at a time constant of four hours. Distributive analysis was done for intima, media, and adventia layers of the vessel using immunohistochemical staining. The staining compared the amount of HSP-70 expression in each layer. Difficulty was encountered cutting the grafts as tissue for slide preparation because the grafts were plastic. Protein analysis was also done to quantify the amount of protein in the endothelial cells. Two static controls were used. The first static control remained at room temperature for four hours before they were frozen. The second static control was tissue placed in a petri dish with whole blood in an incubator at 37 degrees Celsius with 5 percent carbon dioxide. The staining of the tissues is in progress and is part of on-going research in the Vascular Research Lab.

The Flow System:

A 2000 ml Erlenmeyer flask was filled with media and a stop cork was used with holes for the gas-permeable tubing. A pulse dampener and pulsitile pump were used to pump blood into the system. Ti-Cron suture was used to tie the vessels to the connectors. 25 ml pipets were used to keep the vessels straight and stable because curvature would skew the results. The flask was incubated in water at 37 degrees Celsius to be comparable to human body temperature. A trial run was done using water.

1-3 Splitting Cells 05/15/01

4-7 Freezing Cells Back 05/16/01

8-10 Notes on HSP-70 Project 05/21/01

11-12 Protein Isolation 05/22/01

13-15 Protein Isolation Notes 05/22/01

16-21 Protein Assay 05/23/01

22 Notes Protein Extraction 05/24/01

23 Protein Assay Results 05/31/01 *

24 Protein Assay Results 06/01/01

25 Imunohistochemistry 06/05/01

26-27 Product Specification Sheet 06/12/01

28 Notes on sorting AoSMC Project 06/13/01

29-31 H&E staining of tissue slides 06/15/01

32-33 Vector Red / Levamisol conc. (project) 06/20/01

34-35 Cell Counting w/ Hemocytometer 06/21/01

36 Flow Counting 06/21/01

37 Making Vector Red & Levamisol 07/22/01

38 HuASMC → VR & Lev amts & Labels 07/23/01

39-40 Cell Cytometer & Flow 07/24/01

41-42 Vector Red & Levamisole Protocols 07/24/01

43 Vector Red emission & excitation λ 07/27/01

44 Flow / Facs fluorescent λ 07/27/01

45 HSP Project Flow system 07/27/01

46 Vector Red Fibr/SMC sorting → tissues to test 07/27/01

47-48 VR Fibr/SMC sorting retest set-up 07/27/01

49 To plate cells from specimen/tissues 08/10/01

Splitting Cells Tuesday May 15, 01

SOP Number	University of Tennessee Medical Center Vascular Research Lab	Page
VRL-SOP 601	Standard Operating Procedures	Of 1 of 3

Title: Protocol for splitting cells

I. Scope:

This SOP will be followed by personnel engaged in research and clinical studies in the Vascular Research Laboratory of the Department of Surgery.

II. Purpose:

To describe the procedures for proper splitting of cells

III. Procedures:

1. Reagents Required

a. Clonetics trypsin/EDTA (stored frozen in sliver freezer)
b. CMF (in hood)
c. Media with serum (in white refrigerator)
d. 70% Ethanol (next to hood)

2. Equipment Required

a. 10 ml pipette
b. Centrifuge tube
c. Appropriate number of T-75 flasks
d. Aspirator
e. Pipettes

3. **Operation Guidelines**

a. Turn the hood on at least 10 minutes prior to beginning.

b. Place the Clonetics trypsin/EDTA, CMF, and media with serum in the hood and spray with 70% Ethanol.

c. Place the cells in the hood.

d. Aspirate the media off the cells.

e. Place 5 ml of CMF on the cells and slide around flask to cover cells completely.

f. Aspirate the CMF off the cells.

g. Place 5 ml of trypsin/EDTA on the cells.

h. Allow the cells to come off the surface of the flask for about 5 minutes. Watch cells under microscope, tapping gently helps get all of the cells off the surface.

i. Once the cells are off the bottom of the flask, add 5 ml of the media with serum to the flask.

J. Use a 10 ml pipette to pull all of the solution out of the flask to transfer to a 15 ml centrifuge tube. DURING SUCTION INTO 10 ML PIPETTE, THE CELLS ARE NOT TO EXCEED THE COTTON PROTECTION ON THE TOP OF THE PIPETTE. IF THIS OCCURS, THE STERILITY OF THE CELLS HAS BEEN COMPROMISED.

k. Spin the cells in the centrifuge at 1500 rpm for 5 minutes. MAKE SURE CENTRIFUGE IS BALANCED.

(Get new T-75 flasks MARK → type of cell, (IMDM) media, your initials, date, what you're doing)

l. Once the cells have spun, place the tube back in the hood and aspirate off the supernatant. BE CAREFUL NOT TO ASPIRATE THE PELLET IN THE BOTTOM OF THE TUBE.

m. Bring the cells up in a volume suitable for the size of your split with media. (i.e. 3 mls for a 1:3 split, etc.)

n. Place the correct amount of cells in a T-75 flask.

o. Add the remaining volume of media to have 10 mls of media in the flask.

Spread media/cells over side

p. Label the flasks and change the passage number. Loosely replace lids and place flasks in the incubator.

q. Clean up hood/working area with 70% ethanol.

4. **Trouble shooting**

a. If at any point during the procedure, the sterility of the cells are compromised, the cells are to be disposed off or labeled as being

b. contaminated until further instructions for course of action from the lab supervisor.

c. To avoid such problems, the user should sterilize the hood/working area with 70% ethanol before starting the procedure.

d. Be careful not to aspirate the pellet in the bottom of the tubes.

5. **Calibration Instructions**
.NA

6. **Routine Maintenance Instructions and Schedule**
NA

7. **Non-routine maintenance instructions**
NA.

8. **Responsible person(s)**
Laboratory director / senior laboratory technician

III. **References**
None

IV. **Attachments**
None

VRL.SOP.601 / 2000 / AS

Freezing Back Cells

Wednesday, May 16, 2001

5

SOP Number VRL-SOP-602	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 1 of 3

Title: **Freezing Cells Back**

I. **Scope:**
This SOP will be followed by personnel engaged in research and clinical studies in the Vascular Research Laboratory of the Department of Surgery.

II. **Purpose:**
To describe the procedures for proper freezing of cells back.

III. **Procedures:**

1. **Reagents Required**
   a. Trypsin/EDTA (frozen in silver freezer) may be thawed in fridge
   b. CMF (in hood)
   c. Media with serum (in white refrigerator)
   d. Aliquot of FBS (frozen in silver freezer or thawed in white refrigerator) (a portion)
   e. DMSO (in hood in a foiled 15ml tube)
   f. Thawed Mr. Frosty (frozen in -80°C freezer at the end of hall)
   g. 70% Ethanol (next to hood)

2. **Equipment Required**
   a. 15ml centrifuge tube
   b. 1ml ~~cyrovial~~ cryovial
   c. 1cc syringe
   d. Aspirator
   e. 10ml pipette

3. **Operation Guidelines**
   a. Turn on the hood at least 10 minutes to beginning.
   b. Spray the hood/working area with 70% Ethanol.
   c. Place FBS in water bath until thawed, then place in hood. may already be thawed
   d. Place trypsin/EDTA, CMF and media with serum in hood a~~nd spray w~~ith ~~alcoh~~ol.
   e. Place cells in hood and aspirate the media off the cells.

f. Place 5 ml of CMF on the cells and slide around flask to cover cells completely.

g. Aspirate the CMF off the cells.

h. Place 5 ml of trypsin/EDTA on the cells.

(2) may put in incubator
(1) may aspirate (HeLa cells involved) + add 2nd batch of trypsin

i. Allow the cells to come off the surface of the flask for about 5 minutes. Watch cells under microscope, tapping gently helps get all of the cells off the surface.

go ahead & label cell type (HeLa); date, type of media cell was growing in before, initials

j. Once the cells are off the bottom of the flask, add 5 ml of the media with serum to the flask.

(may add 10 mL)

K. Use a 10 ml pipette to pull all of the solution out of the flask to transfer to a 15 ml centrifuge tube. DURING SUCTION INTO 10 ML PIPETTE, THE CELLS ARE NOT TO EXCEED THE COTTON PROTECTION ON THE TOP OF THE PIPETTE. IF THIS OCCURS, THE STERILITY OF THE CELLS HAS BEEN COMPROMISED.

l. Spin the cells in the centrifuge at 1500 rpm for 5 minutes. MAKE SURE CENTRIFUGE IS BALANCED.

m. Once the cells have spun, place the tube back in the hood and aspirate off the supernatant. BE CAREFUL NOT TO ASPIRATE THE PELLET IN THE BOTTOM OF THE TUBE.

n. Resuspend the pellet in 0.9 mls of FBS and place in the cryovial.

if using HeLa cells, may use 3 mL cryovials

o. ~~Obtain a 1 cc syringe~~ Use a micro pipet and place 0.10 cc of DMSO into the vial, dripping it slowly over the cells. (100 µL)

p. Label the cryovial appropriately, place it into the Mr. Frosty, and put in the -80° C freezer at the end of the hall.

q. The next day, remove the Mr. Frosty from the freezer and place the cryovial in liquid nitrogen. Fill out the liquid nitrogen log book so the location of the cells are appropriately documented

4. **Trouble shooting**
   a. If at any point during the procedure, the sterility of the cells are compromised, the cells are to be disposed off or labeled as being contaminated until further instructions for course of action from the lab supervisor.
   b. To avoid such problems, the user should sterilize the hood/working area with 70% ethanol before starting the procedure.
   c. Be careful not to aspirate the pellet in the bottom of the tubes.

5. **Calibration Instructions**
   NA

6. **Routine Maintenance Instructions and Schedule**
   Alcohol in the Mr. Frosty should be changed after 5 uses. Allow the Mr. Frosty to thaw after removing from the freezer.

7. **Non-routine maintenance instructions**
   NA

8. **Responsible person(s)**
   Laboratory director / senior laboratory technician

IV. References
None

V. Attachments
None

5/21/01

approx. 2 weeks left of project

Notes on project w/ Aniket

"Heat Shock Protein Project"

Ex vivo assessment of fluid shear stress on hsp-70 in canine carotid arterial endothelial cells

anything that happens, 1st occurs on endothelial cells

atherosclerosis → autoimmunologic injury

(HSP) Heat Shock Protein expression occurs from stress → heat, fluid-shear stress, etc.

↳ possible auto-antigens

- Trying to find where stress is high & what are local conditions causing it
  a lot of fluid-shear stress → at least initial up-regulation of HSP
- Expose cells (Ex vivo model → dead, outside body, HSP 70)
  ↳ took carotid arteries of dogs & simultaneously exposed to different flow conditions
  put arteries in flow system

Normal physiological stress 15-30 dynes/cm² (1 vessel exposed)
Extra-physiological stress → anything above 60 (1 vessel exposed)
compared

Blood flow, time constant for 4 hrs.

Distributive analysis → (3 layers of vessel: entoma, media, adventia) [via staining: immunohistochemistry] compares amt. in each layer

Protein analysis → quantification of amt. of protein in endothelial cells

immunohistochem. → cut slides of graft, stain, etc.

(over)

fixing → fixing in Carson's fixative (solution for immunohistochem)

cont. HSP project 5-21-01

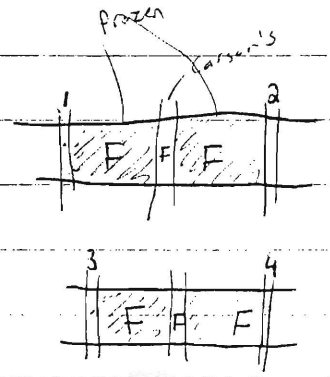
static control - cells not exposed to anything

↳ static w/ time → static for 4hrs. before frozen

↳ static w/ 0 time → immed. after get vessel → cut, 1 sect. fixed, 1 section frozen

Protein		Distributive
Static w/ 0 time	← immediately (0 time) →	Static w/ 0 time
Static w/ time	← petri dishes for 4 hrs →	Static w/ time
Flow	← 4 hr flow stress →	Flow
afterwards, frozen		afterwards, Carson's

(924)



2 sections each (from same dog). if had taken all out time vessel would be small

Extra-physiological

Statics would be same, so only 4 cuts total needed

Normal

Protein analysis

extract protein, protein assay, western blotting, immuno blotting

18 tubes to do

Big Picture → exposing venous vessels to arterial stress

Tuesday, May 22, 2001
Protein Isolation

STRUCTIONS

PIERCE
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

NE-PER™ Nuclear and Cytoplasmic Extraction Reagents

8333 0872w

Product Description

Number	Description
83	**NE-PER™ Nuclear and Cytoplasmic Extraction Reagents** This kit supplies a complete set of lysis reagents that enable the separation of nuclear extract and cytoplasmic fractions from cultured cells and tissue. The optimized reagents and protocol allow non-denatured, active proteins to be purified in less than two hours. This kit contains sufficient cytoplasmic and nuclear extraction reagents for extracting 50 cell pellet fractions with a packed cell volume of 20 µl (a total of ~2.0 g of cell paste). **Kit Contents** **Cytoplasmic Extraction Reagent I (CER I),** ***10 ml*** **Cytoplasmic Extraction Reagent II (CER II),** ***550 µl*** **Nuclear Extraction Reagent (NER),** ***5 ml***

Instructions for Use

Materials

A. Mammalian cells from whole tissue or cultured cells

B. Protease inhibitors:

Nuclear and cytoplasmic extraction can be done without the use of protease inhibitors. For optimal results, however, we recommend using a mixture of the following protease inhibitors at the indicated final concentrations.

Inhibitor	[Stock]	[CER I]	[CER II]	[NER]	
Benzamidine	250 mg/ml	0.5 mg/ml	N/A	0.5 mg/ml	1:500
Aprotinin	2 mg/ml	2 µg/ml	N/A	2 µg/ml	1:1000
Leupeptin	2 mg/ml	2 µg/ml	N/A	2 µg/ml	1:1000
PMSF (Phenlymethlysulfonyl Flouride)	0.2 M	0.75 mM 5µl/ml	N/A	2 mM 10µl/mL	

Protocol

The following protocol reflects reagent volumes based on samples with a packed cell volume of approximately 20 µl (~40 mg of cell paste). Determine the packed cell volume (or mass) of your samples, then substitute volumes as indicated in the table below.

Packed Cell Volume	CER I	CER II	NER
10 µl (20 mg)	100 µl	5.5 µl	50 µl
20 µl (40 mg)	200 µl	11 µl	100 µl
50 µl (100 mg)	500 µl	27.5 µl	250 µl
100 µl (200 mg)	1 ml	55 µl	500 µl

Note: All centrifugation procedures should be done at 4°C. Keep cell samples and extracts on ice.

1. Isolate 20
500 x g fo
as PBS, an
remove the
page 1, an
directly in
50 mg tiss
2. Using a pi
3. Add 200 µ
4. Vortex vig
minutes.
5. Add 11 µl
6. Vortex 5 s
7. Vortex 5 s
(16,000 x
8. Immediate
9. Resuspen
10. Vortex on
minutes, f
11. Centrifug
12. Immediat
13. Store all

Troublesh

Observation
Low cytoplas
Low nuclear
Low concentr
No or low pr
Proteins not
No or low pr fraction

Addition

Number
23235
34080

©Pierce Chem

Tele

281-820-9898
ext 2908

PIERCE

1. Isolate 20 µl packed cell volume (40 mg) of cells by centrifugation in a 1.5 ml microcentrifuge tube by centrifugation at 500 x *g* for 2-3 minutes. **Note:** If using tissue samples, cut the tissue into small pieces, add an appropriate buffer such as PBS, and homogenize in a tissue homogenizer. Pellet the cells by centrifugation at 500 x *g* for 2-3 minutes and remove the supernatant. Estimate the packed cell volume, add the appropriate amount of CER I according the chart on page 1, and continue with Step 4 below. Alternatively, weigh the tissue, cut it into small pieces, dounce homogenize directly in CER I, and proceed to Step 4. Use a 10-fold excess of CER I over the weight of tissue (e.g. 500 µl CER I to 50 mg tissue). In Step 5 of the protocol, use 5.5 µl of CER II per 100 µl of CER I.
2. Using a pipet, carefully remove and discard the supernatant, leaving cell pellet as dry as possible.
3. Add 200 µl of ice-cold CER I to the cell pellet.
4. Vortex vigorously on the highest setting for 15 seconds to fully resuspend the cell pellet. Incubate the tube on ice for 10 minutes.
5. Add 11 µl of ice-cold CER II to the tube.
6. Vortex 5 seconds on the highest setting. Incubate on ice for 1 minute.
7. Vortex 5 seconds on the highest setting. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge (16,000 x *g*).
8. Immediately transfer the supernatant (cytoplasmic extract) fraction to a clean pre-chilled tube. Place this tube on ice.
9. Resuspend the insoluble (pellet) fraction produced in Step 7, which contains nuclei, in 100 µl of ice-cold NER.
10. Vortex on the highest setting for 15 seconds. Return the sample to ice and continue vortexing for 15 seconds every 10 minutes, for a total of 40 minutes.
11. Centrifuge the tube at full speed (16,000 x *g*) in microcentrifuge for 10 minutes.
12. Immediately transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube. Place on ice.
13. Store all extracts at -80°C until use.

Troubleshooting Guide

Observation	Possible Causes	What to do
Low cytoplasmic protein yield	Cells not lysed	Increase amount of CER II Reagent
	Cell pellet not dispersed	Vortex thoroughly
Low nuclear protein yield	Cell pellet not dispersed	Vortex thoroughly
	Incomplete nuclei isolation	Increase time of centrifugation following addition of CER II
Low concentration of protein	Volumes of extraction reagents not appropriate for given packed cell volume	Adjust volumes as directed in protocol table of reagent volumes based on packed cell volume
No or low protein activity detected	Samples not kept cold	Centrifuge at 4°C and keep samples on ice between vortexing steps
	Presence of proteases	Use suggested protease inhibitor cocktail
Proteins not compartmentalized	Incomplete removal of cytoplasmic extract	Carefully remove all cytoplasmic extract prior to nuclear lysis
	Incomplete lysis of cells	Increase vortexing time to be sure cell pellet is dispersed
No or low protein yields in either fraction	Cell line dependent result	May not work with all cell lines

Susan
971 5659

Additional Pierce Products of Interest

Number	Description
23235	Micro BCA™ Protein Assay Reagent Kit
34080	SuperSignal® West Pico Chemiluminescent Substrate

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Telephone: 800-8-PIERCE (800-874-3723) or 815-968-0747 • Fax: 815-968-7316 or 800-842-5007
Internet: http://www.piercenet.com

K9 ~~X~~4 5/22/01

Protein Isolation notes

static 0	static 4hr	normal 4hr	extraphysiological 4 hr
7mm used approx. ½ for protein analysis	10 mm used approx. ½ for protein analysis	17 mm	12mm

What's Going On:

Putting hypotonic solution (CER I) on cells (cells swell)
Add in CER II → detergent → pokes holes in cell membranes → proteins come out, ER, etc (all but nucleus)
nucleus doesn't come out → NER added causing cell to shrink causing nucleus to come out.

1. 1.0 mL of PBS in each
2. 2 tubes (50 mLs) of isopropyl alcohol
   2 tubes (50 mLs) of de-ionized H_2O
3. Homogenizing blade washed w/ H_2O
4. Static 0 homogenized (attempt)
5. blade washed w/ H_2O, alcohol, then PBS
6. more PBS added to static 0 and homogenized
7. blade washed w/ H_2O, alcohol, then PBS
8. Static 4hr homogenized
9. blade washed w/ H_2O, alcohol, then PBS
10. Normal 4hr homogenized
11. blade washed w/ H_2O, alcohol, then PBS
12. Extraphysiological 4hr homogenized
13. blade washed w/ H_2O, alcohol, then PBS
14. Follow instructions on previous 2 pg (11-12)
15. Put tubes in centrifuge @ 1,000 G's for 5 min.
16. Big microfuge tubes (1.5 mL) labeled
17. 500 µL CER 1 (100 µL for each vial + extra for easy calc. & back-up)
18. Addition of protein inhibitors to CER 1 → 1 µL Benzamidine, ½ µL Aprotinin, ½ µL Leupeptin, ~~[illegible]~~ 2.5 µL PMSF

(19) Put CER I on ice (does not have to be sterile)

(20) Taking supernatant off of centrifuged tubes (NOTE: CER I has not yet been added)

(21) Resuspending pellet in ½ mL of PBS and transfering to smaller vials.

(22) Centrifuging vials at 500 Gs for 2 min. (last few steps trying to get down to smaller volume & rid of big chunks)

(23) Take out supernatant

(24) Add CER I (100 µL to each pellet)

(25) Re-suspend via Vortex (highest setting 15 sec)

(26) Incubate in ICE for 10 minutes.

(27) 5.5 µL CER II added to each vial.

(28) Re-suspend via vortex (5 sec. on highest setting)

(29) Incubate on ice for 1 min.

(30) Vortex again for 5 sec.

(31) Centrifuge for 5 min. @ highest setting

(32) Put 4, 0.5 mL tubes on ice (and label)

(33) Supernatant is protein → extract supernatant & store. (do quickly)
↳ be careful not to get too close to pellet (really don't need that much protein anyway)

Not extracting NER (Nuclear proteins) so only went through step 8 on pg. 11-12

05/22/01

Condensed version from pg. 13 & 14

K9 #4 Protein Isolation/Extraction
5/22/01

Static 0 hrs--7mm total length after thawing
Static 4 hrs--10mm "
Normal 4 hrs--17mm "
Extraphysiological 4hrs-- 12mm

1. For static sections, approximately half of total length was used for the PI procedure.
2. Transferred the sections to separate 50ml centrifuge tubes (in ice)
3. Added 1ml PBS to each tube.
4. Homogenized each section
5. At each interval, washed the homogenizer blade with D-water, isopropyl alcohol and PBS respectively
6. Centrifuged tubes @ 1000G for 5 minutes
7. Extracted supernatant and resuspended in 0.5ml PBS.
8. Transferred solution to appropriately labeled 1.5ml microfuge tubes (in ice)
9. Made 500 uL CER I solution and added 1uL benzamidine, 0.5 uL Aprotinin, 0.5 uL Leupeptin and 2.5 uL PMSF and vortexed to mix well.
10. Added 100uL of CER I to each microfuge tube and vortexed for 15 seconds at highest setting
11. Incubtede for 10 minutes on ice.
12. Added 5.5 uL of CER II to each tube.
13. Vortexed for 5 sec, incubated for 1 minuted, vortexed again for 5 secs
14. Centrfuged for 5 minutes at 16000Gs
15. Labeled and put four 0.5 ml microfuge tubes into ice.
16. Transferred the supernatanat which was the protein to 0.5 ml microfuge tubes
17. Got about 100uL of protein in from each tube.

Protein Assay

Wednesday, May 23, 2001

Wednesday 05/23/01 : Protein Assay

Protein Quantitation of lysate

chemical cabinet

IGMA AGNOSTICS®

PROTEIN ASSAY KIT
(Procedure No. P 5656)

not sterile

INTRODUCTION

ɔwry procedure has been found to be the most reliable ɛfactory method for quantitation of soluble proteins. The described here is based on Peterson's modification o-Lowry method and utilizes sodium dodecylsulfate, the Lowry Reagent, to facilitate the dissolution of insoluble lipoproteins.[1]

any proteins, the Lowry reaction can be run directly on solution. However, interference in the direct Lowry s caused by commonly used chemicals, such as Tris, sulfate, EDTA, sucrose, citrate, amino acid and peptide and phenols. The procedure with protein precipitation, es DOC (deoxycholate) and TCA (trichloroacetic acid), l these interferences with the exception of phenols. e amount of various proteins recovered through the n step may vary depending on the particular protein

PRINCIPLE

aline cupric tartrate reagent complexes with the peptide nd forms a purple-blue color when the phenol reagent sorbance is read at a suitable wavelength between 800 nm. The protein concentration is determined alibration curve.

REAGENTS

(For laboratory use only.
Not for drug, household or other uses.)

REAGENT, MODIFIED, Catalog No. L 1013

CORROSIVE. Avoid contact with eyes, skin . Do not breathe dust.

VRY REAGENT SOLUTION is prepared by adding 40 water to a bottle of Lowry Reagent, Modified. Mix well rring to completely dissolve. Do not shake so as to e foaming. Solution is stable at room temperature. T REFRIGERATE.

LUTION, Catalog No. D 5525
eous solution of sodium deoxycholate, 1.5 mg/ml.

OACETIC ACID SOLUTION (TCA),
T 4396
eous solution of trichloroacetic acid, 72% w/v.

CORROSIVE. CAUSES BURNS. Avoid contact with nd clothing.

IOCALTEU'S PHENOL REAGENT,
No. F 9252

CORROSIVE. CAUSES BURNS. Avoid contact with nd clothing. Do not breathe vapor.

FOLIN & CIOCALTEU'S PHENOL REAGENT WORKING SOLUTION is prepared by transferring the contents (18 ml) of Folin & Ciocalteu's Phenol Reagent to the amber glass bottle provided for the Working Solution. Rinse the Folin & Ciocalteu's Phenol Reagent bottle with 10 ml deionized water and add rinsings to Working Solution bottle. Add an additional 80 ml deionized water to the Working Solution bottle and mix well. Store Folin & Ciocalteu's Phenol Reagent Working Solution at room temperature.

PROTEIN STANDARD, Catalog No. P 7656
Prepared from bovine serum albumin, fraction V (BSA).

PROTEIN STANDARD SOLUTION, 400 µg/ml, is prepared by adding to the vial the volume of water indicated on the vial label. Swirl gently to completely dissolve. Store solution in the refrigerator (0–6°C) or freezer (below 0°C). Discard if turbidity develops. Stable at least 3 months when stored refrigerated.

DIRECT PROCEDURE
(Without Protein Precipitation)

* see changes next sheet

1. Prepare STANDARD TUBES by diluting Protein Standard Solution with water to a volume of 1.0 ml in appropriately labeled test tubes:

	Protein Standard Solution (ml)	Water (ml)	Protein Concentration (µg/ml)
1	0.125	0.875	50
2	0.250	0.750	100
3	0.500	0.500	200
4	0.750	0.250	300
5	1.000	0	400

2. Label a test tube BLANK and add 1.0 ml water.
3. Add sample to appropriately labeled test tube and dilute to 1.0 ml with water. Spin if cloudy
4. Add 1.0 ml Lowry Reagent Solution to STANDARD, BLANK and SAMPLE tubes. Mix well.
5. Allow solutions to stand at room temperature for 20 minutes.
6. With rapid and immediate mixing, add 0.5 ml Folin & Ciocalteu's Phenol Reagent Working Solution to each tube.
7. Allow color to develop for 30 minutes.
8. Transfer solutions to cuvets and measure the absorbance of the STANDARDS and SAMPLE tubes vs. the BLANK at a wavelength between 500 and 800 nm. Complete readings within 30 minutes.
9. Plot the absorbance values of the STANDARDS vs. their corresponding protein concentrations to prepare a calibration curve. (See Figure 1 for a typical calibration curve for standards at 750 nm.)
10. Determine the protein concentration of the SAMPLE from the calibration curve. Multiply the result by appropriate dilution factor to obtain the protein concentration in the original sample.

05/23/01

concentrations for standard in Protein Assay

Standard

400 µg/ml Standard

		Standard	H_2O
1	0 µg/µl	0	1000 µl
2	5	12.5 µl	987.5
3	10	25 µl	975
4	25	62.5 µl	937.5
5	50	125 µl	875
6	100	250 µl	750
7	200	500 µl	500

Samples

10 µl sample - 990 µl H_2O.

OD-800 for spectrophotometer

Notes on Protein Assay 05/23/01

Goal is to generate graph of absorbance vs. concentration against the standards (7)

(1) Make 7 standards w/ given concentrations (pg. 18

(2) Start w/ #7 & put water concentration in (7th has lowest H_2O concentration) from 6 to 1

(3) For samples, put 900 µL H_2O in all of them

(4) Put standard in (protein standard) go from lowest volume to highest of standard (1-7)

- can make it from kit

- Just making concentrations H350 (HSP K9 #3 Static 0)

S4 → static 4hr
NO → normal
EP → extrapolated

(5) Labeled vials matched to #ed tubes

1 H350
2 H354
3 H34NO
4 H34EP
5 H450
6 H454
7 H44NO
8 H44EP

Today:
Lowry's Reagent change
on bottles
1 bottle for all HSPs & 1 & 3 standards
1 bottle for 4-7 standards
(did not mix bottles beforehand)
may affect results
(★ make sure you have enough stuff. if you don't when open new bottle may not be exact same... would have to mix both bottles together)

(6) Adding 10 µL of extracted protein according to table

(7) Add 1 mL Lowry Reagent to every tube (standards & HSPs)

(7a) Vortex each tube @ min. speed

(8) Let sit for 20 min.

(9) Add .5 mL folin → quick & immediate mixing → add while vortexing

(10) Wait 30 min., Turn on spectrophotometer NOW

(11) Choose spec. → quantitative, push enter, change parameters (7 standards, print), enter concentrations

(12) Add 1 mL H_2O to ~~each~~ each cuvet

(13) Press auto-zero

(cont.) 5/23/01

(14.) Change front cuvet & add 1 mL each time going thru standards first. (Use new cuvet each time)

(15) Print results (graph of standard, table of unknowns)

- get concentration ($\frac{mass}{volume}$)

- want 30 µg mass

So divide mass by concentration (must change units to match)

** QUANTITATIVE ANALYSIS **

	No.	ABS.	CONC.	
	1	0.147	12.832	24.93 µL
✓	2	0.118	5.5768	53.80 µL
✓	3	0.126	7.2975	41.109 µL
✓	4	0.104	2.3483	127.75
✓	5	0.110	3.7470	80.06
✓	6	0.122	6.4368	46.61
	7	0.270	38.929	7.706
	8	0.215	26.879	11.16

[redacted]

(cont.)

(14.) Change front cuvet & add 1 mL each time going thru stan

(15) Print results (graph of standard, table of

- get concentration (mass / volume)

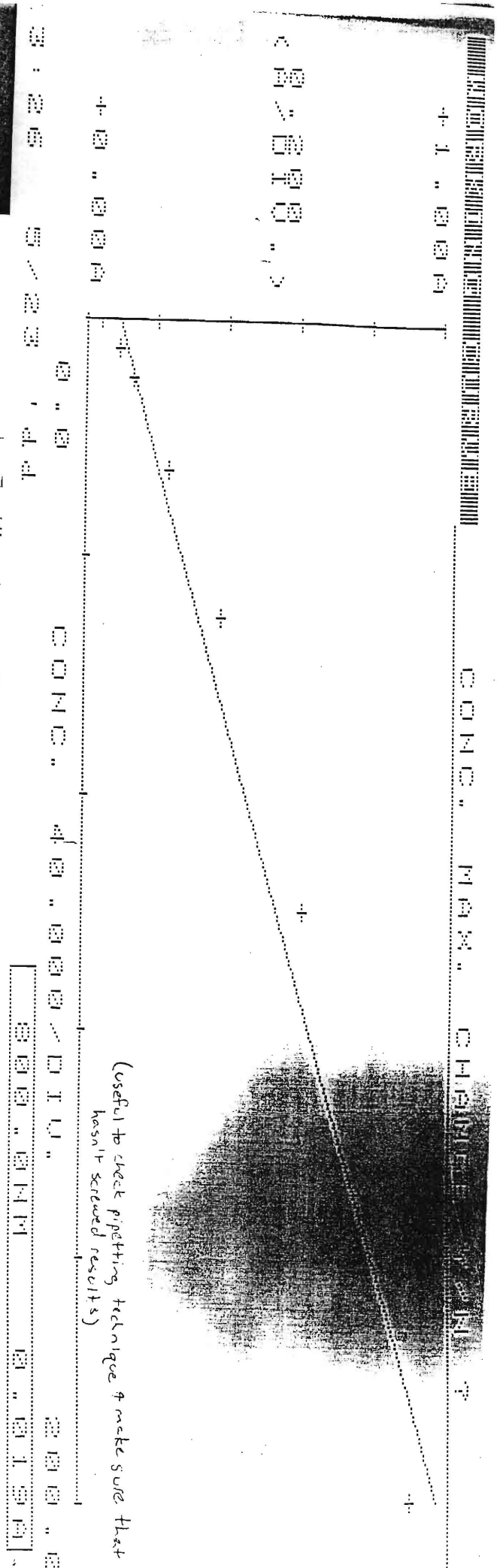
- want 30 µg mass

So divide mass by concentration (m

[redacted]

** QUANTITATIVE ANALYSIS **

No.	ABS.	CONC.	
1	0.147	12.032	24.93 µL
✓ 2	0.118	5.5768	53.80 µL
✓ 3	0.125	7.2975	46.109 µL
✓ 4	0.104	2.3703	127.75
✓ 5	0.110	3.7470	80.06
✓ 6	0.122	6.4788	46.261
7	0.270	38.923	7.706


CONC. MAX. 0.1000
0.0000 / DILU.
0.0000 / 0.0100
0.0000 / 0.0100
0.0000 / 0.0100

5/23/01

(16) 2, 3, 5, 46 will have to re-concentrate to get small enough amt for gel.

Re-concentration

Use microconcentrators (have filters)

rub frozen vials around fingers to thaw

Added all (90 µL) of each to microconcentrators

Spin down

Label

(4)

(9)

(10)

(11)

(12)

Su

Re

ce

Lo

Sc

Vo

Ti

(13)

(14)

(15)

Labeled vial 4

05/24/01 Nhts Protein extraction (again) (like protein isolation pg. 11)

(4) H34EP - protein extracted again

(9) H20S

(10) H24S

(11) H24NO

(12) H24EP

Supernatant Extracted.

Resuspended in .5 mL PBS

centrifuged

1mL of CER I made.

Supernatant discarded

Vortex (15s)

Incubate in ice (10 min)

5/24/01

(13) H10S

(14) H14NO

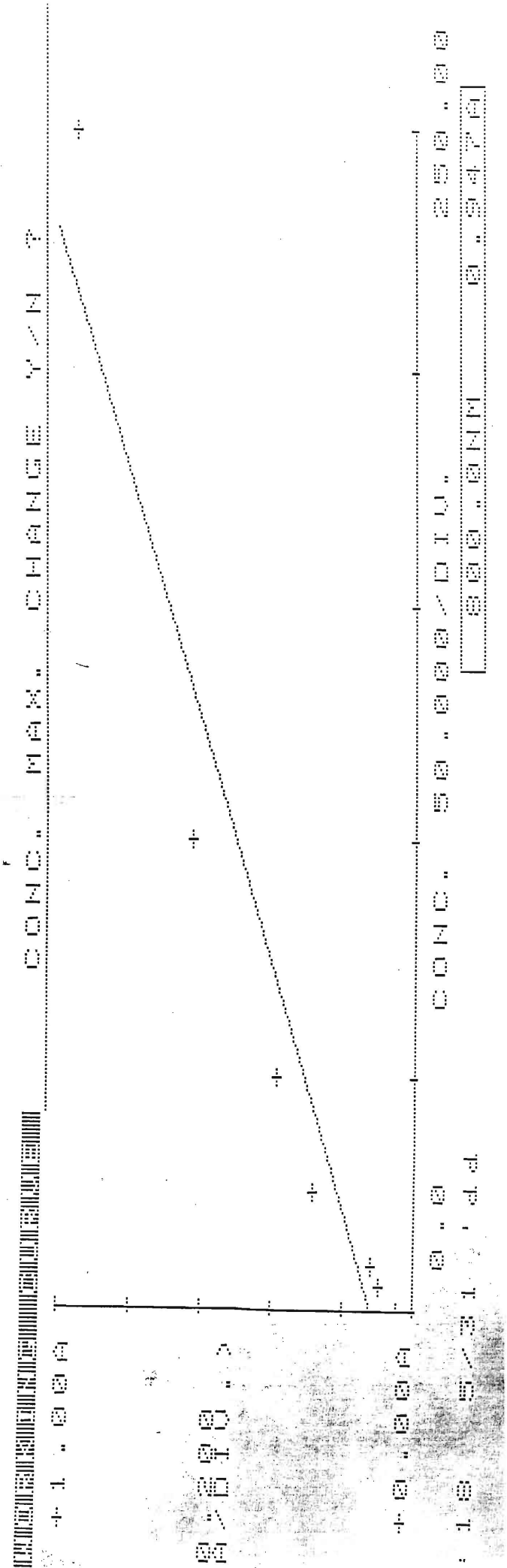
(15) H14EP

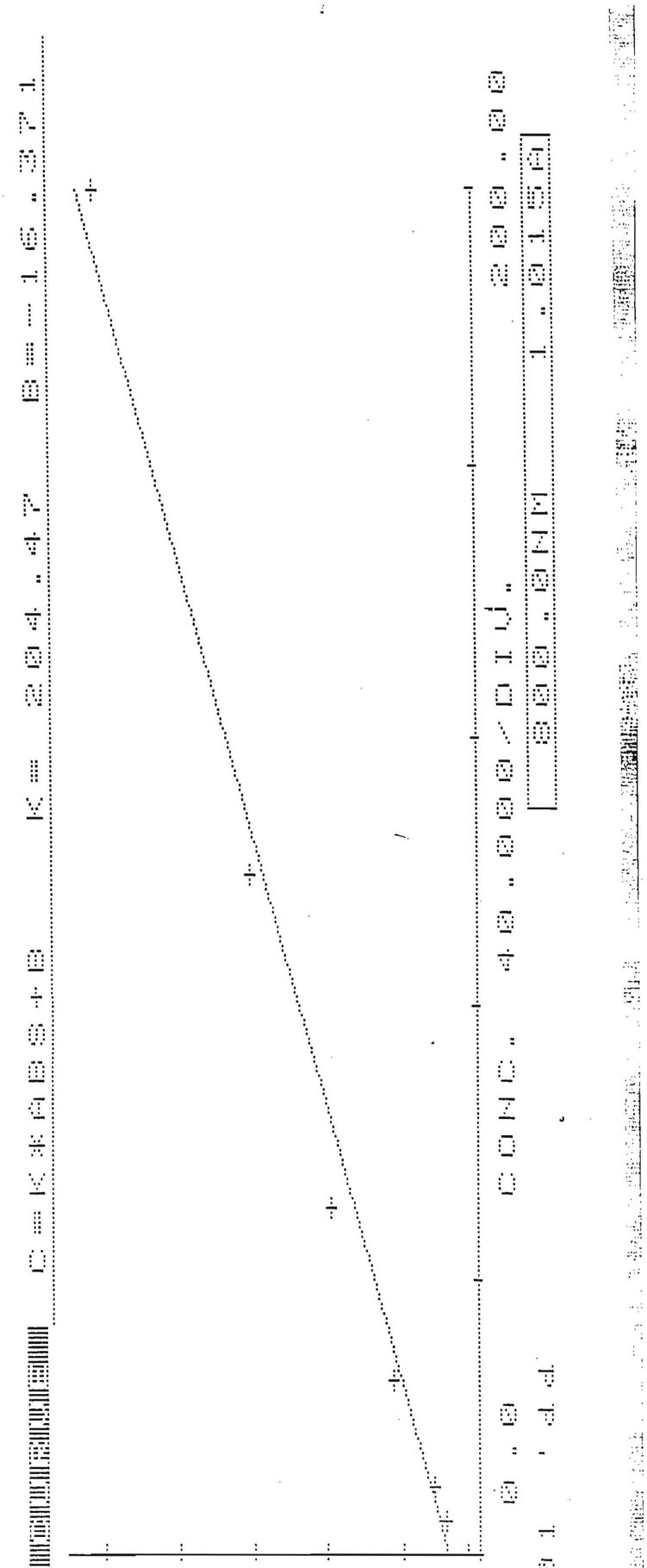
5/31/01 Protein Assay

QUANTITATIVE ANALYSIS

No.	ABS.	CONC.
1	0.101	-4.7948
2	0.095	-6.3286
3	0.117	-0.5768
4	0.239	31.585
5	0.069	-13.103
6	0.109	-2.6219
7	0.196	20.257
8	0.151	8.4982
9	0.107	-3.1331
10	0.149	7.8592
11	0.090	-7.4789
12	0.167	12.716
13	0.104	-4.8279
14	0.133	3.7698
15	0.143	6.3253

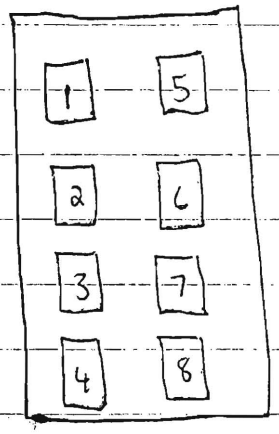
Protein Assay (5/31/01)


CONC. MAX. CHANGE Y/N ?
0.000
0.000
0.000


0.000
0.010
0.020
0.030
0.040
0.050
0.060
0.070
0.080
0.090
0.100
0.110
0.120
0.130
0.140
0.150
0.160
0.170
0.180
0.190
0.200
0.210
0.220
0.230
0.240
0.250
0.260
0.270
0.280
0.290
0.300
0.310
0.320
0.330
0.340
0.350
0.360
0.370
0.380
0.390
0.400

Immunohistochemistry

06-05-01



Protocol

(1-4 wells)	(5-8 wells)
wash PBS (15 min)	① Wash PBS 5 min
	② add protease antigen retrieval 10 min
	③ add PBS wash 5 min.
④ add protein block (10 min)	④ add protein block (10 min)
⑤ add 1° HSP Ab 1hr	⑤ add 1° HSP Ab for 1hr
add 1:90 to well 1	1:90 to well 5
1:180 to well 2	1:180 to well 6
1:360 to well 3	1:360 to well 7
(-) control well 4	(-) control well 8
⑥ wash PBS 5 min	⑥ wash PBS 5 min
⑦ Anti mouse 2° Ab (Link) 30 min.	⑦ Anti mouse 2° Ab Link 30 min.

06-12-01 Aniket

PRODUCT SPECIFICATION SHEET

HUMAN FIBRONECTIN

Fibronectin is a broad range natural cell adhesion factor. It is a 440-500 kDa dimeric glycoprotein consisting of two similar 220-250 kDa subunits linked by two disulfide bonds. It is found as a dimer in plasma and in multimeric form in the extracellular matrix and on cell surfaces. Its primary function is related to cell adhesion to the extracellular matrix which occurs via the Arg-Gly-Asp-Ser (RGDS) sequence of fibronectin with the appropriate transmembrane integrin receptor of the cells.[1] Other domains of fibronectin are also involved with this adhesion process and may involve interaction with collagen, heparin and other cell surface glycosaminoglycans.[1] The conformation and orientation of adsorbed fibronectin is also important and has an effect on cell spreading and strength of adhesion of endothelial cells.[2] Fibronectin addition to serum free medium promotes cell adhesion.[3] More significant effects are observed with BHK, CHO and other cell lines by coating of cultureware with fibronectin at 1-5 ug/cm^2. Details of fibronectin structure, properties, distribution, cellular expression, interaction with other proteins, matrix properties, cell interactions and adhesion and effects on differentiation can be found in two excellent books by Hynes or Mosher.[4,5]

PRODUCT:	FIBRONECTIN, Human	
CATALOG NUMBER:	354008	LOT NUMBER: 001760
SOURCE:	Human plasma **NOTE: The source plasma was tested and found nonreactive for hepatitis B surface antigen (HB,Ag) and negative for antibody to human immunodeficiency virus (HIV). Nevertheless, this product should be handled using the same safety precautions used when handling potentially infectious material.**	
QUANTITY & PHYSICAL FORM:	1 milligram per vial, lyophilized.	
FORMULATION:	100 mM CAPS, 0.15M NaCl, 1 mM calcium chloride, pH 11.0.	
RECONSTITUTION & USE:	Equilibrate vial to room temperature. Resuspend in one milliliter sterile distilled water. Allow 30 minutes for material to go into solution. **DO NOT AGITATE OR SWIRL.** If entire amount of material is not to be used immediately, transfer into appropriate aliquots and store at -20°C. It is recommended that solubilized product is used within two weeks. **DO NOT STORE IN FROST-FREE FREEZER. AVOID MULTIPLE FREEZE THAWS.** Human Fibronectin is generally used in the concentration range of 1-5 micrograms per cm^2 of growth surface for attachment or at 5 micrograms per ml as a media additive. Please see reverse for coating directions.	
MOLECULAR WEIGHT:	440,000 in non-reduced form.	

BECTON DICKINSON

Becton Dickinson Labware
Two Oak Park
Bedford, MA 01730
(781) 275-0004 Fax: (781) 275-0043
(800) 343-2035

27

(cont.) 06-12-01

QUALITY CONTROL: $\geq$ 99% by 4-12% SDS-PAGE under reducing conditions.

Human Fibronectin has been tested for its ability to promote attachment and spreading using BHK-21 cells.

Fibronectin has been tested and found negative for the presence of bacteria, fungi and mycoplasma.

STABILITY: Stable for a minimum of 3 months from day of shipment when stored at 2-8°C.

REFERENCES:

1. Aota, S., et.al., J. Biol. Chem., **266**:15938 (1991).
2. ~~Iuliano, D.J., et.al., J. Biomed. Mater. Res., 27:1103 (1993).~~
3. Barnes, D., and Sato, G., Cell, **22**:649 (1980).
4. Hynes, R.O., Fibronectins, Springer-Verlag, New York (1990).
5. Mosher, D.F. (ed), Fibronectin, Academic Press, New York (1989).

Coating Procedure

Use these recommendations as guidelines to determine the optimal coating conditions for your culture system.

1) Dilute fibronectin to desired concentration using serum-free culture Ca^{++}, Mg^{++} free medium or buffer at pH 7-9. The final solution should be sufficiently dilute so that the volume added will cover the surface evenly.

   Example: If the final coating concentration will be 5 ug/cm^2, dilute the material to 50 ug/ml and add 1 ml/35 mm dish, 3 ml/60 mm dish, etc.

   NOTE: Because of the CAPS component in the HFN preparation, buffers of media containing Ca^{++} and/or Mg^{++} added to the HFN may result in the formation of insoluble metal hydroxides. This will not occur if the buffering capacity of the diluent brings the pH to 8.0 or lower.

2) Add appropriate amount of diluted fibronectin to culture surface.

3) Incubate at room temperature for 1 hour.

4) Aspirate remaining material.

5) Rinse plates carefully with dH_2O - avoid scraping bottom surface.

6) Plates are ready for use. They may also be stored at 2-8°C damp or air dried if sterility is maintained.

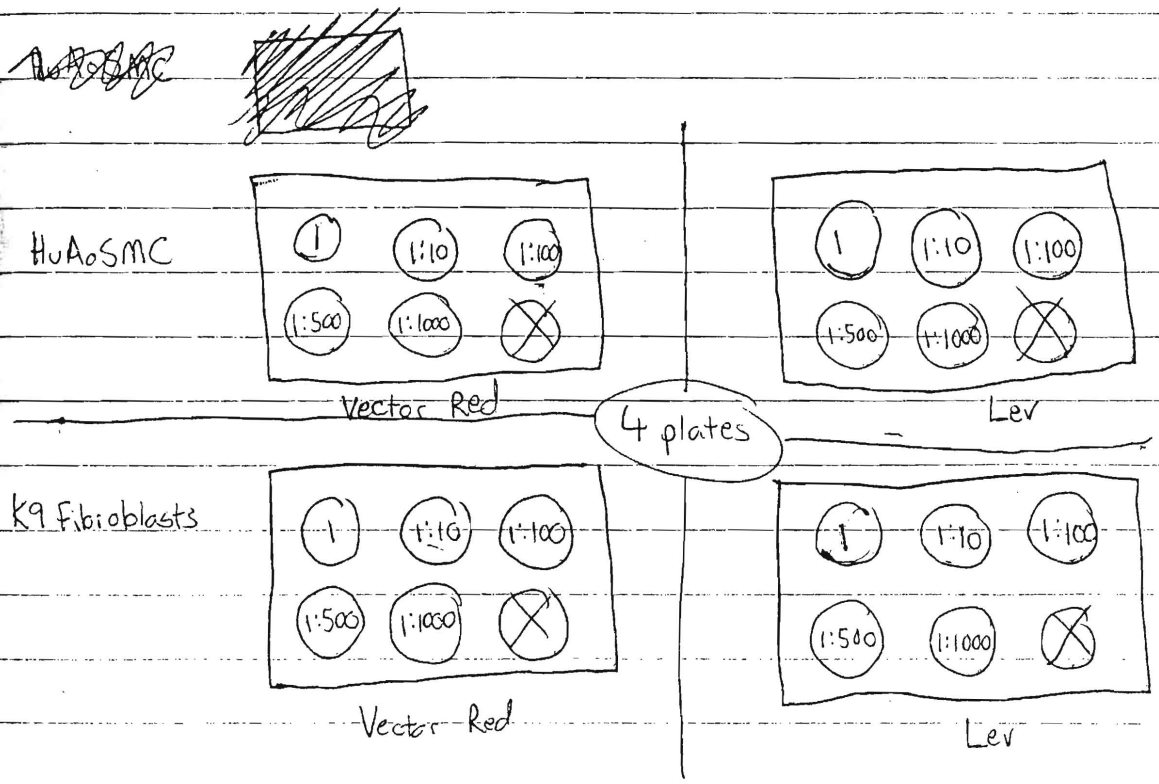
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Notes on Sorting Aortic Muscle Cell Experiment 06-13-01

Growing (1) K9 Fibroblasts

(2) Human Aortic Smooth Muscle Cells (HuAoSMC)

- pulled out from freezer, began growing



V.R. - makes 3 mLs

- figure out optimal ratios

Levamisole

29

H&E staining of tissue slides 06-15-01

after cut rabbit kidney (paraffin embedded)

SOP Number VRL-SOP.307	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 1 of 3

Title: H&E staining of paraffin embedded tissue slides

I. Scope:
This SOP will be followed by personnel engaged in research and clinical studies in the Vascular Research Laboratory of the Department of Surgery.

II. Purpose:
To describe the procedures for regressive hematoxylin and eosin histochemical staining of paraffin embedded tissue slides.

III. Procedures:

1. **Reagents Required**
   a. 1% acid alcohol
   b. Ammonia water
   c. Working eosin solution
   d. Hematoxylin stain
   e. 100% ethanol
   f. 95% ethanol
   g. Xylene (or equivalent)

2. **Equipment required**
   a. Solution containers (staining boats).
   b. Multi-well slide staining station.
   c. Exhaust fume hood.

3. **Protocol Guidelines**
   a. Deparaffinize and hydrate tissue to deionized H_2O (VRL.SOP.)
   b. Stain in Richard-Allen II hematoxylin for 1 minute at room temperature (RT). (Harris hematoxylin substituted for 12 minutes at RT is an acceptable substitution)
   c. Rinse in tap water until glass is clear.
   d. Differentiate by dipping 5-10 times in 1% acid alcohol.
   e. Place in ammonia water to blue. (<10 sec)
   f. Rinse in warm running water for 30 sec.
   g. Place in 80 % Alcohol for 1 min.
   h. Place in 1 % working eosin for 30 sec.
   i. Serially place in 3 changes of 95 % Alcohol for 30 sec each change.
   j. Place in absolute alcohol for 1 min.

under Stacy's supervision on rabbit kidney

(cont) 06-15-01

SOP Number VRL-SOP.307	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 2 of 3

k. Clear in 3 changes of Xylene for 2 min each change.
l. Coverslip with Permount and coverslip in fume hood.

4. **Expected results**
   a. Nuclei stain blue
   b. Erythrocytes stain reddish pink
   c. Cytoplasm stains pink

5. **Calibration Instructions**
   Study pathologist will approve acceptability of staining quality

6. **Routine Reagents needed for working reagents solutions**
   a. Deionized H2O
   b. 100 % ethanol
   c. Ammonium hydroxide (VRL.SOP.20)
   d. Stock eosin solution (VRL.SOP.20)

7. **Routine working solution maintenance**
   a. Alcohol station solutions should be changed every twenty-one days
   b. Ammonia water station solutions should be changed after 5 uses.
   c. Hematoxylin solution should be filtered every third day or if crystallization is observed.

8. **Trouble shooting**
   a. Hematoxylin solution contains crystals: filter hematoxylin using Fisher[R] filter paper.
   b. Nuclear staining is too intense: incrementally increase differentiating in acid alcohol solution.
   c. Nuclear staining is too pale: incrementally increase hematoxylin staining time.
   d. Cytoplasmic staining is too intense. Incrementally decrease staining time in eosin working solution.
   e. Substitution of this procedure by automated staining (subcontracted outside lab) is acceptable.

9. **Responsible person(s)**
   Laboratory director / laboratory supervisor

31

(cont) 06-15-01

SOP Number VRL-SOP.307	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 3 of 3

IV. References

Carson, FL. Histotechnology: a self instructional text. ASCP Press, Chicago, 1990

V. Attachments

NA.

VRL.SOP.307 2000 / RLD

A

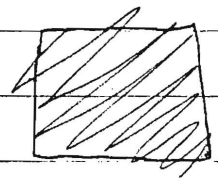
B

forget

Vector Red for sorting project 06-20-01

checking for toxicity & flow

Fibroblasts in Vector Red



A

Make 3 mLs of Vector Red

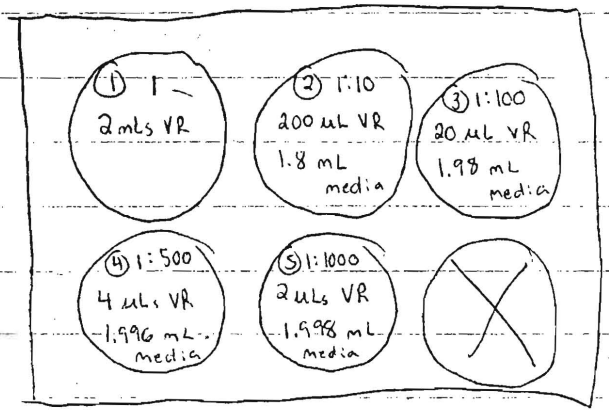
Well (1) → ~~2~~ 1.5 mLs Vector Red

1:10 (2) → 200 µLs VR + 1.8 mL media

1:100 (3) → 20 µLs VR + 1.98 mL media

1:500 (4) → 4 µLs VR + 1.996 mL media

1:1000 (5) → 2 µLs VR + 1.998 mL media



B

How many ULs is one drop?
38 µLs

Levamisol 1 drop = 1 mL

forget old ratios for Lev

1:1 (1) → 2 drops Lev + 2 mLs media

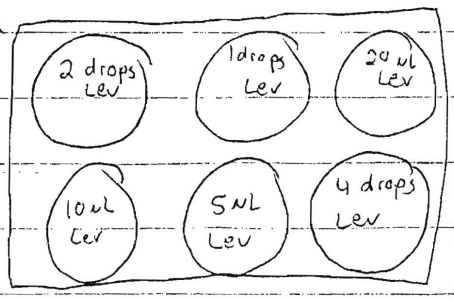
~~1:10~~ (2) → 1 drop Lev + 2 mLs

(3) → 20 µLs Lev + 2 mLs

(4) → 10 µLs Lev + 2 mLs

(5) → 5 µLs Lev + 2 mLs

(6) → 4 drops Lev + 2 mLs



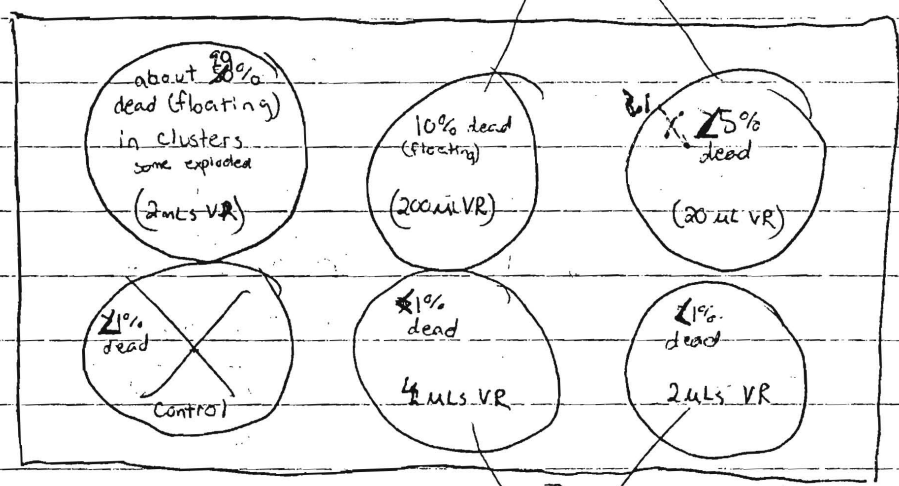
Jun 21, 01 Vector Red + Levamisol on fibroblasts

After 16 hrs. (8:30 a.m. Jun 21)

under microscope

(A) Vector Red (fibro)

not confluent although areas are

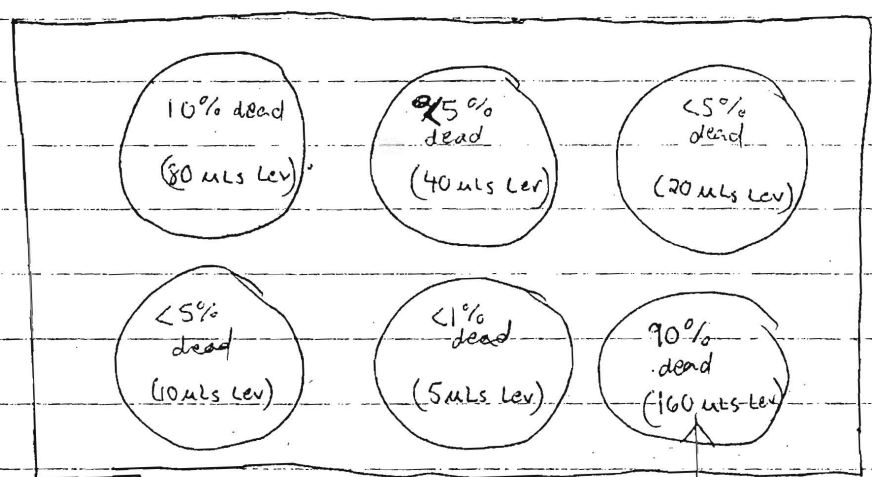


seed to flow →

+ B #5 (w/ least amt Lev b/c control had so few cells to begin with)

dead probably more b/c wells are confluent

(B) Levamisol



had most Lev in it. (about 160 mls)

June 21, 01

separately 4 at a time Cell Counting W/ Hemocytometer

trypsinize each well (1mL trypsin)

spin-down

re-suspend in 1mL of media

SOP Number VRL-SOP-607	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 1 of 2

Title: Cell Counting with a Hemocytometer

I. Scope:
This SOP will be followed by personnel engaged in research and clinical studies in the Vascular Research Laboratory of the Department of Surgery.

II. Purpose:
To describe the procedures for counting cells with a hemocytometer.

III. Procedures:

1. **Reagents Required**
   a. All reagents used to split cells (refer to VRL.SOP.601)
   b. Trypan Blue

2. **Equipment required**
   a. All equipment required to split cells (refer to VRL.SOP.601)
   b. Hemocytometer, cover slips

3. **Operation Guidelines**
   a. Follow steps 3a-3l from cell splitting procedure (refer to VRL.SOP.601)
   b. Resuspend cells in one ml of media.
   c. In a 1.5ml tube, add 500µl Trypan Blue, 475µl of CMF and 25µl of the media.
   d. Dry hemacytometer and place coverslip on top.
   e. Add approximately 20 µl of suspension into both sides of hemacytometer.
   f. Calculate the cell count using the equation: cells/ml = (n) x 10^4,
      where n = the average cell count per square of the four corner squares counted. For example: cells/ml = (n) x 10^4 **or** cells/ml = 30 x 10,000 = 300,000 cells/ml.
   g. Cells that land on the outside the lines, do not count. All cells on the inside of lines count.
   h. Cells that take up the blue color are dead and they do not count.

4. **Trouble shooting**
   Same as VRL.SOP.601

June 21, 01

(cont) Cell Counting w/ Hemocytometer

SOP Number VRL-SOP-607	University of Tennessee Medical Center Vascular Research Lab Standard Operating Procedures	Page 2 of 2

5. **Calibration Instructions**
   NA

6. **Routine Reagent**
   NA

7. **Responsible person(s)**
   Laboratory director / laboratory supervisor

IV. **References**
VRL.SOP.601

V. **Attachments**
NA

10/50

9308

Sandra
Branton

VR – Fluorescent – VR treated
SS
FS

VR control
#5 levamisole

} FACS

Making Vector Red 7-22-01 need 285 mLs

100 mM Tris - HCL

for every 2.5 mL add 1 drop A, B, & C solutions

10 mL 1M + 90 PBS

syringe filter sterilized (using .02 micron filter) acrodisc

Making Levamisol

2 drops of Levamisol per 2.5 mLs of 100 mM Tris - HCL

80 mLs

7-23-01

Smooth Muscle Cells

16 hrs.

Tagging Markers

7/1 VR Vector Red 8/2 VR 9/3 VR

do first, incubate immediately
do in dark, b/c VR reacts
w/ light

200 uL VR 1.8 mL SMGM2	50 uL VR 1.95 mL media	10 uL 1.990 mL media
5 uL 1.995 mL SMGM2	2 uL 1.998 mL SMGM2	Control 2 mL media

FACS

Trypan blue

10/4 VR 11/5 VR 12/6 VR

1/1L 2/2L 3/3L

Levamisol

50 uL Lev 1.95 mL media	10 uL Lev 1.990 mL SMGM2	5 uL Lev 1.995 mL SMGM2
2 uL Lev 1.998 SMGM2	10 uL VR 10 uL Lev 1.78 mL SMGM2	Control 2 mL media

4/4L

5/5L

6/6L

PI

FACS

Trypan Blue

mixed accidentally

Cell Cytometer

Trypan Blue & Flow 7-24-01

~~Trypsinized~~
~~Re-suspend in media~~
~~aspirate~~
~~re-suspend in paraformaldehyde~~

Trypsinize
Dilute in media → SMGM2
Take sample for trypan blue - 25 µL sample
spin, fix
1500 rpm
paraformaldehyde

Aspirate media, wash w/ CMF, aspirate, strip w/ 1mL trypsin, dilute in SMGM2
- take sample for trypan blue 25 µL → Results
- Spin remaining in centrifuge 1500 rpm for 5 min.
- Aspirate
- Bring up pellet in 1 mL paraformaldehyde
- Transfer to flow tubes
- Take to flow

FACS

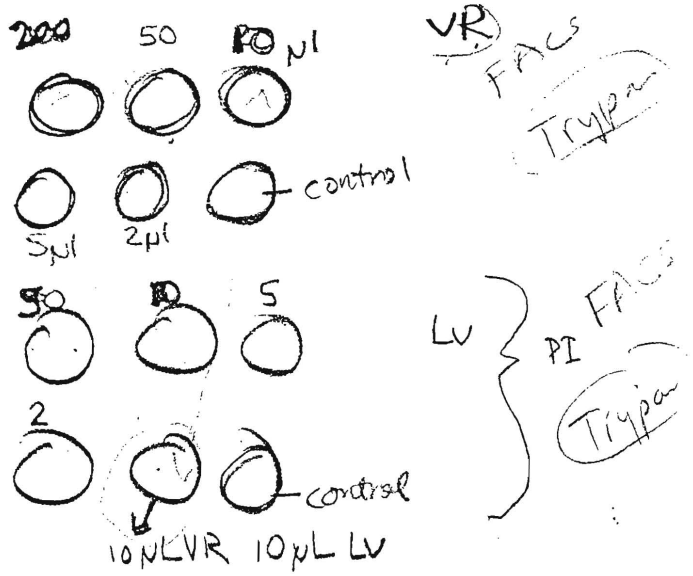
Fluorescent Activated Cell Sorter/Scanner

7-24-01

Cell Cytometer Results (Trypan Blue)

	Cell #	Concentration	Viable	Non-viable
VR	1	200 ul VR	0	3
	2	50	2	0
	3	10	1	0
	4	5	1	3
	5	2	3	1
	6	control	2	2
Lev	1	50 ul Lev	1	1
	2	10	2	3
	3	5	1	0
	4	2	1	2
	5	10uL VR, 10 uL Lev	0	3
	6	control	1	0

X 10,000



VECTOR

Vector® Red Alkaline Phosphatase Substrate Kit I

Cat. No. SK-5100

The Vector® Red substrate kit contains all of the reagents necessary (except buffer) to prepare a working solution for either immunocytochemical or membrane-based applications. Vector® Red produces a red reaction product that can be seen using either brightfield or fluorescent microscopy. Vector® Red can be permanently mounted in non-aqueous media or mounted aqueously in VECTASHIELD® Mounting Medium.

DISPENSING REAGENTS:

For convenience, the reagents are supplied in dropper bottles. When dispensing drops, hold the bottle in an inverted vertical position and squeeze gently. To prevent evaporation, secure the opaque caps on the bottles when they are not in use. DO NOT PIPET REAGENTS DIRECTLY FROM BOTTLES. Drop volumes of each component may be different due to solvent characteristics. Proper concentrations of substrate components in the working solution are assured only by using the drop dispensers.

INSTRUCTIONS FOR USE:

Prepare the Vector® Red substrate working solution ***immediately*** before use.

1. To 5 ml of 100 mM Tris-HCl, pH 8.2 - 8.5 buffer*, add 2 drops of Reagent 1. Mix well.
2. Add 2 drops of Reagent 2 and mix well.
3. Add 2 drops of Reagent 3 and mix well.

* ***This kit contains a modified formulation to improve stability. It is important to make the working solution in 100 mM - 200 mM Tris-HCl buffer, pH 8.2 - pH 8.5***

Incubate tissue sections or membranes with substrate solution at room temperature until suitable staining develops. Development times should be determined by the investigator but generally 20-30 minutes provides good staining intensity. Improved staining may be obtained by developing the substrate in the dark.

Wash sections in assay buffer for 5 minutes. Rinse in water.

For permanent mounting: Rinse in tap water, counterstain, if desired (see chart on reverse), dehydrate, clear and mount in a permanent mounting medium, such as **VectaMount**™, Catalog No. H-5000.

For aqueous mounting in VECTASHIELD® Mounting Medium : Tap excess buffer from sections and mount. Before mounting, slides may be rinsed for 2-5 minutes in 100% ethanol to increase the intensity of Vector® Red fluorescence.

The Vector® Red reaction product is a highly fluorescent, non-fading, bright red precipitate when viewed with Texas Red® or rhodamine excitation and emission filter systems. Vector® Red fluorescence may also be visible with fluorescein or AMCA filter systems using broad band emission filters.

NOTES:

1. Use the working solution of Vector® Red within 15 minutes of preparation or decreased sensitivity may result. Increasing the incubation time in substrate solution beyond 45 minutes will not increase sensitivity, unless freshly made substrate solution is reapplied to sections.
2. Do not heat Vector® Red substrate kit components or working solution. Heating decreases staining sensitivity.
3. The reagents should be stored at 4 °C and protected from light whenever possible. Occasionally a precipitate may form in some reagents upon prolonged storage. This will have no effect on the quality or intensity of the staining. Do not filter the reagents or working solution.
4. Do not put sodium azide in the buffer used for the Vector® Red working solution; it will prevent staining.
5. For tissue, using 0.1% Tween® 20 in the Vector® Red working solution may increase the sensitivity and crispness of staining especially when non-enzymatic antigen retrieval methods are used in the immunostaining protocol. Tween® 20 should not be added to the Vector® Red substrate solution for membrane applications.
6. When using neural tissue, Vector® Red is not recommended for visualizing processes, fibers, or terminals (inadequate staining may occur).
7. Endogenous alkaline phosphatase activity (other than the intestinal isoenzyme) can be inhibited by the addition of levamisole (Cat. No. SP-5000) to the buffer prior to the preparation of the working solution. Intestinal alkaline phosphatase activity can be inhibited, before immunostaining, with several tissue pretreatments (Bulman AS and Heyderman E; J. Clin. Pathol. 34, 1349-1351,1981).

IMPORTANT: Little is known about the toxicity and carcinogenicity of the substrate components. Care should be taken in the handling and disposing of all the reagents.

Tween® 20 is a registered trademark of Atlas Chemical Industries
Texas Red® is a registered trademark of Molecular Probes Inc.

Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010 U.S.A., • (650) 697-3600 • Fax: (650) 697-0339

VECTOR
LABORATORIES

Levamisole Solution

Cat. No. SP-5000

Concentration 125 mM

Storage Refrigerate

REMARKS:

Levamisole will inhibit most forms of alkaline phosphatase other than the intestinal isoenzyme. Since the intestinal form of the enzyme is used as the marker enzyme in the VECTASTAIN® ABC-AP Kit, levamisole can be used to inhibit endogenous alkaline phosphatase in most cases. Because the inhibition produced by levamisole is reversible, the inhibitor must be added to the substrate solution.

This lot of levamisole was tested on two frozen tissues and was found to completely inhibit endogenous alkaline phosphatase activity.

INSTRUCTIONS FOR USE:

When used in conjunction with Vector Laboratories' Alkaline Phosphatase Substrate Kits, add one drop of Levamisole solution to 5 ml of alkaline phosphatase substrate buffer and mix well. Then add substrate reagents and continue as outlined in the instructions for the Alkaline Phosphatase Substrate Kit.

43

Vector Red Excitation & Emission Levels

Donnell, Robert L.

From: Vector Laboratories [vector@vectorlabs.com]
Sent: Thursday, July 26, 2001 3:20 PM
To: Donnell, Robert L.
Subject: Re: Vector Red Excitation and Emission Levels

488
ARG

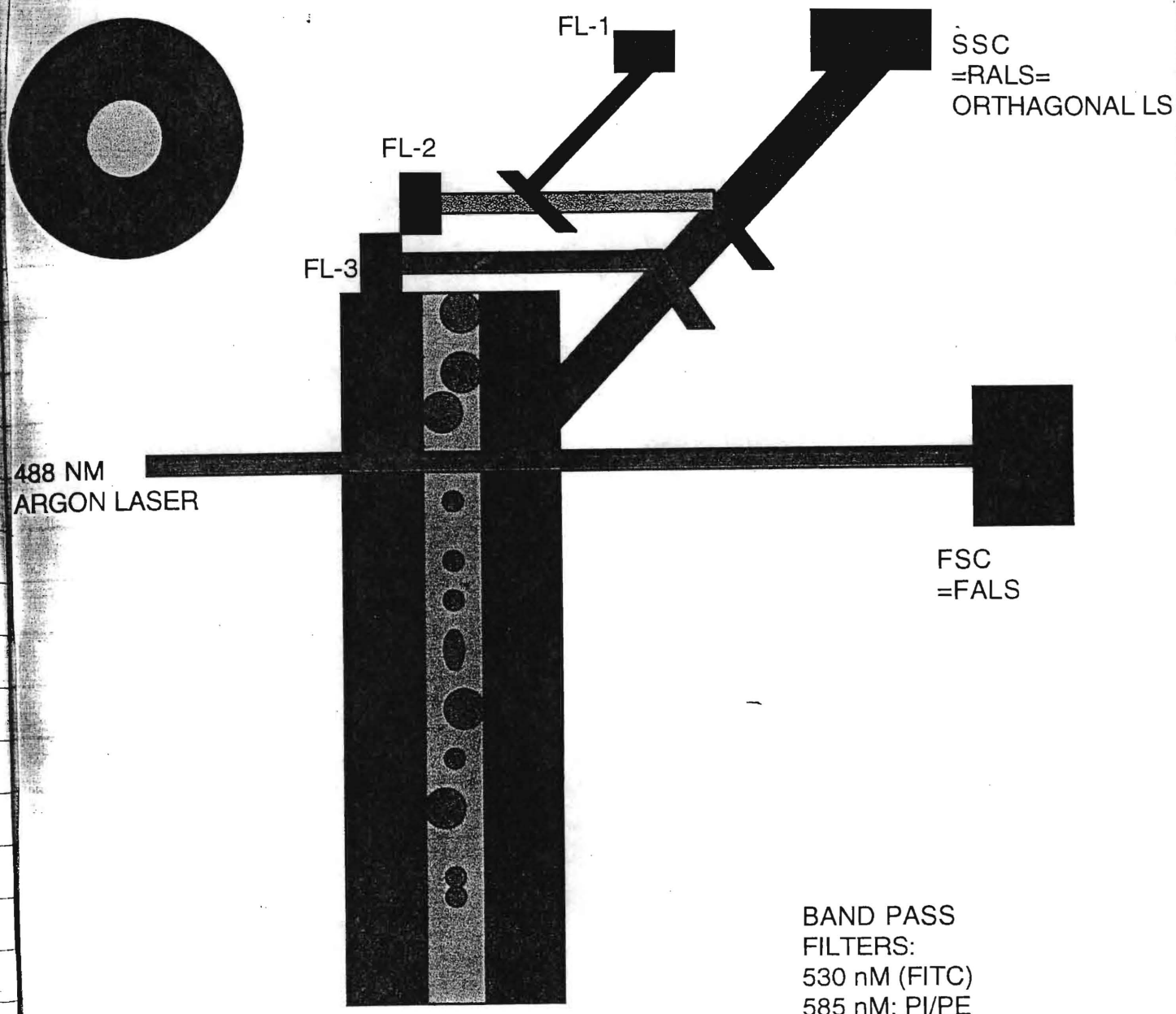
Thanks for your email and phone inquiry from Frances Kirkland. The exictation is broad and seems to be from 360nm through to 560nm. The emission spectrum seems to be above 560nm.

Sincerely,

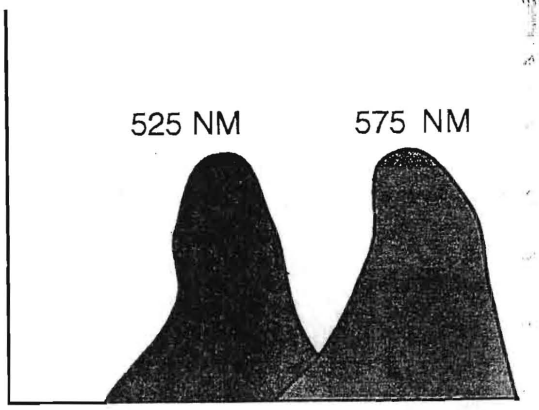
Technical Service
Vector Labs.
----- Original Message -----
From: "Donnell, Robert L." [redacted]
To: <vector@vectorlabs.com>
Sent: Thursday, July 26, 2001 11:35 AM
Subject: Vector Red Excitation and Emission Levels

> We currently use your Vector Red Alkaline Phosphatase Substrate Kit and
are
> wondering what the excitation and emission wavelengths on the Vector Red
> Stain are.
> Sincerely,
>
> Robert L. Donnell, DVM, PhD
> Director of Surgical Research
> Department of Surgery, UTMCK
> [redacted]
> [redacted]

3 Fluorescent wavelengths



BAND PASS
FILTERS:
530 nM (FITC)
585 nM: PI/PE
>650 nM: RED



HSP Project → Flow System 07/27/01

2000 mL Erlenmeyer flask

gas permeable tubing

2000 mL media

pump → pulsitile pump

pulse dampener

stopper cork

connectors

pressure transducer

computer program

cut-off 25 mL pipets used to keep vessel stable

→ no curvature desired

ti-cron → to tie vessels to connectors

must incubate flask in water to keep temp. of system @ 37°

07/27/01

Want from ATLS K9

SMC
fibroblasts
> from: Aorta, Carotid, Iliac } endothelial

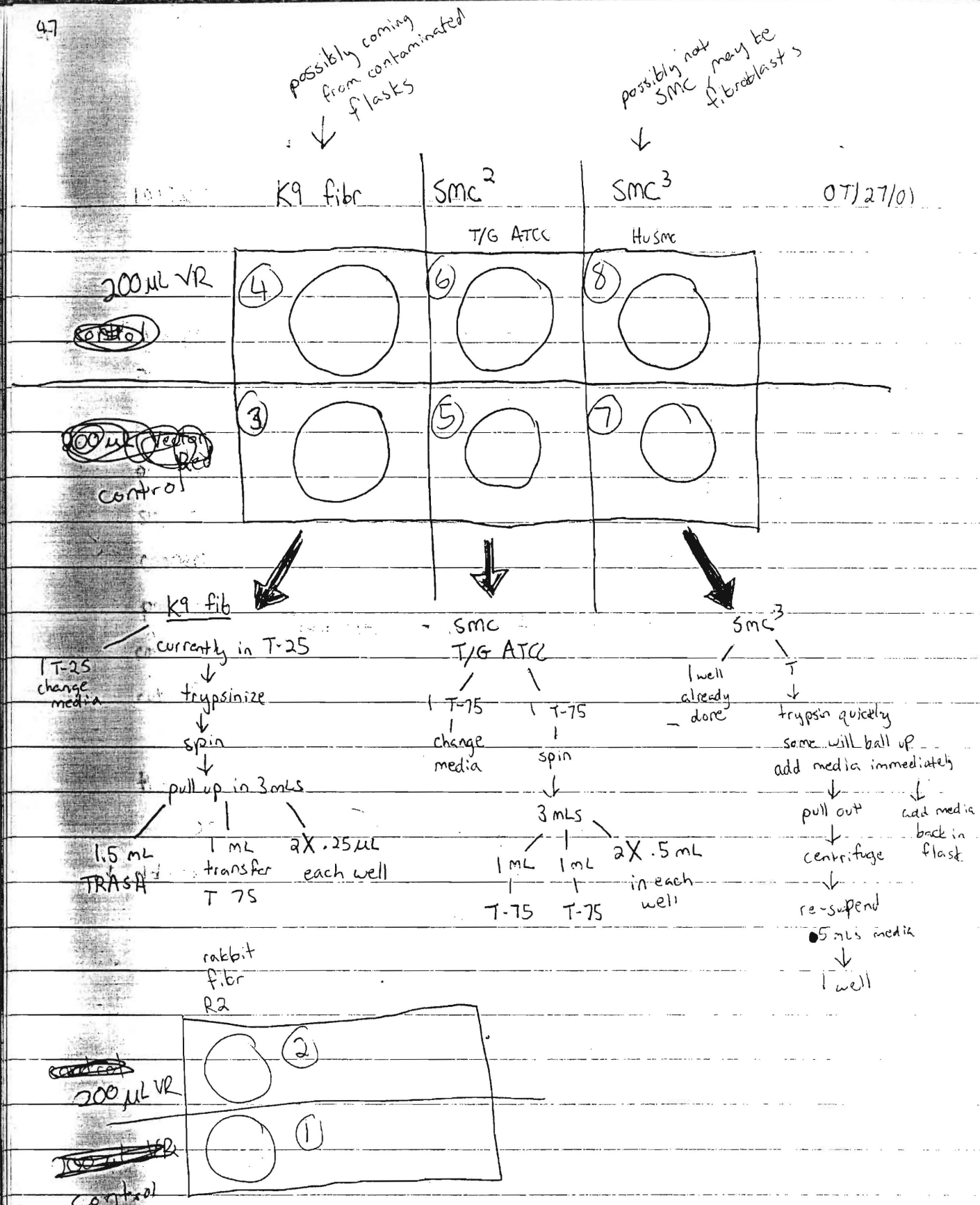
↓

may be mixed or sorted.

↓

Try FACS/Flow test using Vector Red

- Want to eventually try to sort fibro & SMC on arteriolers b/c so thin nearly impossible to separate at this time


possibly coming from contaminated flasks
possibly not SMC may be fibroblasts
K9 fibr
SMC2
SMC3
07/27/01
T/G ATCC
HuSMC
200 µL VR
control
4
6
8
3
5
7
K9 fib
currently in T-25
1 T-25 change media
trypsinize
spin
pull up in 3 mLs
1.5 mL TRASH
1 mL transfer T 75
2X .25 µL each well
SMC T/G ATCC
1 T-75 change media
1 T-75 spin
3 mLs
1 mL T-75
1 mL T-75
2X .5 mL in each well
SMC3
1 well already done
T
trypsin quickly
some will ball up
add media immediately
pull out
centrifuge
re-suspend .5 mLs media
1 well
add media back in flask
rabbit fibr R2
200 µL VR
control
2
1

07/27/01

	concentration	cells		media
1	control	rabbit fibro		DMEM
2	200uL VR	rabbit fibro		DMEM
3	control	K9 fibro	did not grow well ↓ no FBS in media	DMEM
4	200 uL VR	K9 fibro	did not grow well ↓ no FBS in media	DMEM
5	control	smc^2 T/G ATCC		SMGM2
6	200uL VR	smc^2 T/G ATCC		SMGM2
7	control	smc^3 HuSMC	possibly not SMC, may be fibro	IMDM
8	200uL VR	smc^3 HuSMC	possibly not SMC, may be fibro	IMDM

media in flask

8-10-01

1 To plate cells from specimen/tissues:

2 Get about a 1 cm^2 block.

3 Mense it with a scalpel.

a - If wanting mostly smooth muscle cells from an artery, scrape the outer and inner layer

b - put specimen in sterile petri dish

c - put tools (scalpel, scissors, clamps, forceps) in other side of petri dish

d - use sterile gloves

4 Put pieces of mensed tissue in 6-well plates

5 Place 1 drop of FBS on each piece of tissue

6 Turn over (Invert) well-plate

7 Place in incubator upside-down for 1 hr.

8 Take out, re-invert

9 Place only enough media to barely cover bottom of well plate.

10 Put media in so that it does not touch the specimen but comes very close as it surrounds it

11 Let sit overnight in incubator

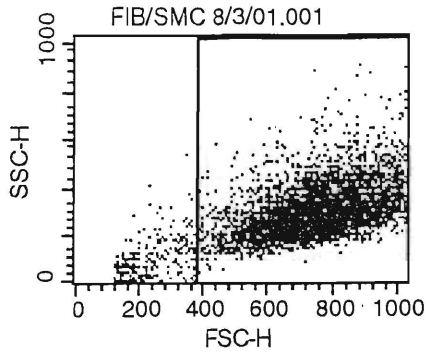
12 Add enough media to just cover specimen

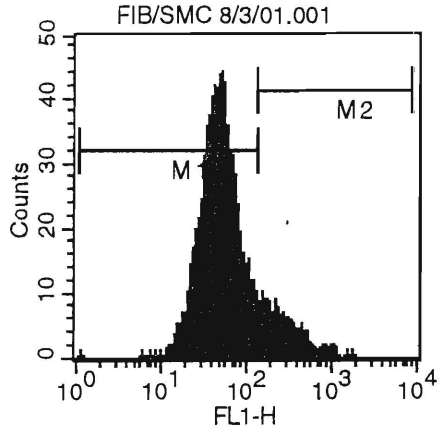
13 Let sit for 3 days or until specimen un-attaches from bottom of well

14 Watch under microscope for cells to grow

☆ Typically, use IMDM for media

rabbit fibroblasts

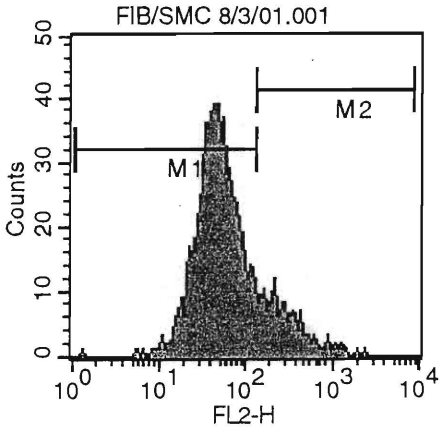




Histogram Statistics

File: FIB/SMC 8/3/01.001 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4730
Total Events: 5000

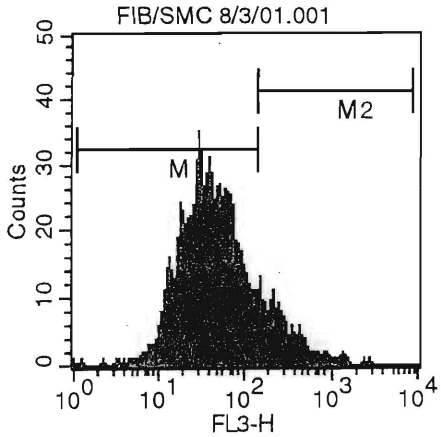
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4730	100.00	94.60	74.74	50
M1	1, 132	4221	89.24	84.42	49.88	50
M2	132, 8582	514	10.87	10.28	279.42	142



Histogram Statistics

File: FIB/SMC 8/3/01.001 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4730
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4730	100.00	94.60	84.97	42
M1	1, 132	4104	86.77	82.08	50.87	42
M2	132, 8582	634	13.40	12.68	306.27	198

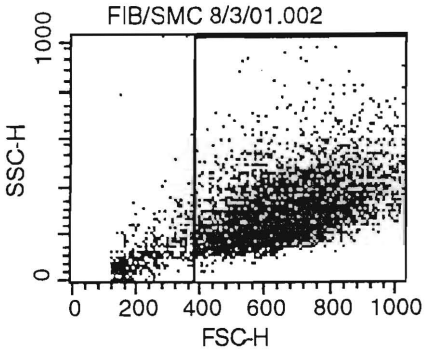


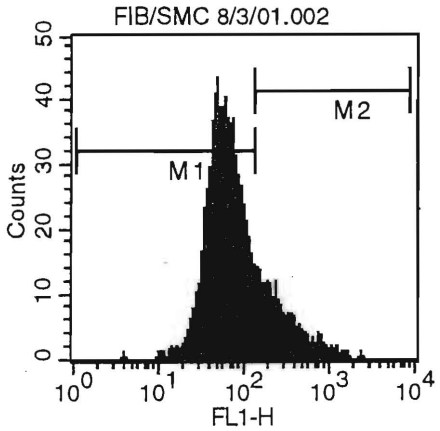
Histogram Statistics

File: FIB/SMC 8/3/01.001 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4730
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4730	100.00	94.60	73.68	27
M1	1, 132	4162	87.99	83.24	43.92	27
M2	132, 8582	573	12.11	11.46	290.61	139

[illegible] vector red
rabbit fibroblasts

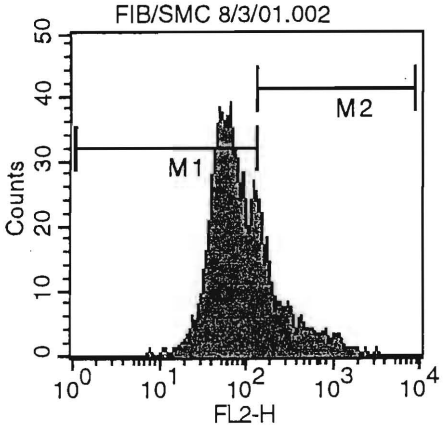




Histogram Statistics

File: FIB/SMC 8/3/01.002 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4548
Total Events: 5000

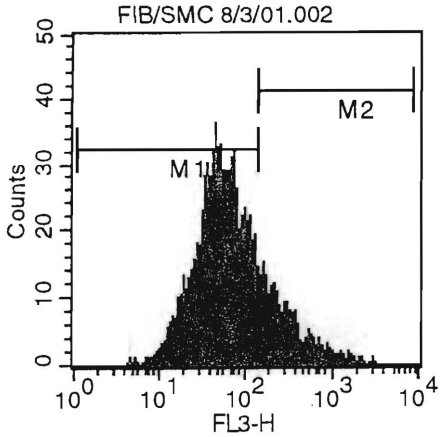
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4548	100.00	90.96	97.73	47
M1	1, 132	3782	83.16	75.64	61.34	47
M2	132, 8582	780	17.15	15.60	274.79	132



Histogram Statistics

File: FIB/SMC 8/3/01.002 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4548
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4548	100.00	90.96	116.37	61
M1	1, 132	3603	79.22	72.06	64.43	61
M2	132, 8582	955	21.00	19.10	312.50	137

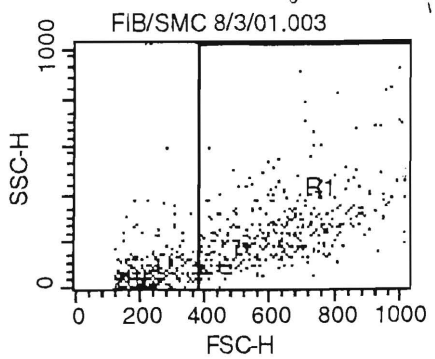


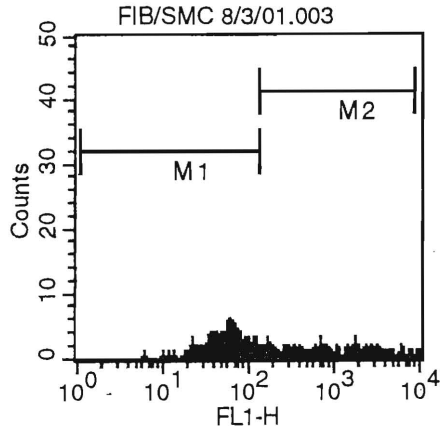
Histogram Statistics

File: FIB/SMC 8/3/01.002 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4548
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4548	100.00	90.96	98.14	40
M1	1, 132	3779	83.09	75.58	53.88	40
M2	132, 8582	777	17.08	15.54	313.71	146

control RT fibroblasts
(did not grow well - no FBS in media)



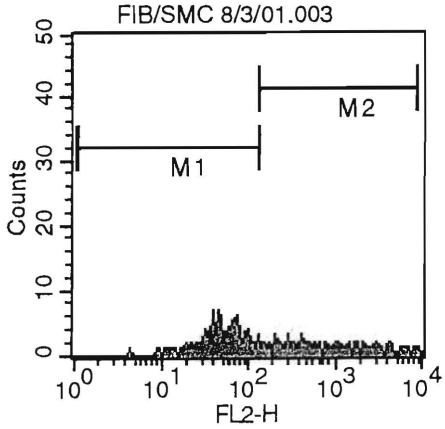


Histogram Statistics

File: FIB/SMC 8/3/01.003
Patient ID: TAG=TUBE
Gate: G1
Total Events: 750

Sample ID: FIBROBLAST VECTOR RED
Acquisition Date: 3-Aug-1
Gated Events: 462

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	462	100.00	61.60	473.44	58
M1	1, 132	313	67.75	41.73	57.05	58
M2	132, 8582	149	32.25	19.87	1348.13	150

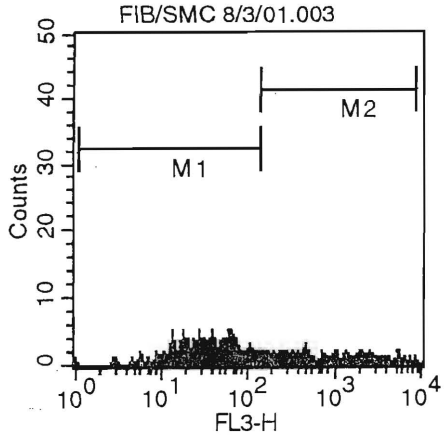


Histogram Statistics

File: FIB/SMC 8/3/01.003
Patient ID: TAG=TUBE
Gate: G1
Total Events: 750

Sample ID: FIBROBLAST VECTOR RED
Acquisition Date: 3-Aug-1
Gated Events: 462

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	462	100.00	61.60	476.76	37
M1	1, 132	310	67.10	41.33	55.94	37
M2	132, 8582	152	32.90	20.27	1335.03	195



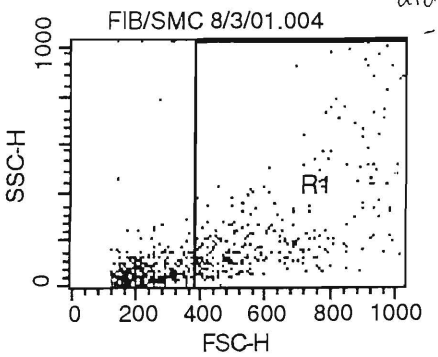
Histogram Statistics

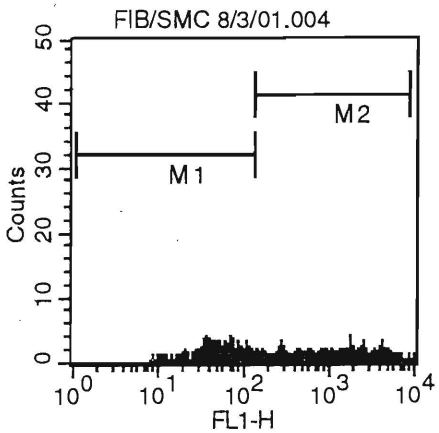
File: FIB/SMC 8/3/01.003
Patient ID: TAG=TUBE
Gate: G1
Total Events: 750

Sample ID: FIBROBLAST VECTOR RED
Acquisition Date: 3-Aug-1
Gated Events: 462

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	462	100.00	61.60	383.66	13
M1	1, 132	324	70.13	43.20	43.01	13
M2	132, 8582	137	29.65	18.27	1192.10	429

200 uL Vector Red
K9 fibroblasts did not grow well
- no FBS in media

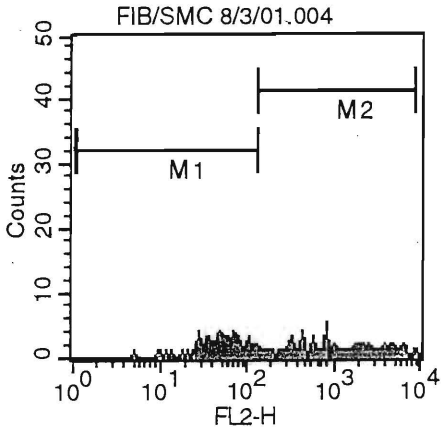




Histogram Statistics

File: FIB/SMC 8/3/01.004 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 360
Total Events: 720

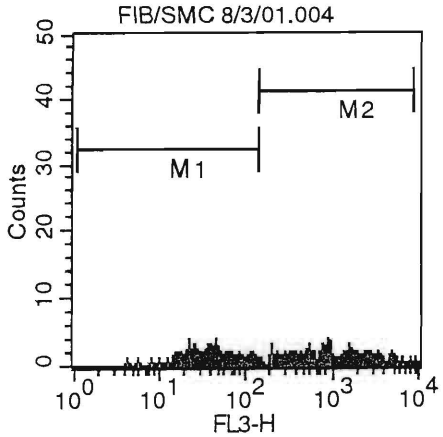
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	360	100.00	50.00	767.09	34
M1	1, 132	178	49.44	24.72	52.28	34
M2	132, 8582	181	50.28	25.14	1424.69	1611



Histogram Statistics

File: FIB/SMC 8/3/01.004 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 360
Total Events: 720

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	360	100.00	50.00	813.38	743
M1	1, 132	176	48.89	24.44	54.67	27
M2	132, 8582	184	51.11	25.56	1539.10	743

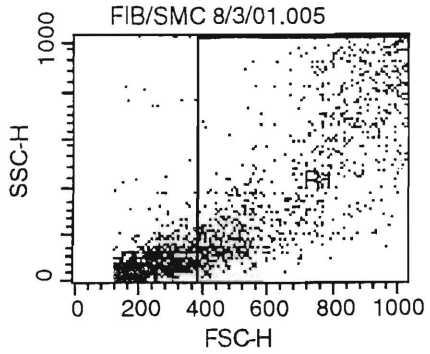


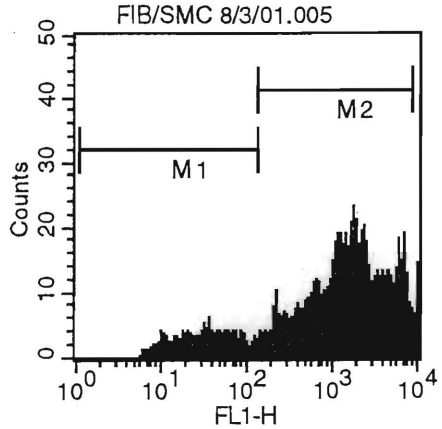
Histogram Statistics

File: FIB/SMC 8/3/01.004 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 360
Total Events: 720

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	360	100.00	50.00	690.35	21
M1	1, 132	187	51.94	25.97	43.71	21
M2	132, 8582	173	48.06	24.03	1389.31	813

SMC² T/G ATCC

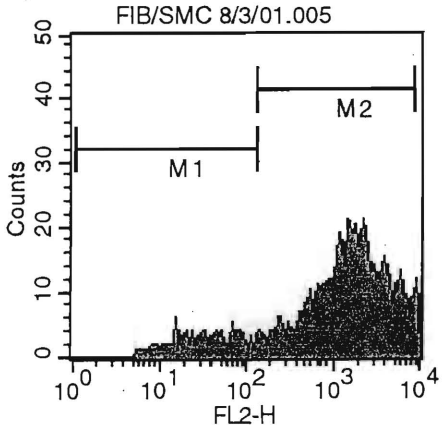




Histogram Statistics

File: FIB/SMC 8/3/01.005 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3740
Total Events: 5000

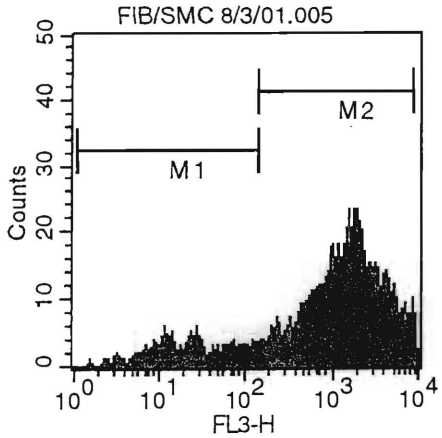
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3740	100.00	74.80	2073.54	1596
M1	1, 132	418	11.18	8.36	46.06	35
M2	132, 8582	3253	86.98	65.06	2175.98	1596



Histogram Statistics

File: FIB/SMC 8/3/01.005 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3740
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3740	100.00	74.80	2042.56	1298
M1	1, 132	416	11.12	8.32	44.93	15
M2	132, 8582	3326	88.93	66.52	2291.26	1298

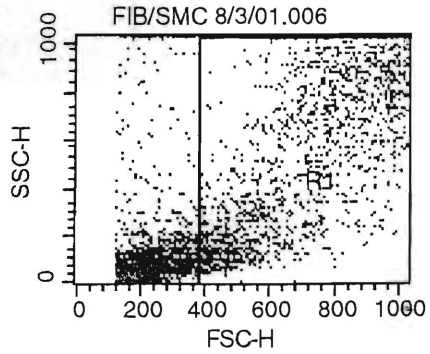


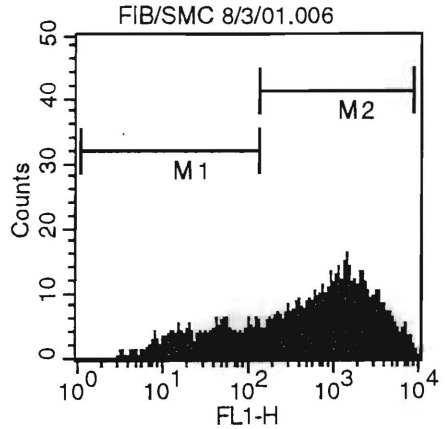
Histogram Statistics

File: FIB/SMC 8/3/01.005 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3740
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3740	100.00	74.80	1703.18	1370
M1	1, 132	469	12.54	9.38	37.92	11
M2	132, 8582	3273	87.51	65.46	1940.84	1370

300 µL vector Red
SMC² T/G ATCC

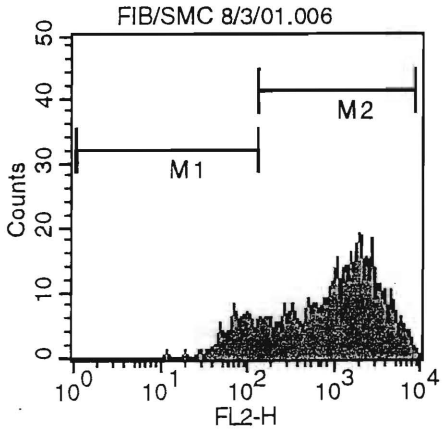




Histogram Statistics

File: FIB/SMC 8/3/01.006 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 2785
Total Events: 5000

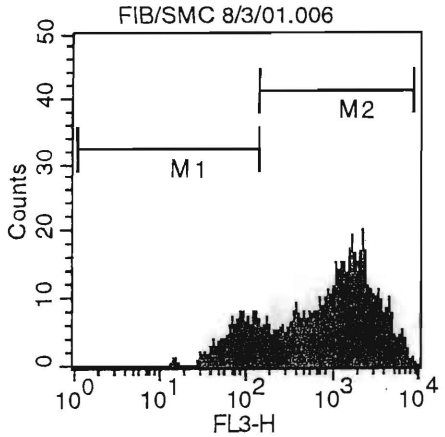
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2785	100.00	55.70	1129.67	1241
M1	1, 132	599	21.51	11.98	48.53	41
M2	132, 8582	2186	78.49	43.72	1425.91	1241



Histogram Statistics

File: FIB/SMC 8/3/01.006 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 2785
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2785	100.00	55.70	1463.40	1715
M1	1, 132	355	12.75	7.10	78.96	66
M2	132, 8582	2432	87.32	48.64	1664.39	1715



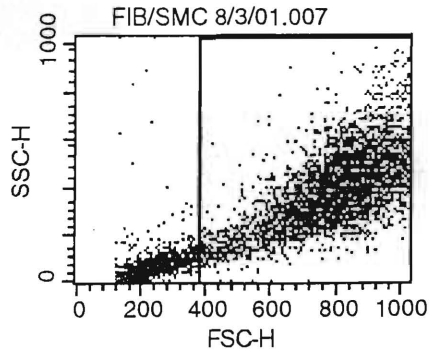
Histogram Statistics

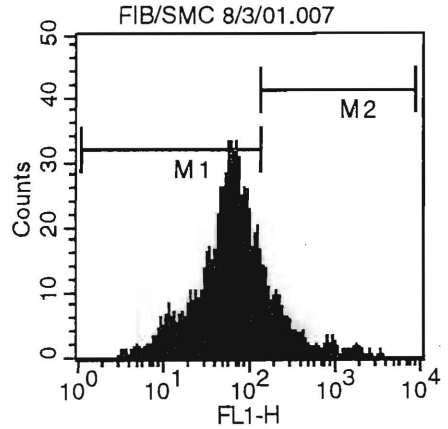
File: FIB/SMC 8/3/01.006 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 2785
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2785	100.00	55.70	1318.56	1963
M1	1, 132	407	14.61	8.14	77.28	81
M2	132, 8582	2384	85.60	47.68	1527.49	1963

Control

SMC³ HuSMC?

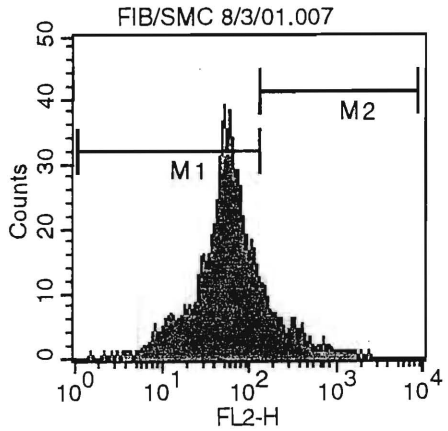




Histogram Statistics

File: FIB/SMC 8/3/01.007 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3766
Total Events: 5000

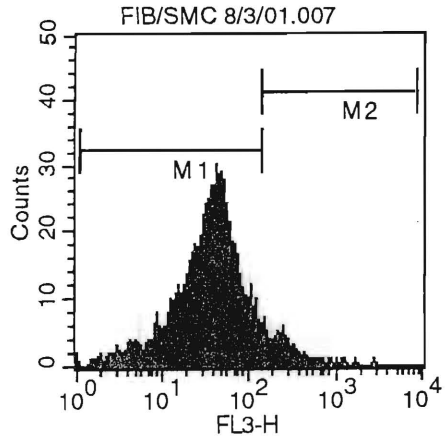
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3766	100.00	75.32	90.53	54
M1	1, 132	3218	85.45	64.36	57.86	54
M2	132, 8582	558	14.82	11.16	279.69	136



Histogram Statistics

File: FIB/SMC 8/3/01.007 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3766
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3766	100.00	75.32	80.35	50
M1	1, 132	3355	89.09	67.10	54.73	50
M2	132, 8582	418	11.10	8.36	286.82	141

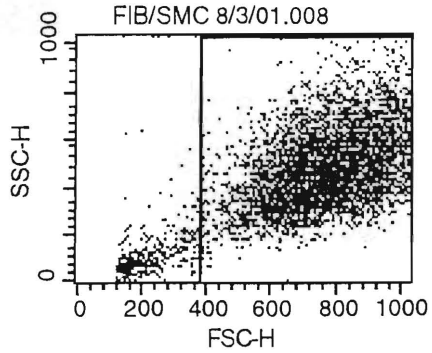


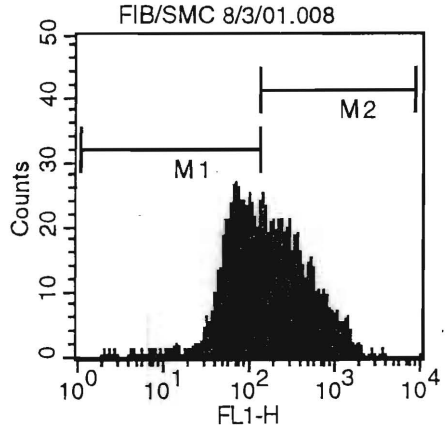
Histogram Statistics

File: FIB/SMC 8/3/01.007 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 3766
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3766	100.00	75.32	53.28	38
M1	1, 132	3544	94.11	70.88	39.06	38
M2	132, 8582	221	5.87	4.42	282.27	137

SmC3 HuSMC?

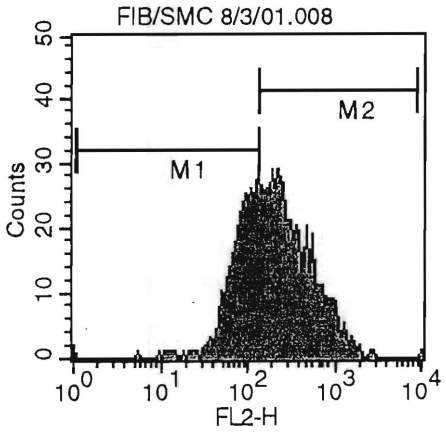




Histogram Statistics

File: FIB/SMC 8/3/01.008 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4648
Total Events: 5000

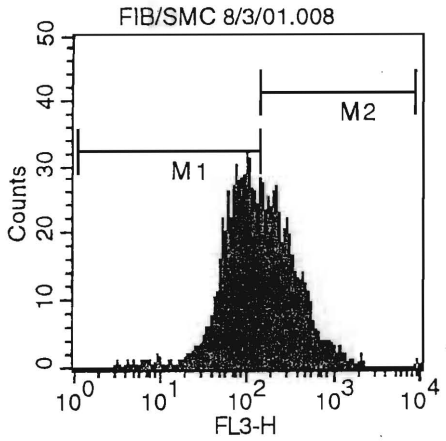
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4648	100.00	92.96	228.75	66
M1	1, 132	2295	49.38	45.90	76.34	66
M2	132, 8582	2368	50.95	47.36	372.01	138



Histogram Statistics

File: FIB/SMC 8/3/01.008 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4648
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4648	100.00	92.96	247.50	181
M1	1, 132	1822	39.20	36.44	87.59	123
M2	132, 8582	2841	61.12	56.82	349.54	181



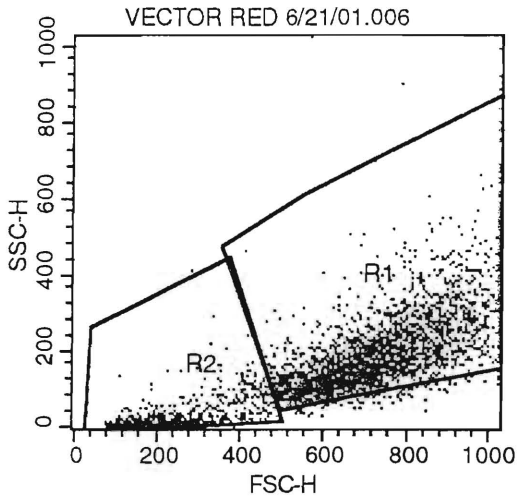
Histogram Statistics

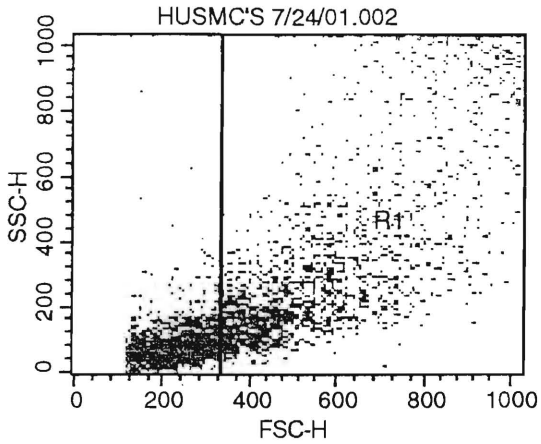
File: FIB/SMC 8/3/01.008 Sample ID: FIBROBLAST VECTOR RED
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1
Gate: G1 Gated Events: 4648
Total Events: 5000

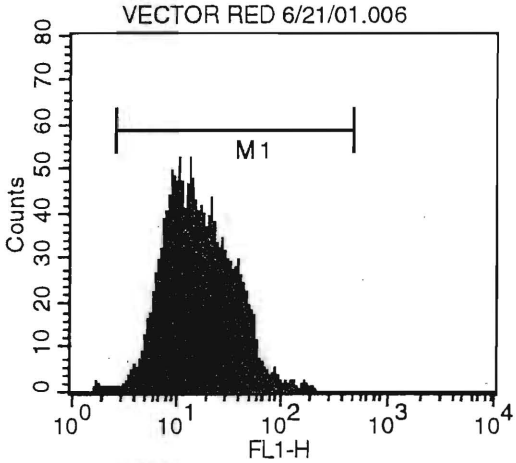
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4648	100.00	92.96	172.75	90
M1	1, 132	2539	54.63	50.78	78.87	90
M2	132, 8582	2128	45.78	42.56	284.41	139

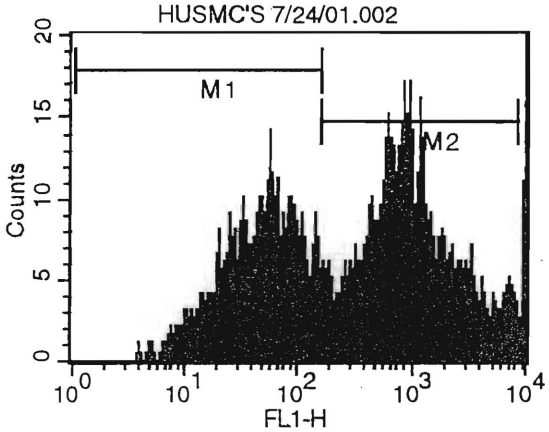
K9 fibroblasts
10 uLs Levamisol

HuAoSMC
10 uLs Levamisol



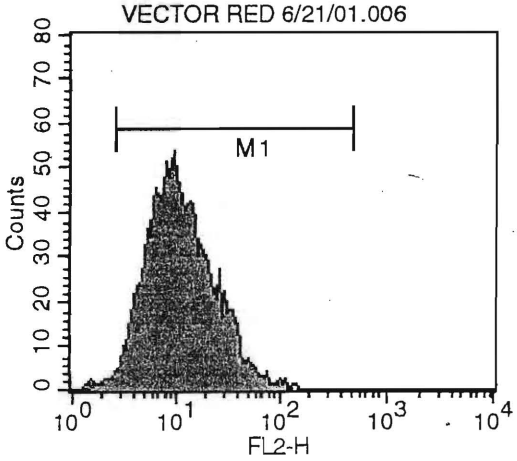


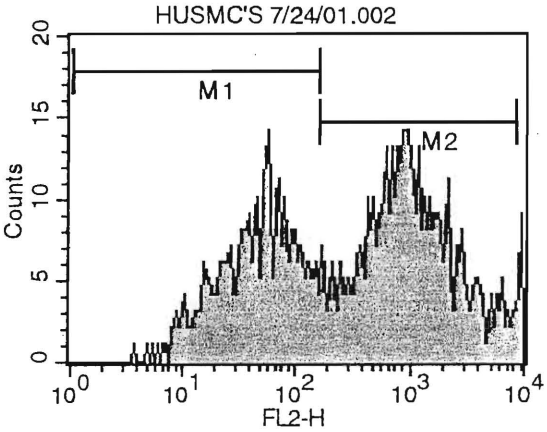




File: HUSMC
Patient ID: T
Gate: G1
Total Events

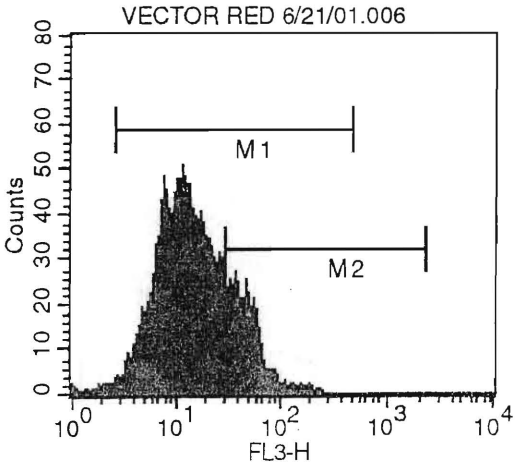
Marker	Lef
All	
M1	
M2	15

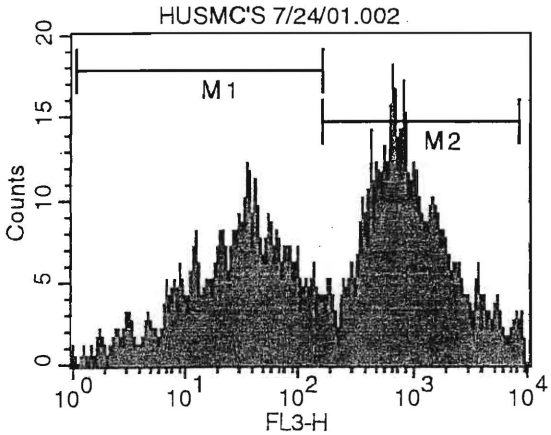




File: HUSMC
Patient ID: T
Gate: G1
Total Events

Marker	Lef
All	
M1	
M2	15



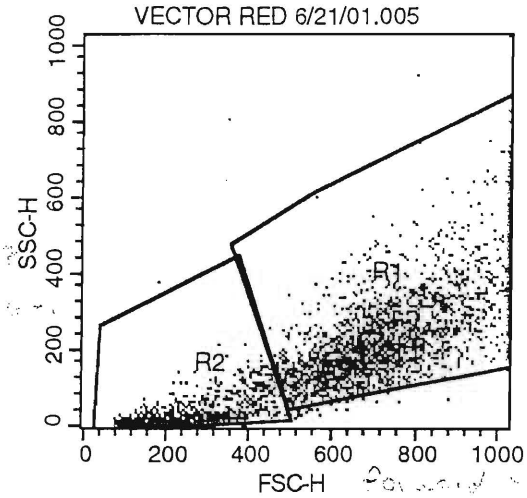


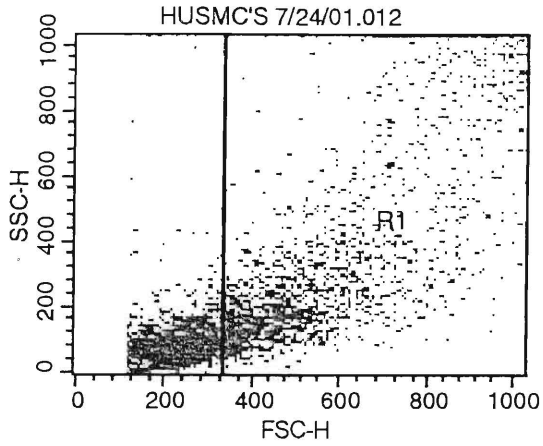
File: HUSM
Patient ID: T
Gate: G1
Total Event

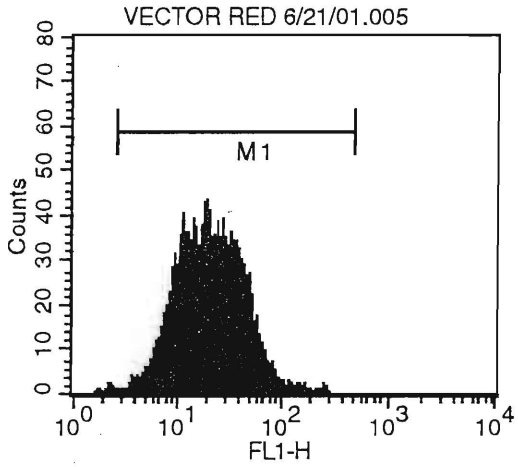
Marker	Le
All	
M1	
M2	15

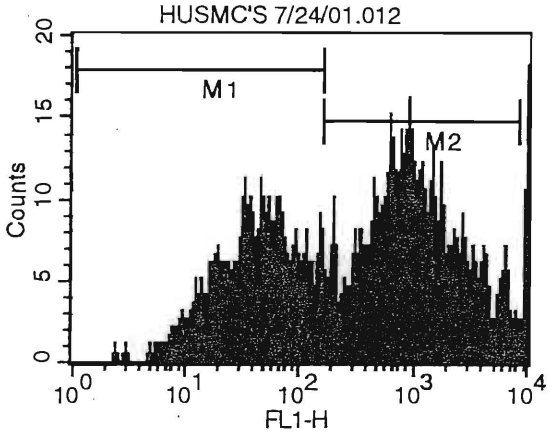
K9 fibroblasts
2uLs Vector Red

HUAoSMC
2uLs Vector Red



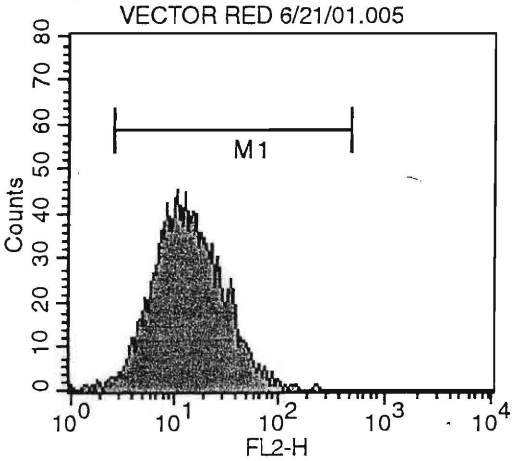


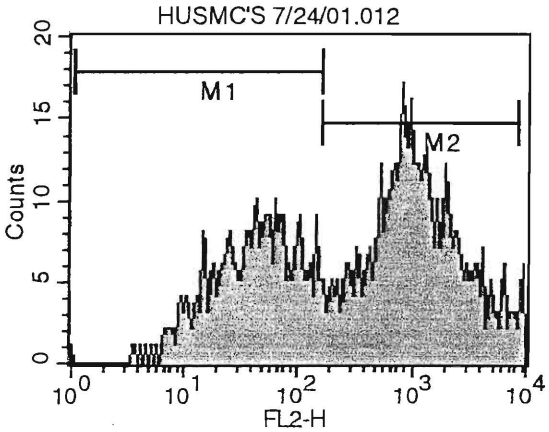




File: HUSMC
Patient ID: T.
Gate: G1
Total Events

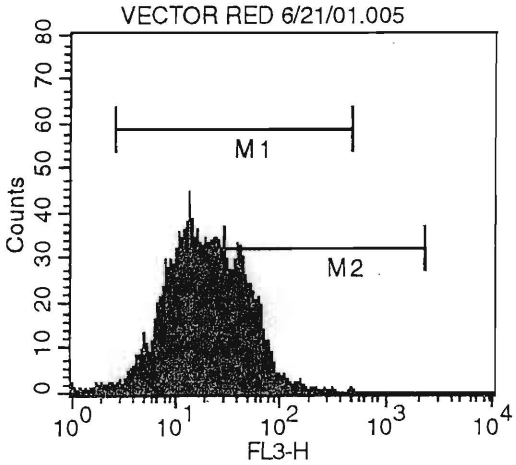
Marker	Left
All	
M1	
M2	158

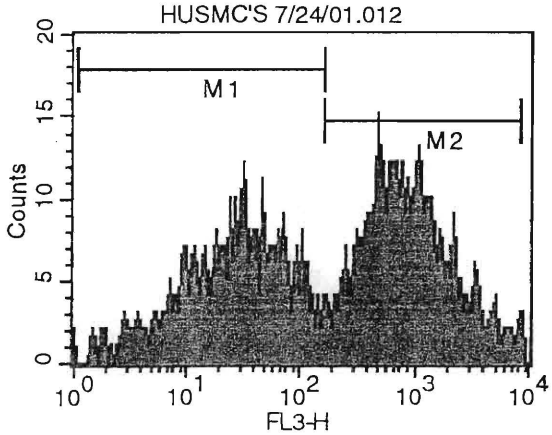




File: HUSMC
Patient ID: T/
Gate: G1
Total Events:

Marker	Left
All	1
M1	
M2	158



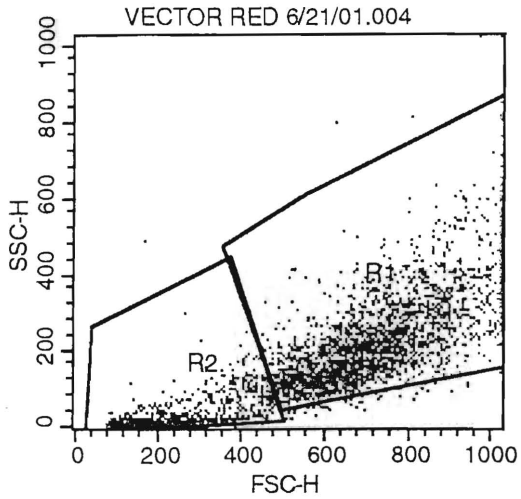


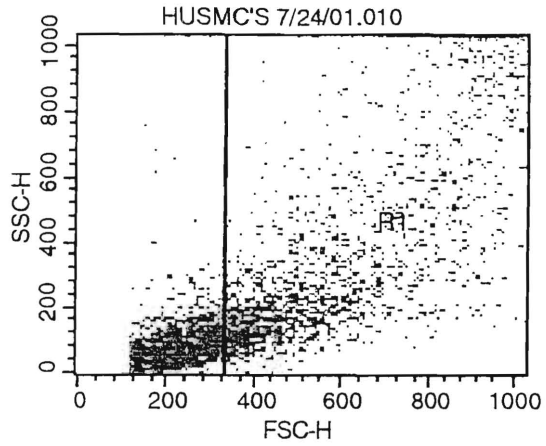
File: HUSMC
Patient ID: T
Gate: G1
Total Events

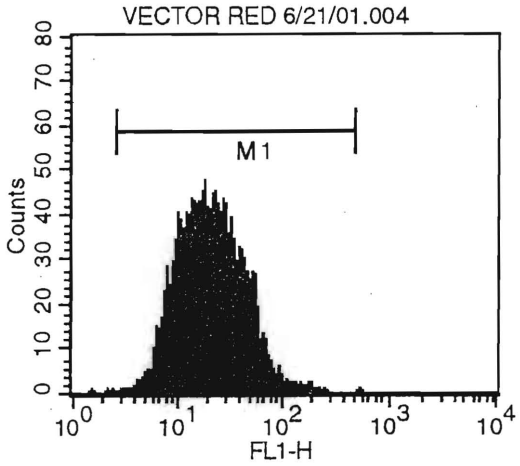
Marker	Lef
All	
M1	
M2	15

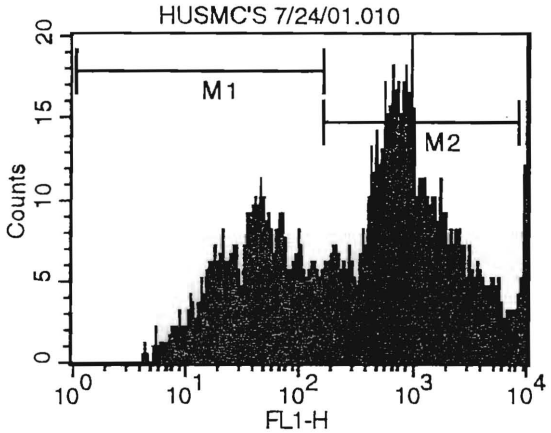
K4 fibroblasts
4 µLs Vector Red

HuAoSMC
5 µLs VR



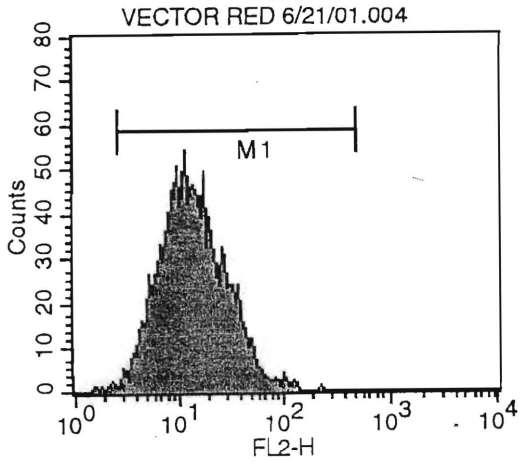


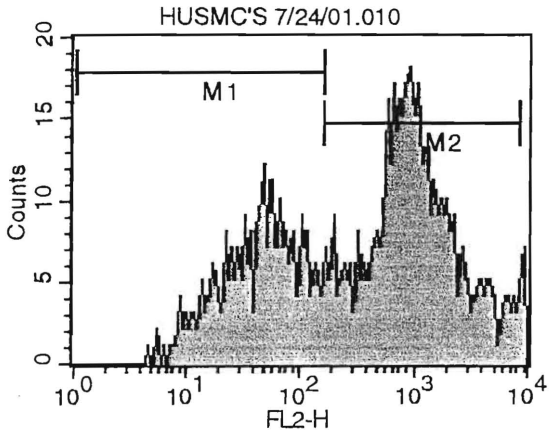




File: HUSM
Patient ID: T
Gate: G1
Total Event

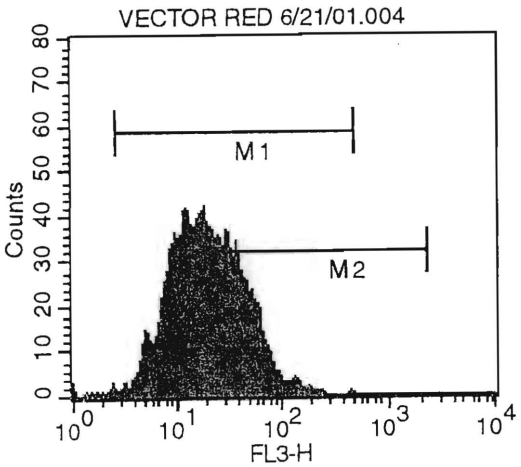
Marker	Le
All	
M1	
M2	15

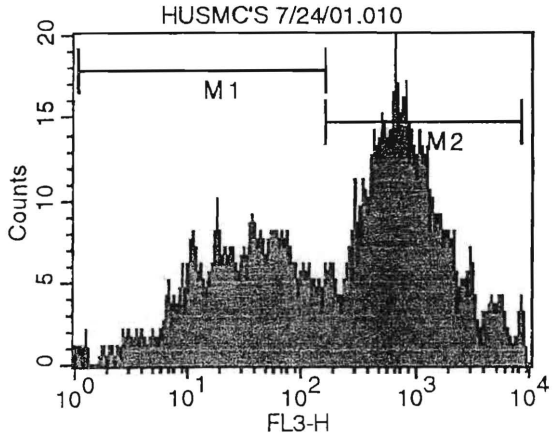




File: HUSMC
Patient ID: T
Gate: G1
Total Events

Marker	Lef
All	
M1	
M2	15



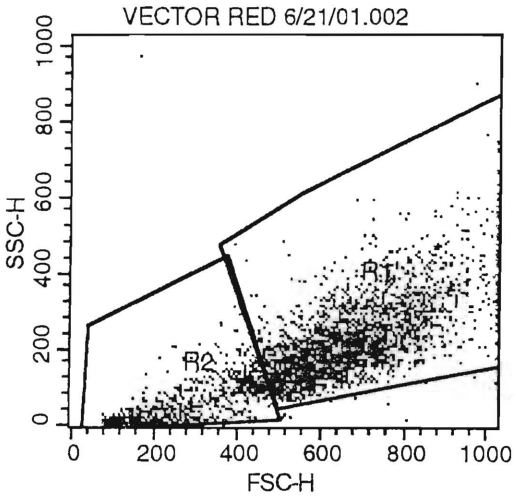


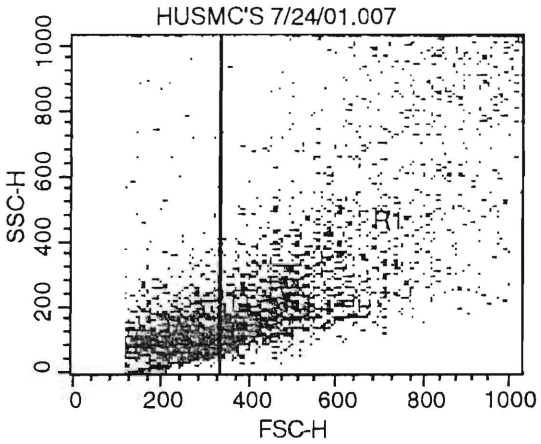
File: HUSM
Patient ID: T
Gate: G1
Total Event

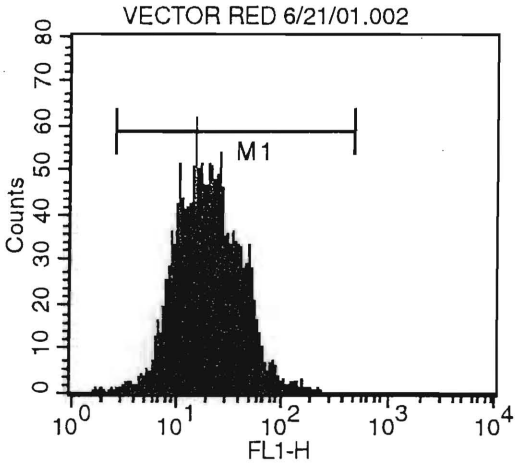
Marker	Le
All	
M1	
M2	15

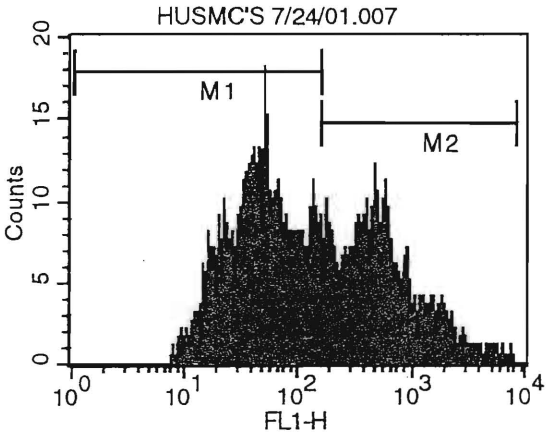
K9 fibroblasts
200 uLs Vector Red

HuAoSMC
200 uLs Vector Red



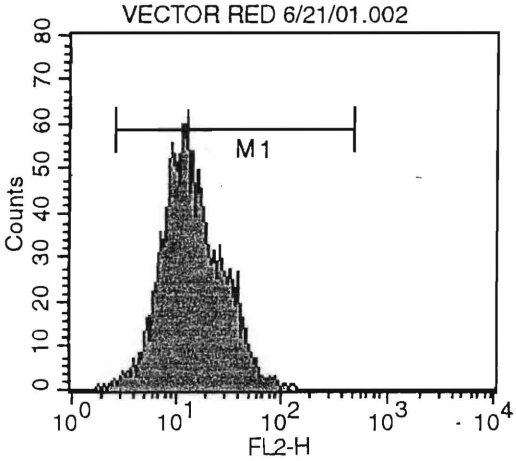


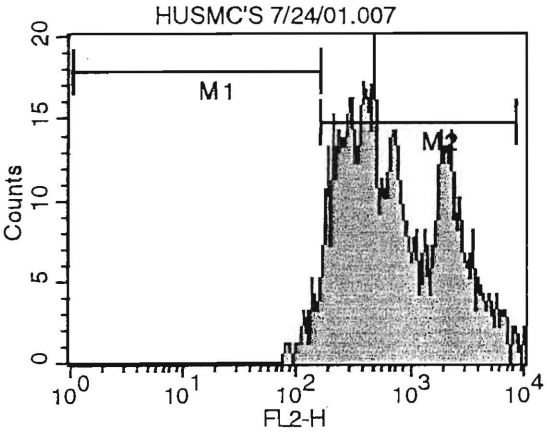




File: HUSMC'S 7
Patient ID: TAG=
Gate: G1
Total Events: 50

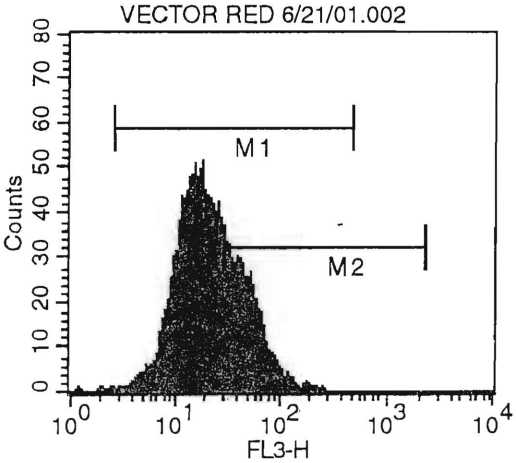
Marker	Left, Ri
All	1, 9
M1	1,
M2	158, 8

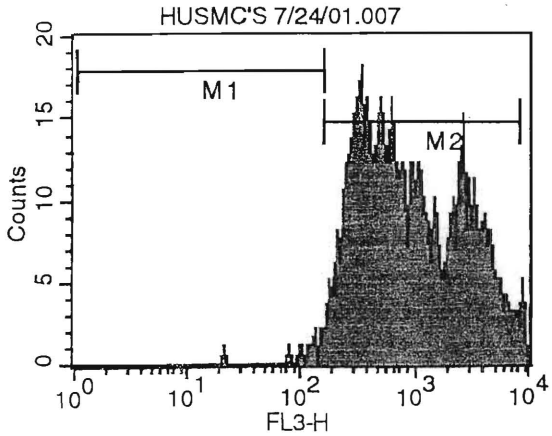




File: HUSMC'S 7
Patient ID: TAG=
Gate: G1
Total Events: 50

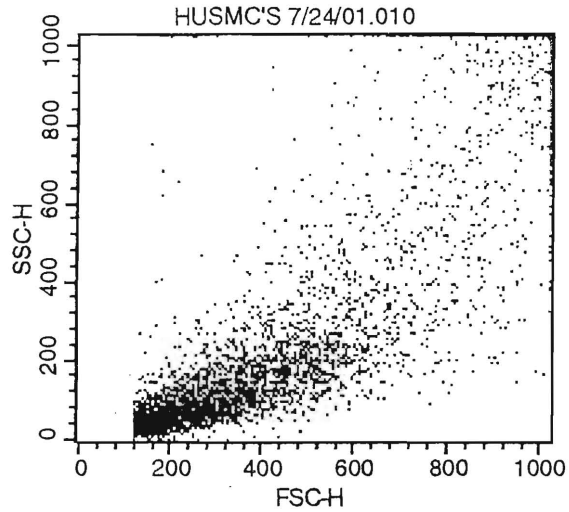
Marker	Left, Ri
All	1, 9
M1	1,
M2	158, 8

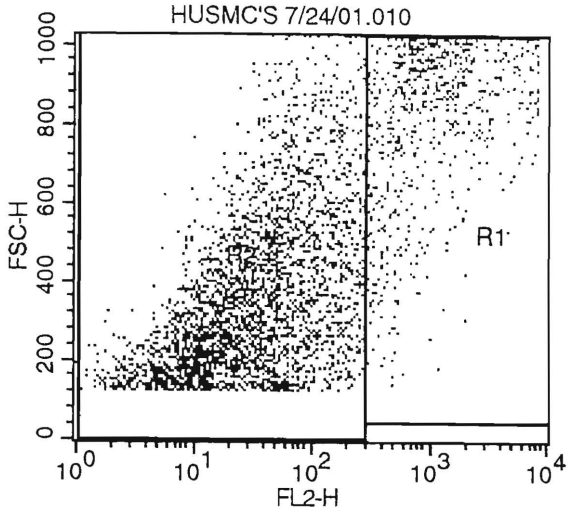


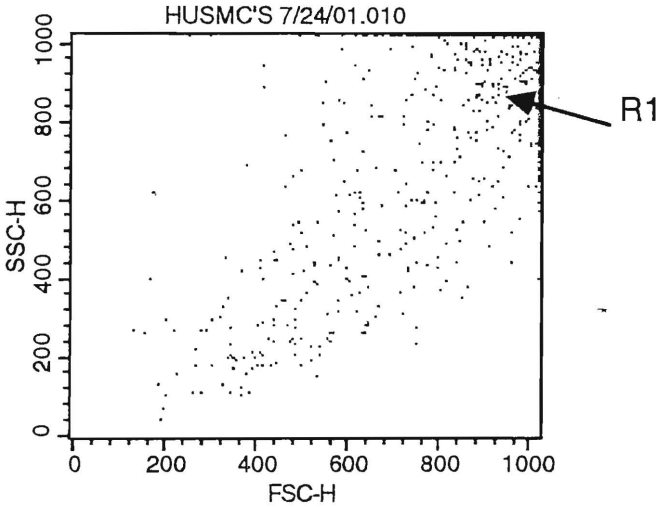


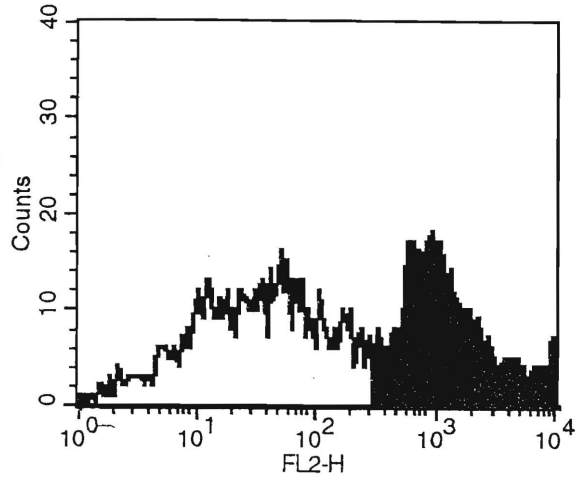
File: HUSMC'S
Patient ID: TAG=
Gate: G1
Total Events: 5

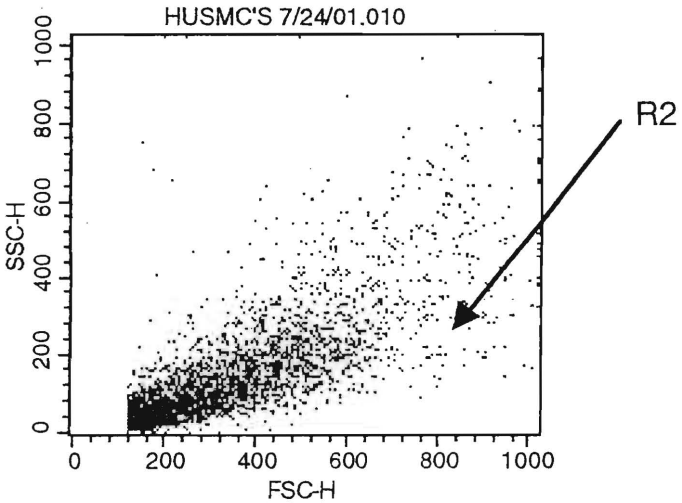
Marker	Left, R
All	1,
M1	1,
M2	158,





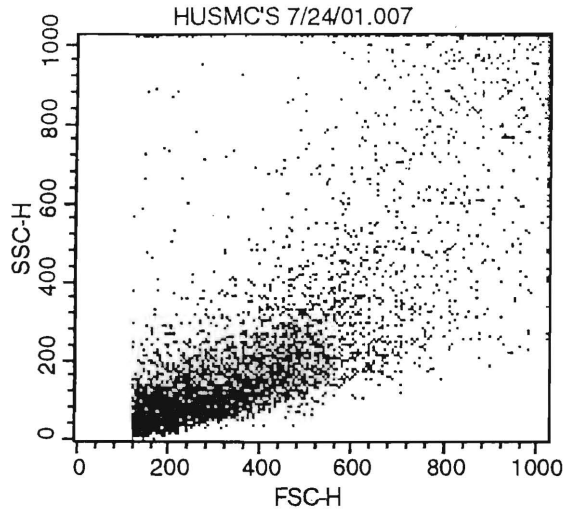


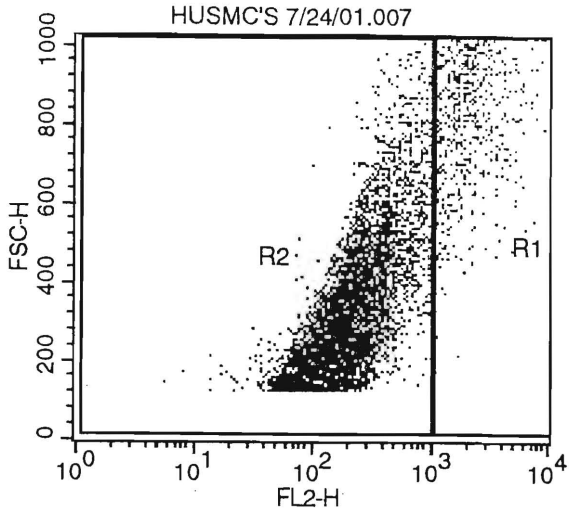


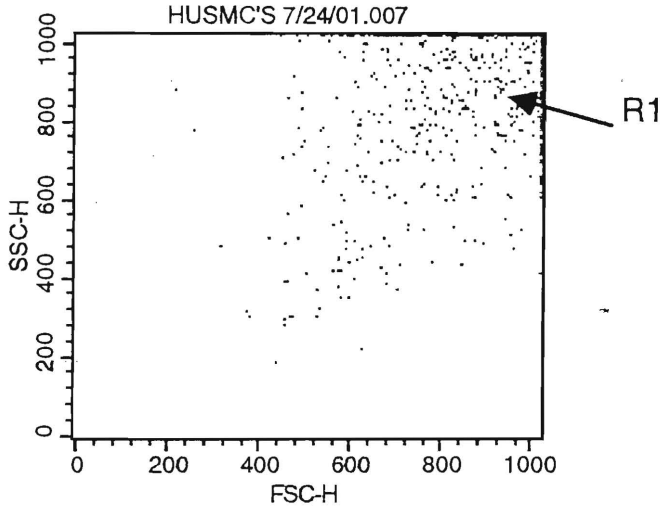


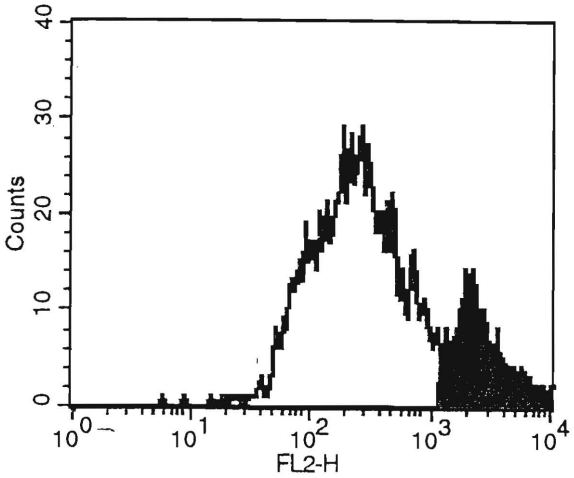
HuAoSMC

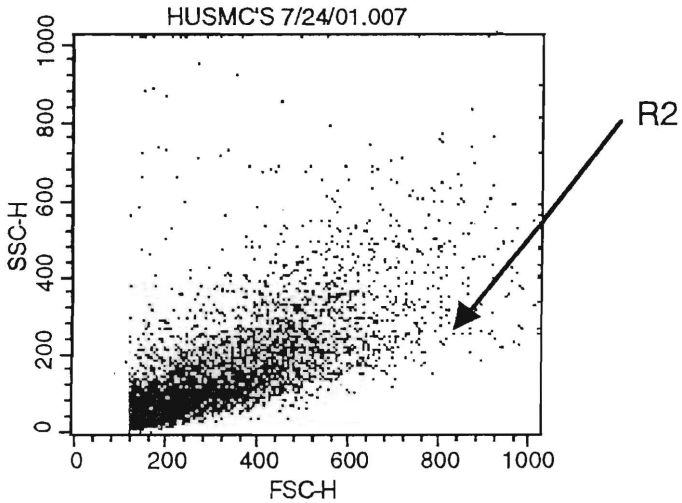
5uLs Vector Red



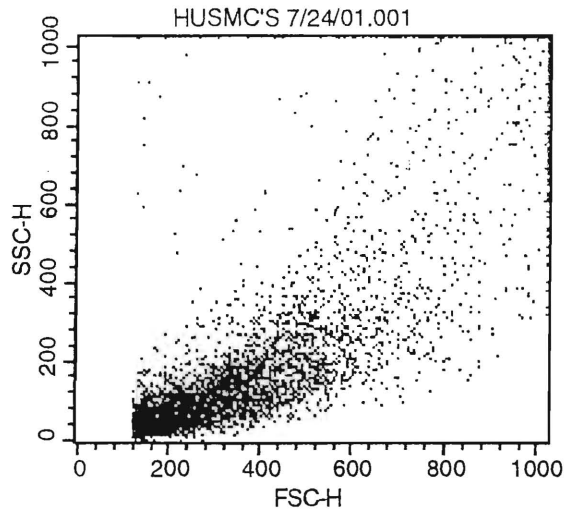


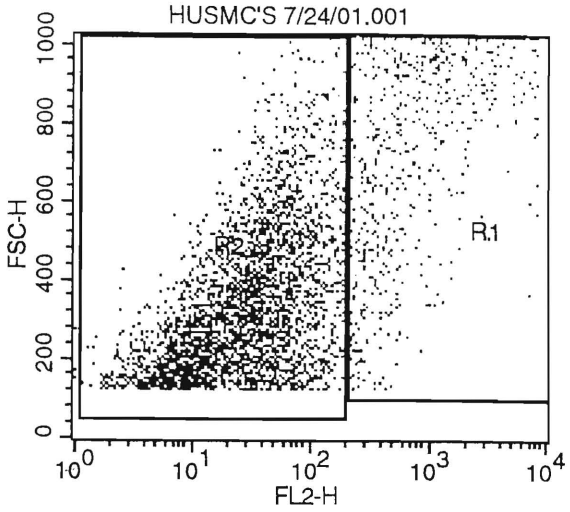


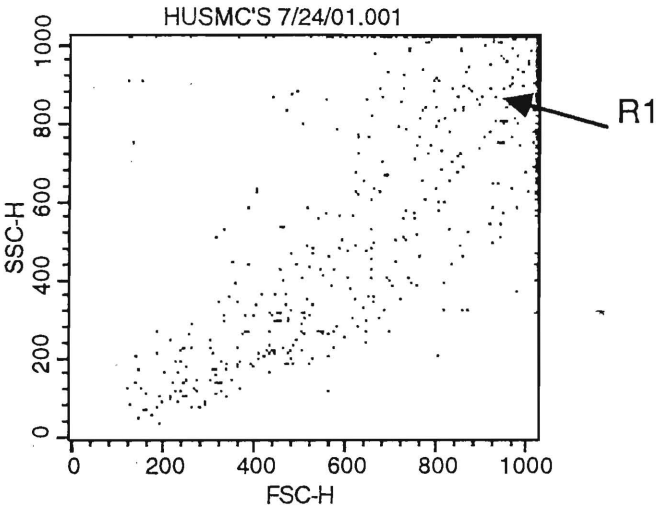


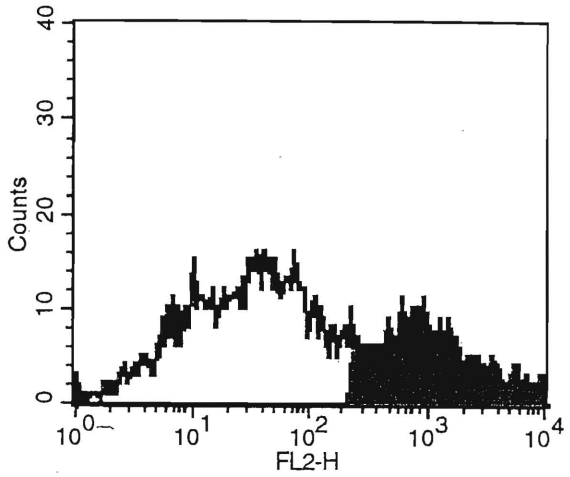


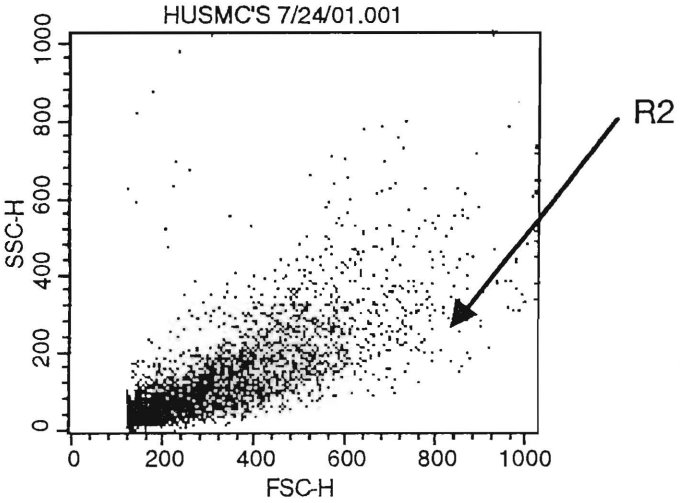
HuAoSMC
200 µLs Vector Red









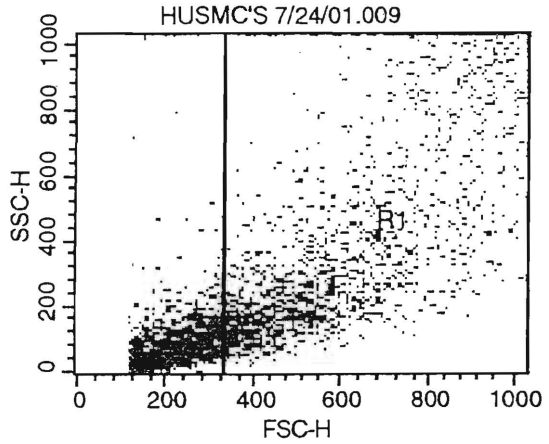


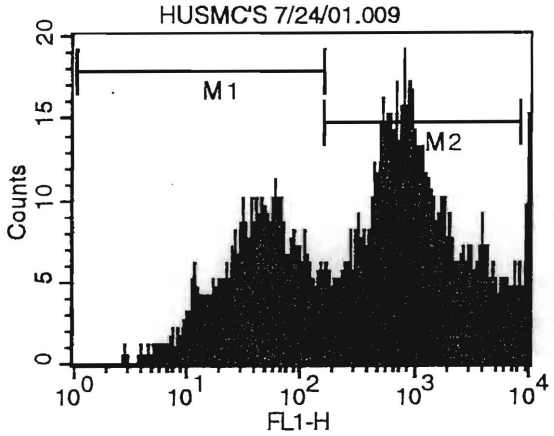
HuAoSMC (L1)

50 uLs Levamisol

Hu Ao SMC
10 uLs VR

10 uLs VR

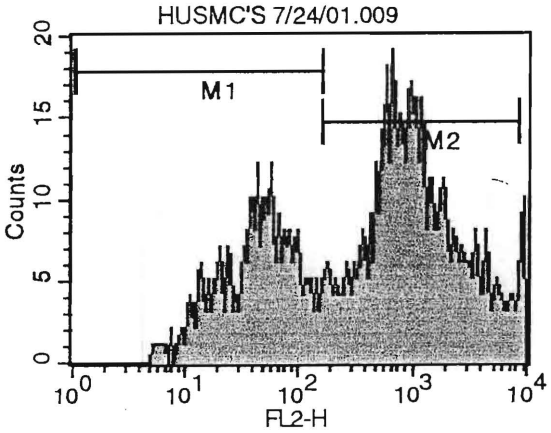




Histogram Statistics

File: HUSMC'S 7/24/01.009
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3423
Total Events: 5000

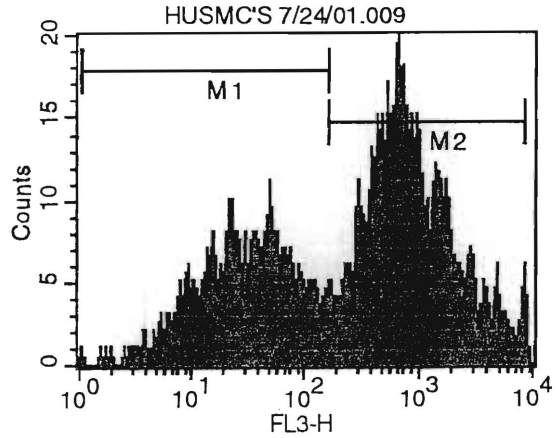
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3423	100.00	68.46	1076.34	743
M1	1, 158	1110	32.43	22.20	55.12	58
M2	158, 8429	2271	66.35	45.42	1414.76	743



Histogram Statistics

File: HUSMC'S 7/24/01.009
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3423
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3423	100.00	68.46	1044.53	610
M1	1, 158	1103	32.22	22.06	56.41	42
M2	158, 8429	2314	67.60	46.28	1478.12	610



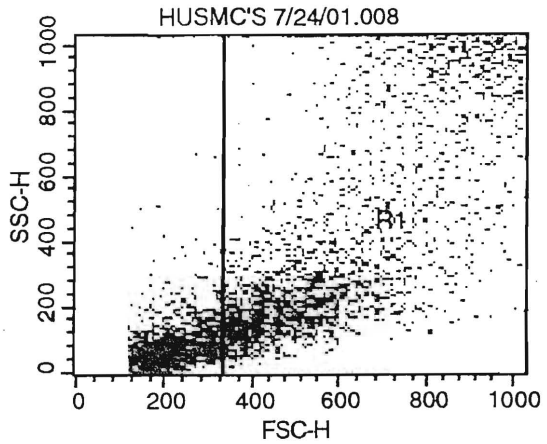
Histogram Statistics

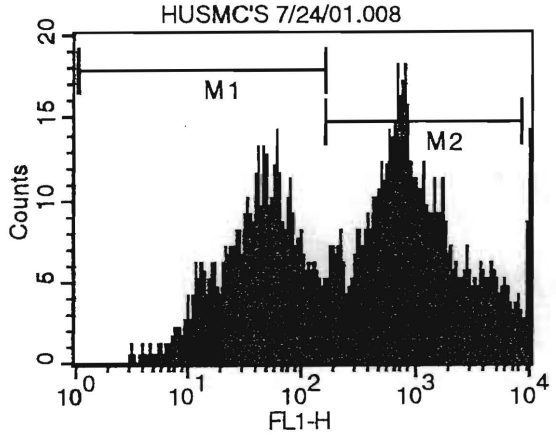
File: HUSMC'S 7/24/01.009
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3423
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3423	100.00	68.46	749.14	594
M1	1, 158	1242	36.28	24.84	45.22	46
M2	158, 8429	2186	63.86	43.72	1147.73	594

Hu Ao SMC
50 uLs VR

50 uLs VR



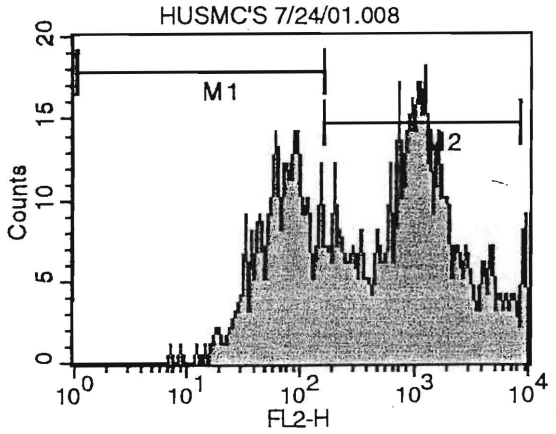


Histogram Statistics

File: HUSMC'S 7/24/01.008
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3393

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3393	100.00	67.86	901.02	632
M1	1, 158	1302	38.37	26.04	53.17	57
M2	158, 8429	2060	60.71	41.20	1311.03	632

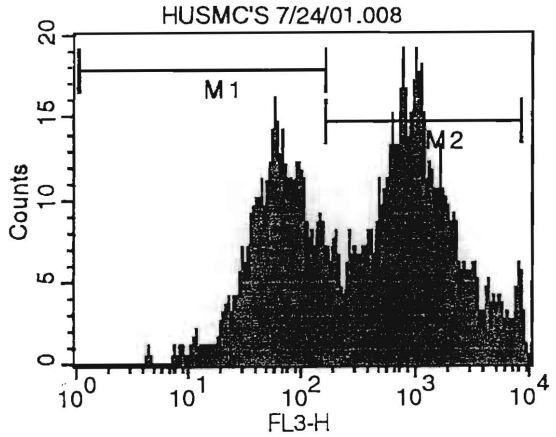


Histogram Statistics

File: HUSMC'S 7/24/01.008
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3393

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3393	100.00	67.86	1020.62	1155
M1	1, 158	1174	34.60	23.48	74.79	58
M2	158, 8429	2211	65.16	44.22	1488.17	1155



Histogram Statistics

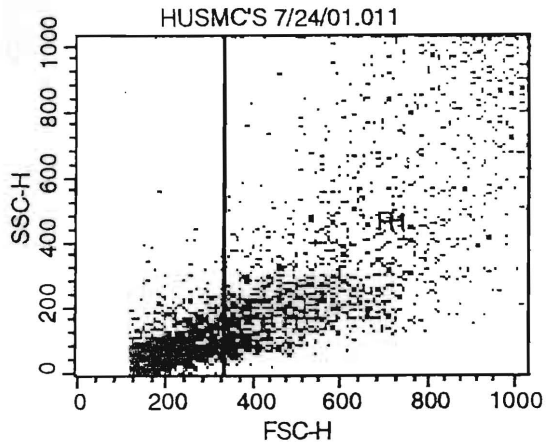
File: HUSMC'S 7/24/01.008
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

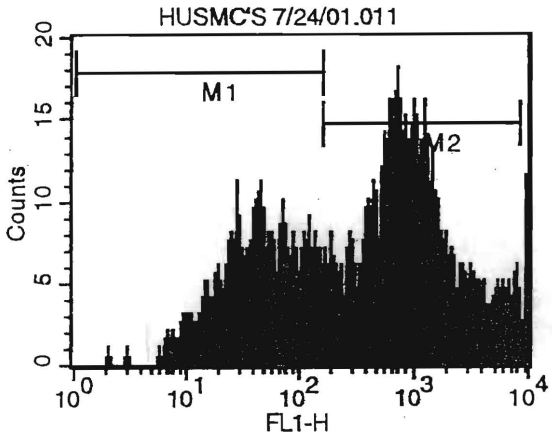
Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3393

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3393	100.00	67.86	796.27	704
M1	1, 158	1303	38.40	26.06	67.71	54
M2	158, 8429	2091	61.63	41.82	1245.87	704

control
HuAoSMC

control

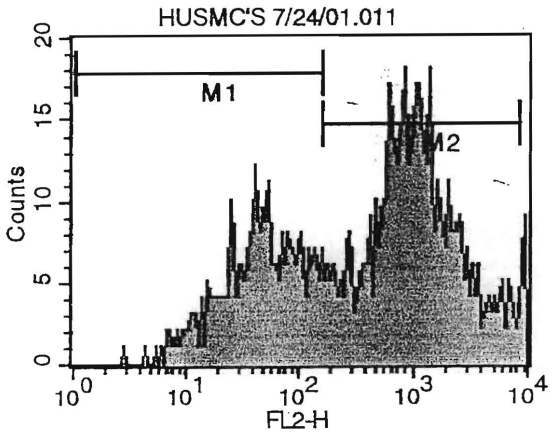




Histogram Statistics

File: HUSMC'S 7/24/01.011 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3356
Total Events: 5000

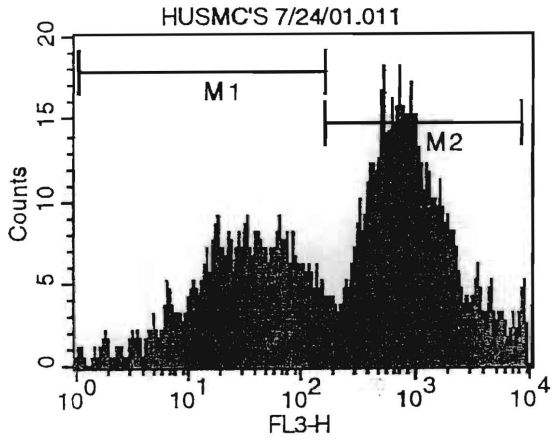
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3356	100.00	67.12	1046.29	9058
M1	1, 158	1115	33.22	22.30	59.10	27
M2	158, 8429	2200	65.55	44.00	1379.40	685



Histogram Statistics

File: HUSMC'S 7/24/01.011 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3356
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3356	100.00	67.12	1014.18	813
M1	1, 158	1116	33.25	22.32	59.18	38
M2	158, 8429	2233	66.54	44.66	1460.51	813



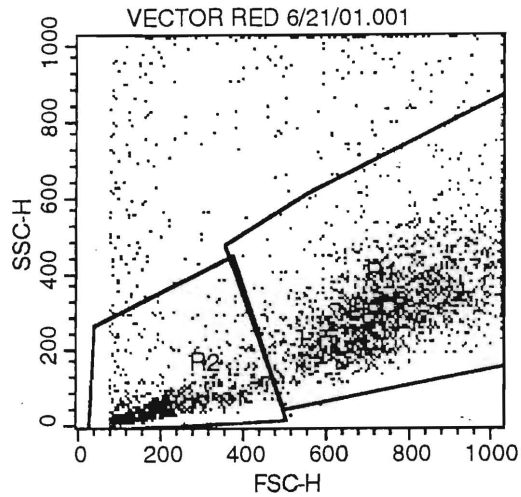
Histogram Statistics

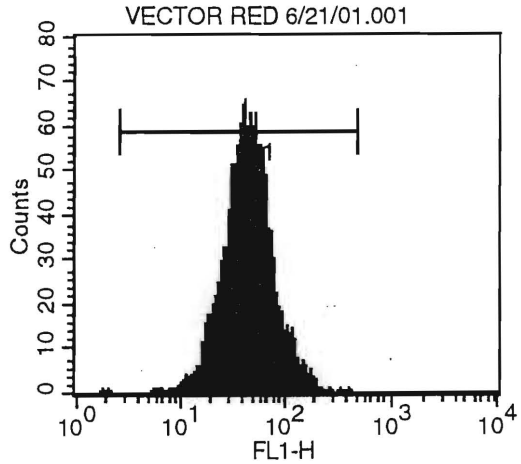
File: HUSMC'S 7/24/01.011 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3356
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3356	100.00	67.12	707.39	483
M1	1, 158	1269	37.81	25.38	44.55	18
M2	158, 8429	2090	62.28	41.80	1109.06	483

K9 fibroblasts
2 mL VR

2 mLs VR



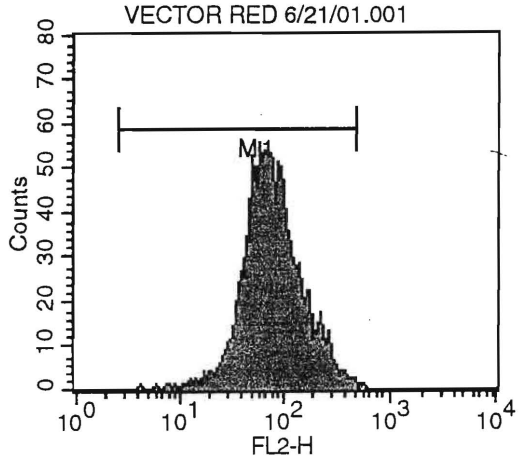


Histogram Statistics

File: VECTOR RED 6/21/01.001
Patient ID: A1
Gate: G1
Total Events: 10000

Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 6389
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	6389	100.00	63.89	46.80	39
M1	3, 470	6387	99.97	63.87	46.81	39

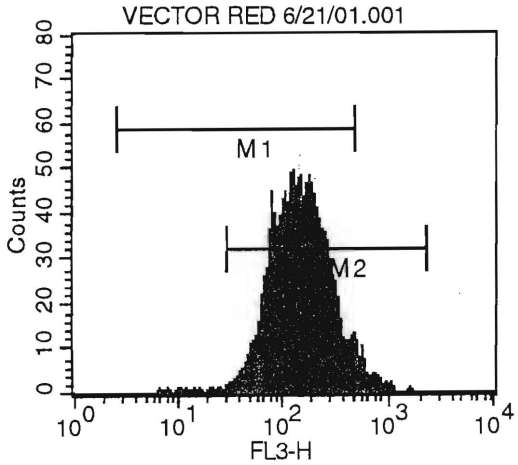


Histogram Statistics

File: VECTOR RED 6/21/01.001
Patient ID: A1
Gate: G1
Total Events: 10000

Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 6389
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	6389	100.00	63.89	84.33	53
M1	3, 470	6387	99.97	63.87	84.20	53



Histogram Statistics

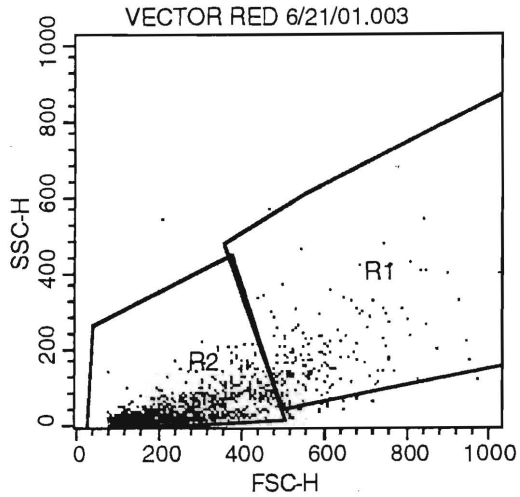
File: VECTOR RED 6/21/01.001
Patient ID: A1
Gate: G1
Total Events: 10000

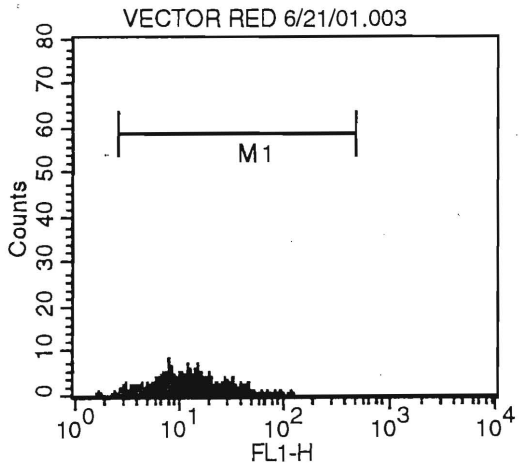
Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 6389
X Parameter: FL3-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	6389	100.00	63.89	164.55	119
M1	3, 470	6234	97.57	62.34	153.49	119
M2	28, 2288	6374	99.77	63.74	164.90	119

K9 fibroblasts
20 µLs VR

20 µLs VR



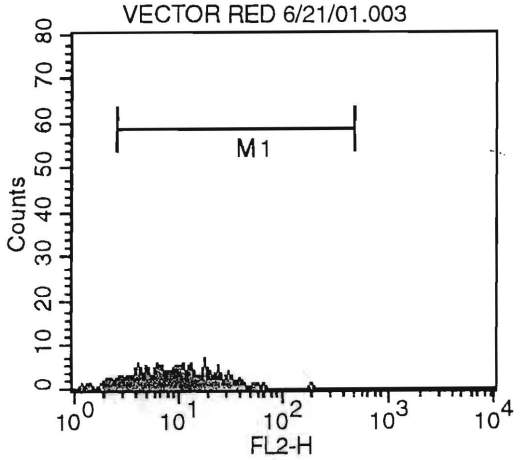


Histogram Statistics

File: VECTOR RED 6/21/01.003
Patient ID: A3
Gate: G1
Total Events: 5190

Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 517
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	517	100.00	9.96	14.28	8
M1	3, 470	513	99.23	9.88	14.37	8

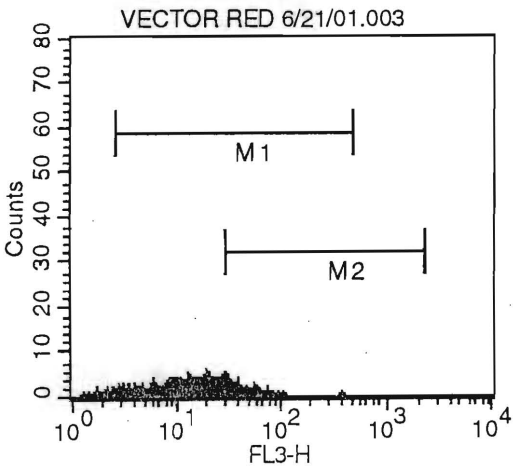


Histogram Statistics

File: VECTOR RED 6/21/01.003
Patient ID: A3
Gate: G1
Total Events: 5190

Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 517
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	517	100.00	9.96	11.07	18
M1	3, 470	494	95.55	9.52	11.49	18

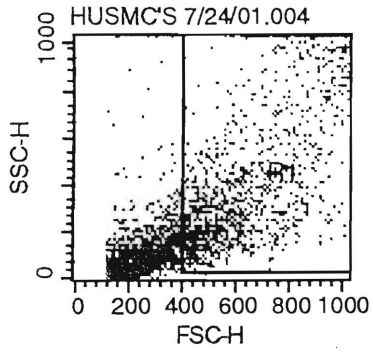


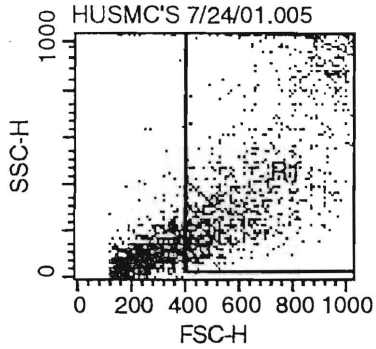
Histogram Statistics

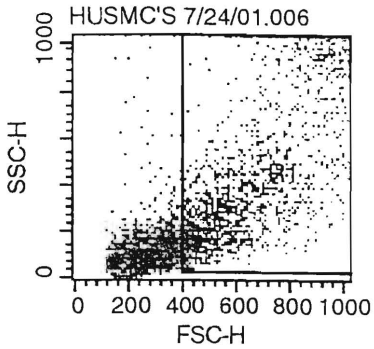
File: VECTOR RED 6/21/01.003
Patient ID: A3
Gate: G1
Total Events: 5190

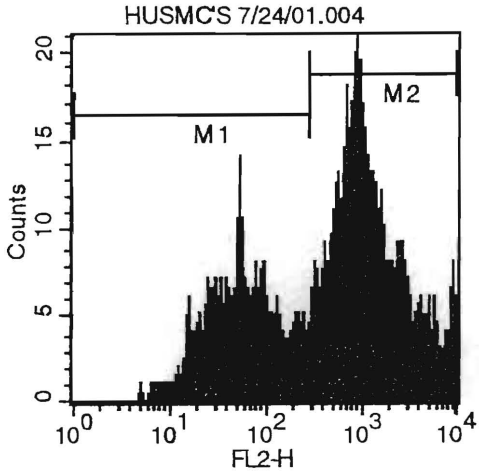
Sample ID: VECTOR RED
Acquisition Date: 21-Jun-1
Gated Events: 517
X Parameter: FL3-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	517	100.00	9.96	17.83	18
M1	3, 470	499	96.52	9.61	18.41	18
M2	28, 2288	84	16.25	1.62	45.62	29







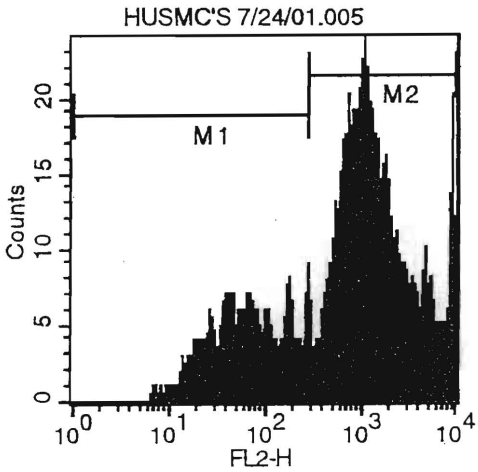


Histogram Statistics

File: HUSMC'S 7/24/01.004
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3002

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3002	100.00	60.04	1124.17	858
M1	1, 289	970	32.31	19.40	79.62	50
M2	289, 9910	2034	67.75	40.68	1621.49	858



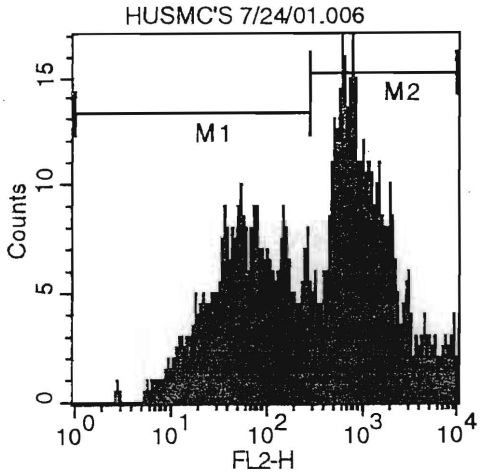
Histogram Statistics

File: HUSMC'S 7/24/01.005
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3479

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3479	100.00	69.58	1398.67	1027
M1	1, 289	832	23.91	16.64	87.29	269
M2	289, 9910	2649	76.14	52.98	1809.71	1027

accidentally mixed

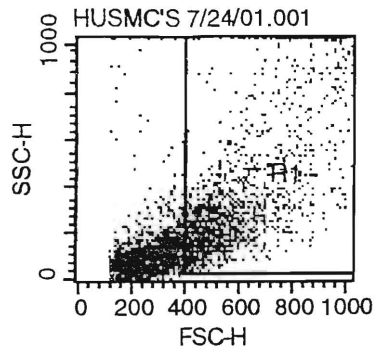


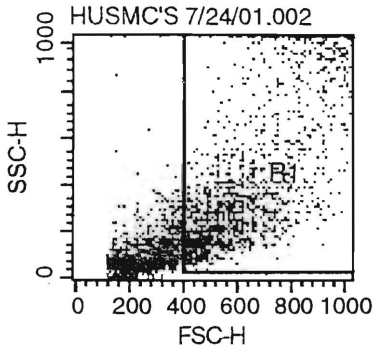
Histogram Statistics

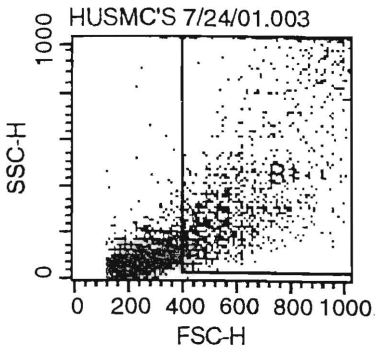
File: HUSMC'S 7/24/01.006
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

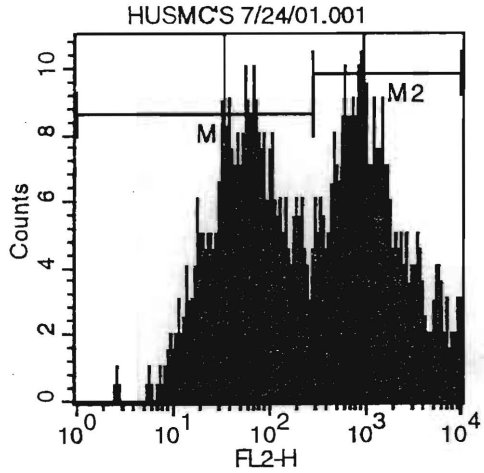
Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2591

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2591	100.00	51.82	778.40	578
M1	1, 289	1159	44.73	23.18	87.16	51
M2	289, 9910	1433	55.31	28.66	1337.13	578







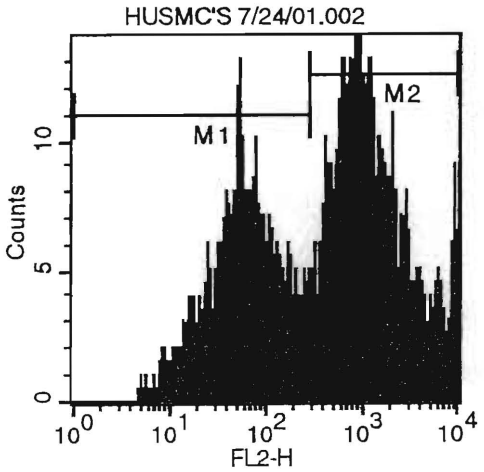


Histogram Statistics

File: HUSMC'S 7/24/01.001
Patient ID: TAG=TUBE
Gate: G1
Total Events: 4425

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2133

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2133	100.00	48.20	778.00	34
M1	1, 289	1055	49.46	23.84	81.92	34
M2	289, 9910	1082	50.73	24.45	1454.91	874

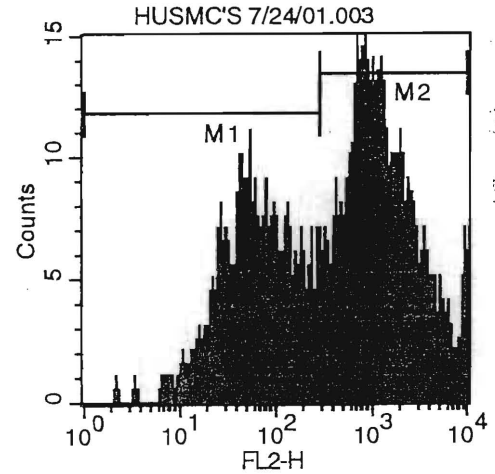


Histogram Statistics

File: HUSMC'S 7/24/01.002
Patient ID: TAG=TUBE
Gate: G1
Total Events: 4785

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2757

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2757	100.00	57.62	976.76	813
M1	1, 289	1082	39.25	22.61	81.27	53
M2	289, 9910	1675	60.75	35.01	1555.22	813

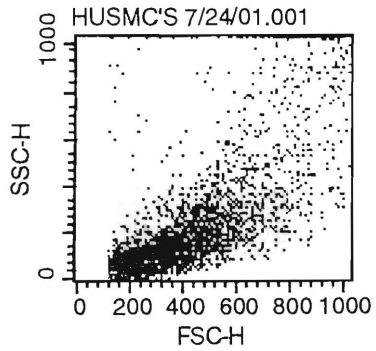


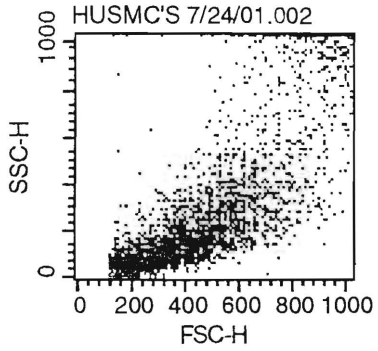
Histogram Statistics

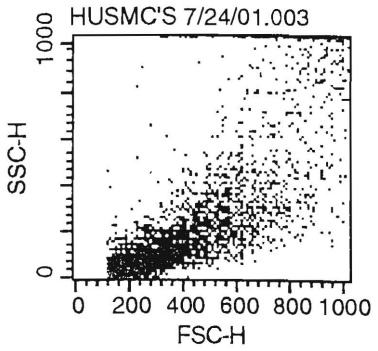
File: HUSMC'S 7/24/01.003
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5235

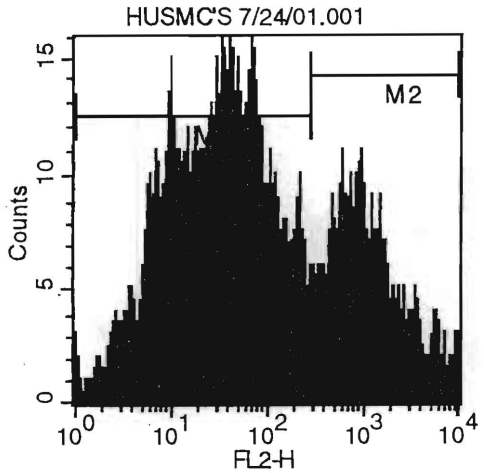
Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2905

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2905	100.00	55.49	1046.99	667
M1	1, 289	1094	37.66	20.90	88.79	50
M2	289, 9910	1813	62.41	34.63	1624.34	667







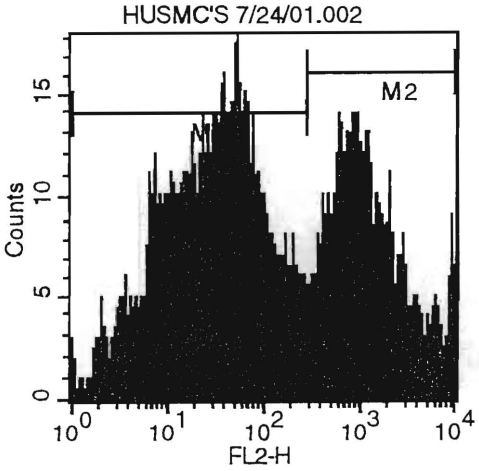


20 uL Lev

Histogram Statistics

File: HUSMC'S 7/24/01.001
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 4425
Total Events: 4425

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4425	100.00	100.00	402.17	34
M1	1, 289	3296	74.49	74.49	52.57	34
M2	289, 9910	1134	25.63	25.63	1417.81	573

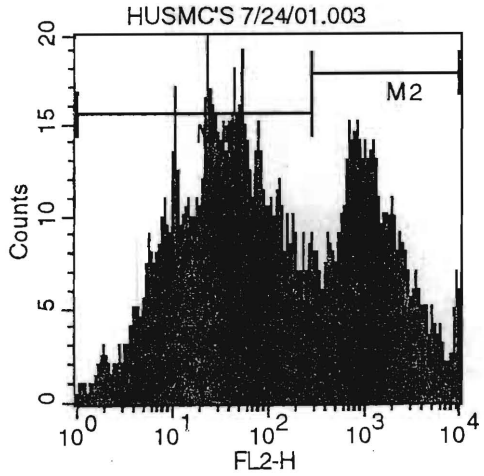


10 uL Lev

Histogram Statistics

File: HUSMC'S 7/24/01.002
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 4785
Total Events: 4785

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	4785	100.00	100.00	584.11	53
M1	1, 289	3060	63.95	63.95	53.41	53
M2	289, 9910	1725	36.05	36.05	1525.53	583

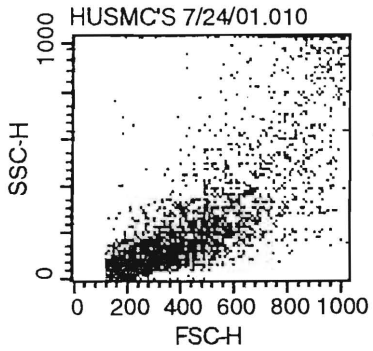


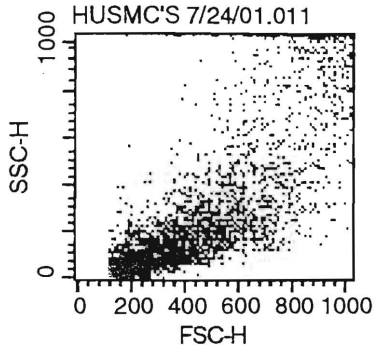
5 uL Lev

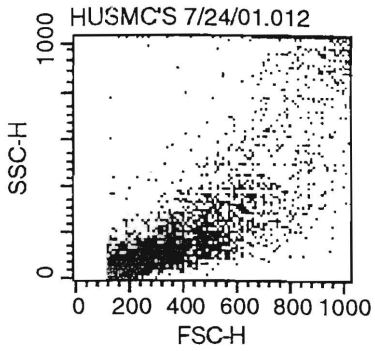
Histogram Statistics

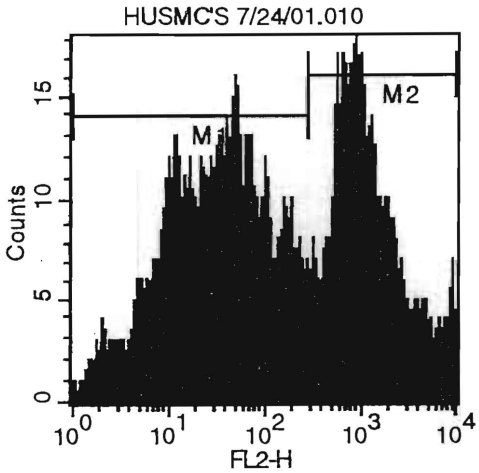
File: HUSMC'S 7/24/01.003
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5235
Total Events: 5235

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5235	100.00	100.00	604.34	23
M1	1, 289	3374	64.45	64.45	57.51	23
M2	289, 9910	1865	35.63	35.63	1592.94	667







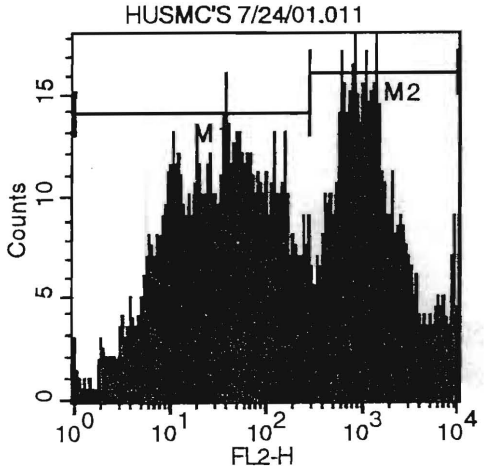


5 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.010
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5000
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	687.22	851
M1	1, 289	2927	58.54	58.54	56.78	49
M2	289, 9910	2077	41.54	41.54	1574.89	851

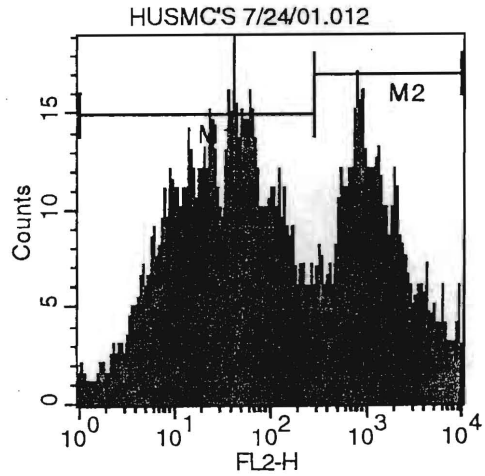


5 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.011
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5000
Total Events: 5000

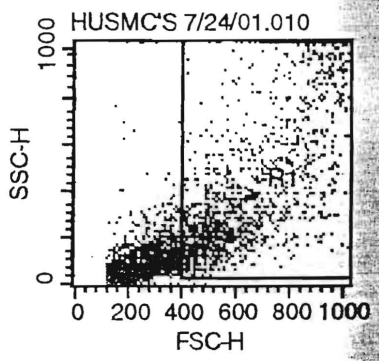
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	696.53	813
M1	1, 289	2925	58.50	58.50	59.41	38
M2	289, 9910	2078	41.56	41.56	1592.74	813

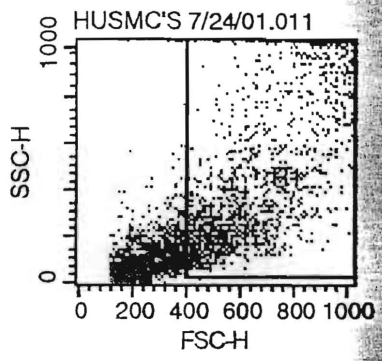


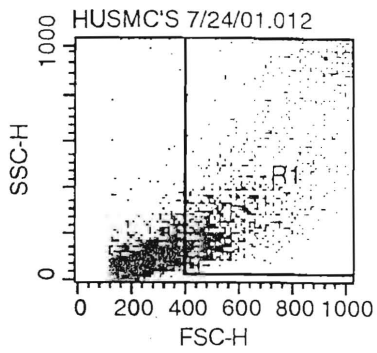
Histogram Statistics

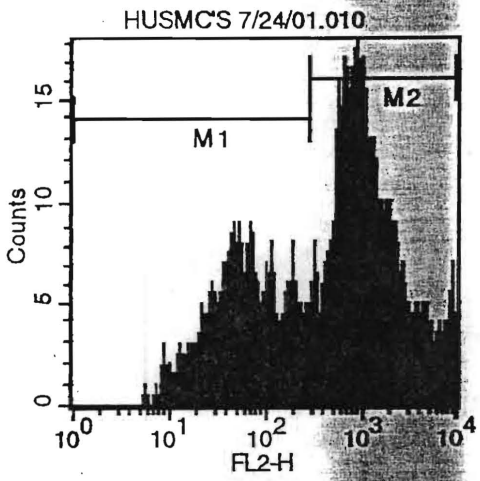
File: HUSMC'S 7/24/01.012
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5000
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	593.30	40
M1	1, 289	3253	65.06	65.06	53.45	40
M2	289, 9910	1750	35.00	35.00	1596.29	743









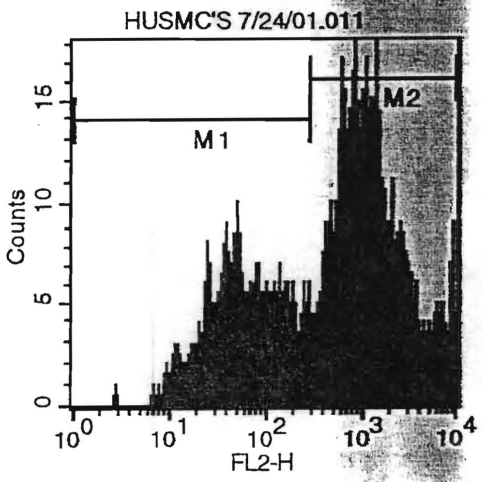
5 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.010
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3079

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3079	100.00	61.58	1084.18	851
M1	1, 289	1050	34.10	21.00	87.17	45
M2	289, 9910	2031	65.96	40.62	1598.84	851



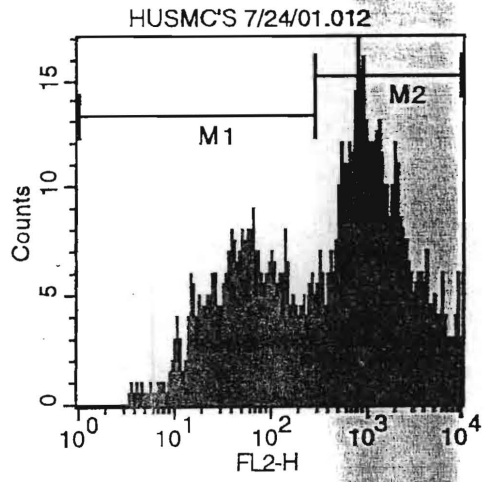
2 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.011
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3019

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3019	100.00	60.38	1116.03	813
M1	1, 289	996	32.99	19.92	89.03	49
M2	289, 9910	2026	67.11	40.52	1619.69	813



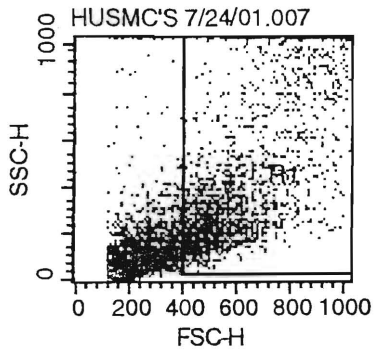
control

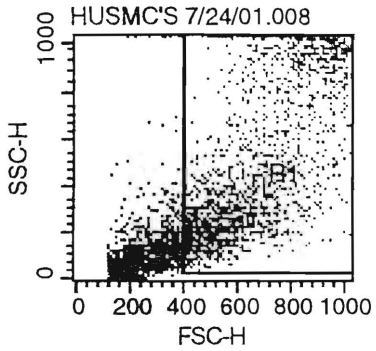
Histogram Statistics

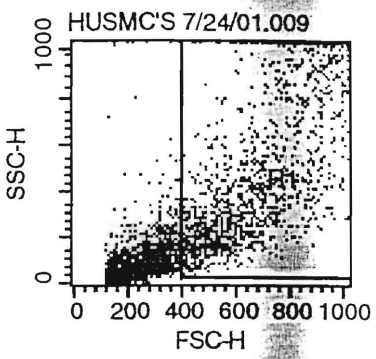
File: HUSMC'S 7/24/01.012
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

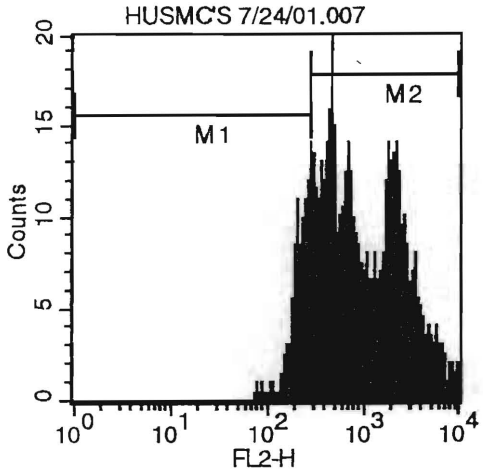
Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2752

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2752	100.00	55.04	1035.76	743
M1	1, 289	1056	38.37	21.12	80.99	62
M2	289, 9910	1698	61.70	33.96	1628.65	743









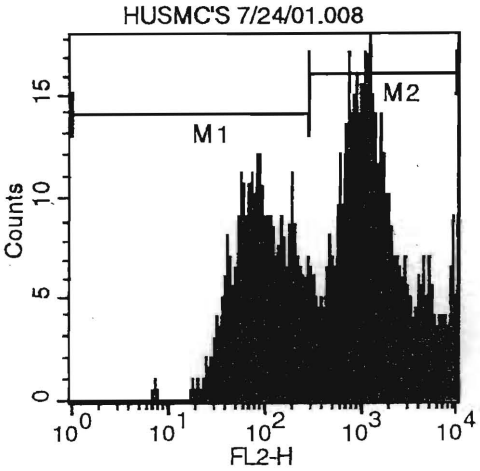
200 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.007
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 2181

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2181	100.00	43.62	1243.24	433
M1	1, 289	364	16.69	7.28	230.25	276
M2	289, 9910	1830	83.91	36.60	1437.96	433



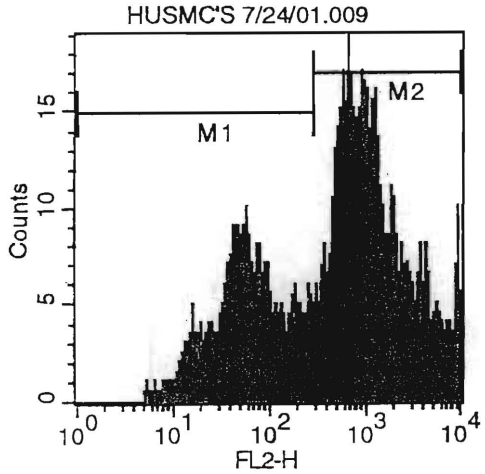
50 uL VR

Histogram Statistics

File: HUSMC'S 7/24/01.008
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3042

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3042	100.00	60.84	1125.72	1155
M1	1, 289	1105	36.32	22.10	108.28	82
M2	289, 9910	1940	63.77	38.80	1703.94	1155



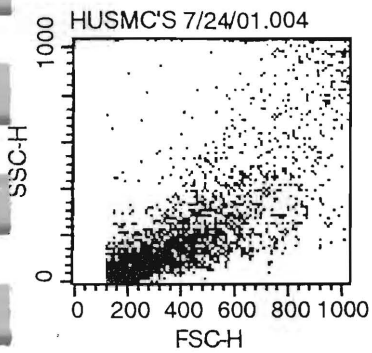
10 uL VR

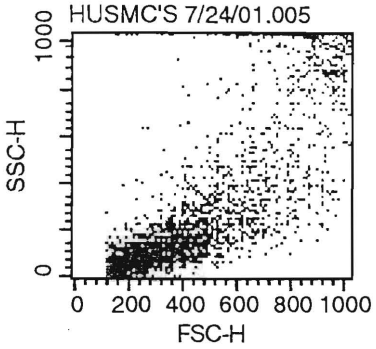
Histogram Statistics

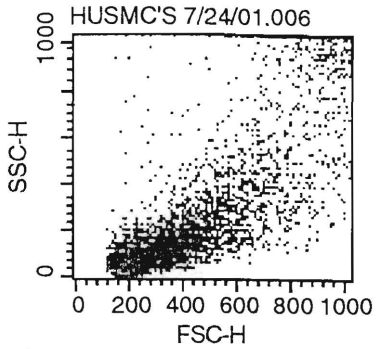
File: HUSMC'S 7/24/01.009
Patient ID: TAG=TUBE
Gate: G1
Total Events: 5000

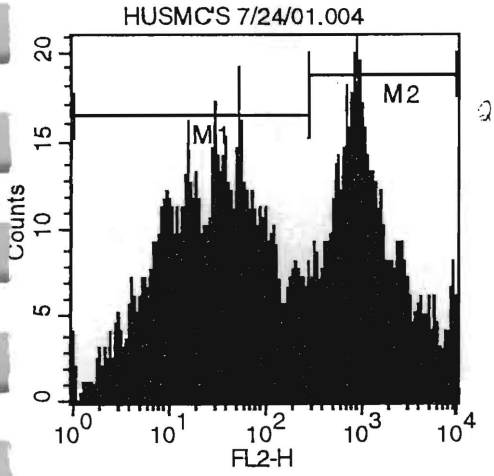
Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 3120

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3120	100.00	62.40	1137.90	610
M1	1, 289	1011	32.40	20.22	88.81	55
M2	289, 9910	2110	67.63	42.20	1640.16	610









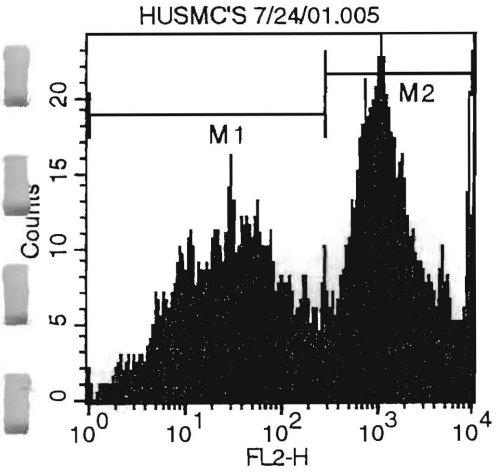
2 uL Lev

Histogram Statistics

File: HUSMC'S 7/24/01.004
Patient ID: TAG=TUBE
Gate: No Gate
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	695.06	858
M1	1, 289	2913	58.26	58.26	52.52	50
M2	289, 9910	2089	41.78	41.78	1590.65	858



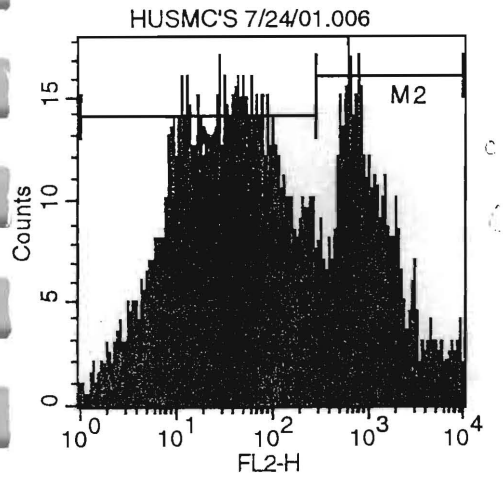
10 uL VR
10 uL Lev

Histogram Statistics

File: HUSMC'S 7/24/01.005
Patient ID: TAG=TUBE
Gate: No Gate
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	989.87	1027
M1	1, 289	2301	46.02	46.02	56.01	29
M2	289, 9910	2701	54.02	54.02	1784.91	1027



control

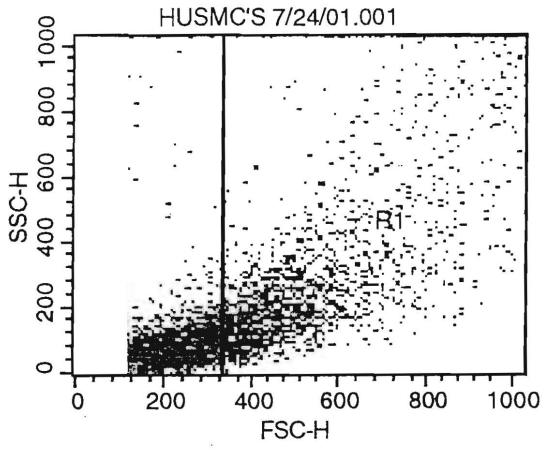
Histogram Statistics

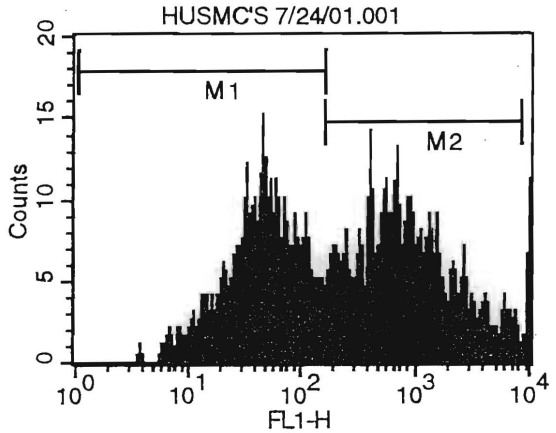
File: HUSMC'S 7/24/01.006
Patient ID: TAG=TUBE
Gate: No Gate
Total Events: 5000

Sample ID: HUMAN AORTIC SMC'S
Acquisition Date: 24-Jul-1
Gated Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	5000	100.00	100.00	429.38	578
M1	1, 289	3492	69.84	69.84	55.75	28
M2	289, 9910	1509	30.18	30.18	1293.91	578

HuAoSMC
50 uLs Lev

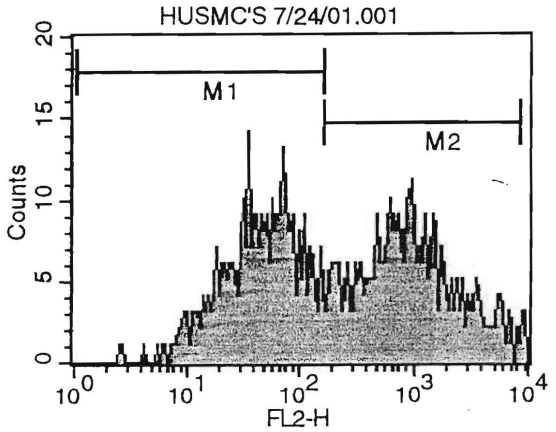




Histogram Statistics

File: HUSMC'S 7/24/01.001 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2494
Total Events: 4425

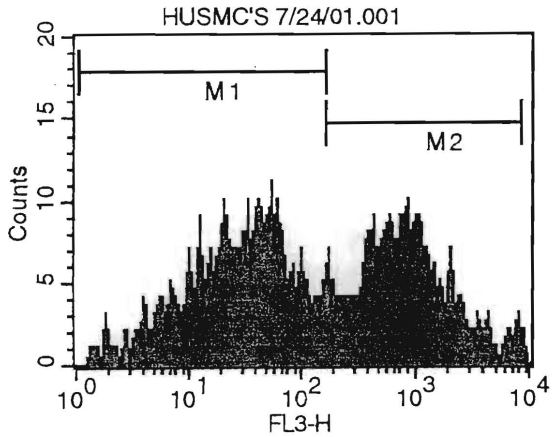
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2494	100.00	56.36	709.29	43
M1	1, 158	1212	48.60	27.39	55.80	43
M2	158, 8429	1263	50.64	28.54	1190.09	385



Histogram Statistics

File: HUSMC'S 7/24/01.001 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2494
Total Events: 4425

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2494	100.00	56.36	680.25	34
M1	1, 158	1228	49.24	27.75	55.72	34
M2	158, 8429	1262	50.60	28.52	1243.00	874

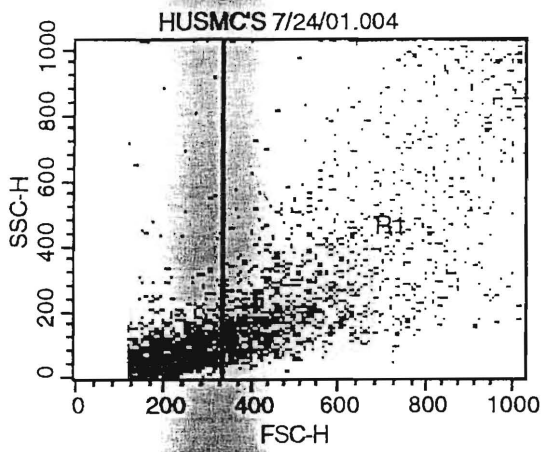


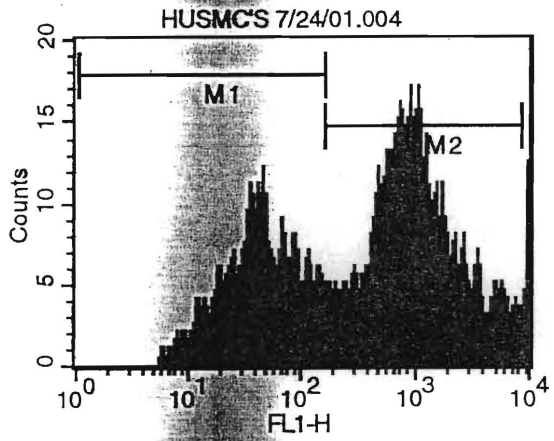
Histogram Statistics

File: HUSMC'S 7/24/01.001 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2494
Total Events: 4425

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2494	100.00	56.36	495.12	52
M1	1, 158	1356	54.37	30.64	41.29	52
M2	158, 8429	1139	45.67	25.74	1035.11	791

HuAoSMC
2 [redacted] uL Lev

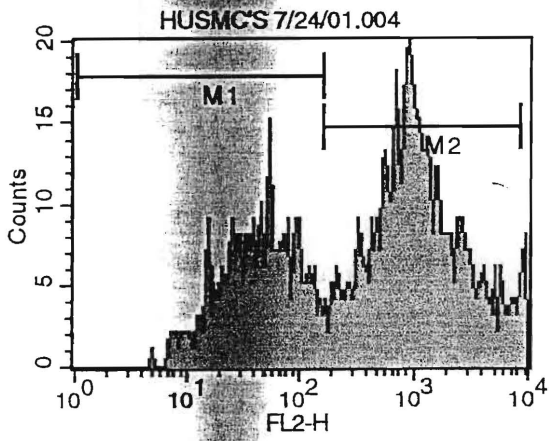




Histogram Statistics

File: HUSMC'S 7/24/01.004 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3294
Total Events: 5000

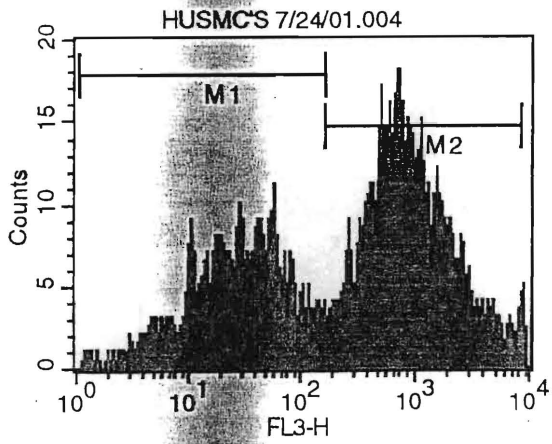
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3294	100.00	65.88	1079.18	9140
M1	1, 158	1087	33.00	21.74	54.24	43
M2	158, 8429	2160	65.57	43.20	1418.84	843



Histogram Statistics

File: HUSMC'S 7/24/01.004 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3294
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3294	100.00	65.88	1031.80	858
M1	1, 158	1094	33.21	21.88	53.94	50
M2	158, 8429	2192	66.55	43.84	1477.19	858



Histogram Statistics

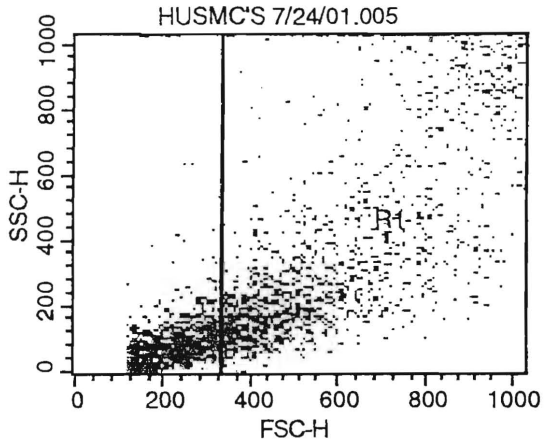
File: HUSMC'S 7/24/01.004 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 3294
Total Events: 5000

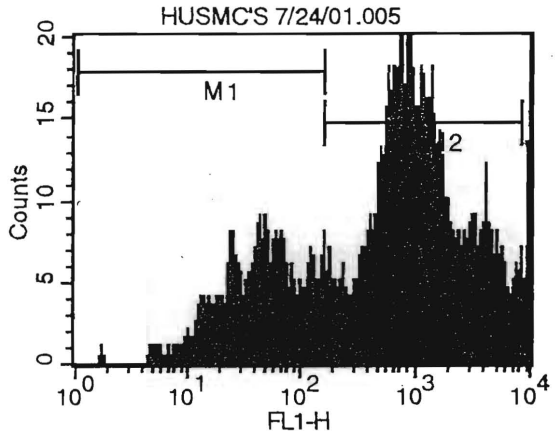
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3294	100.00	65.88	732.91	632
M1	1, 158	1206	36.61	24.12	39.99	55
M2	158, 8429	2090	63.45	41.80	1132.19	632

HuAoSMC

~~[illegible]~~

10 uL VR ; 10 uL Lev
(accidentally mixed w/ control)

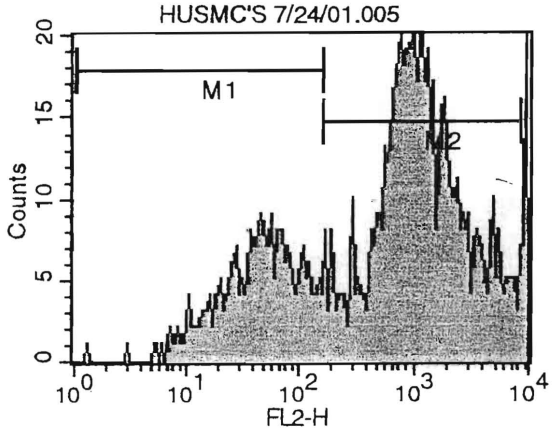




Histogram Statistics

File: HUSMC'S 7/24/01.005
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3739
Total Events: 5000

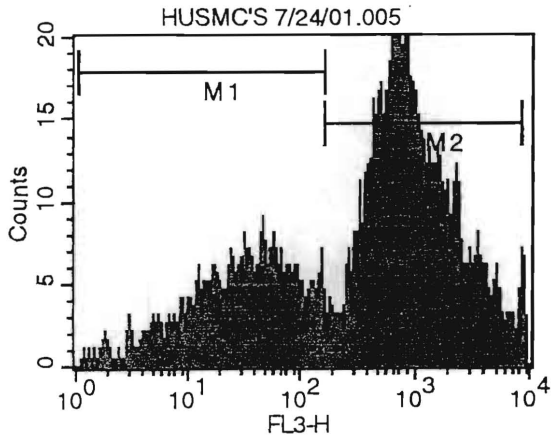
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3739	100.00	74.78	1365.73	9140
M1	1, 158	908	24.28	18.16	55.97	42
M2	158, 8429	2754	73.66	55.08	1580.77	710



Histogram Statistics

File: HUSMC'S 7/24/01.005
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3739
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3739	100.00	74.78	1307.94	1027
M1	1, 158	911	24.36	18.22	56.64	44
M2	158, 8429	2807	75.07	56.14	1651.17	1027



Histogram Statistics

File: HUSMC'S 7/24/01.005
Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE
Acquisition Date: 24-Jul-1
Gate: G1
Gated Events: 3739
Total Events: 5000

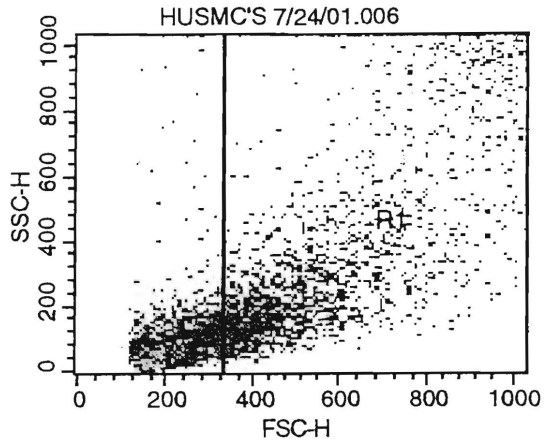
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	3739	100.00	74.78	935.87	661
M1	1, 158	1027	27.47	20.54	43.42	43
M2	158, 8429	2713	72.56	54.26	1273.42	661

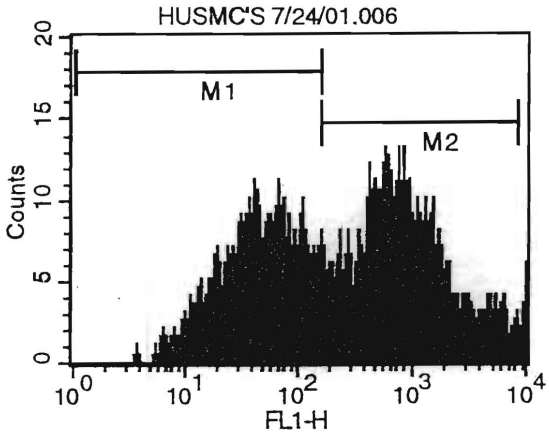
HuAoSMC

control

2 mL media

(accidentally mixed w/ 10 uL VR/10 uL Le

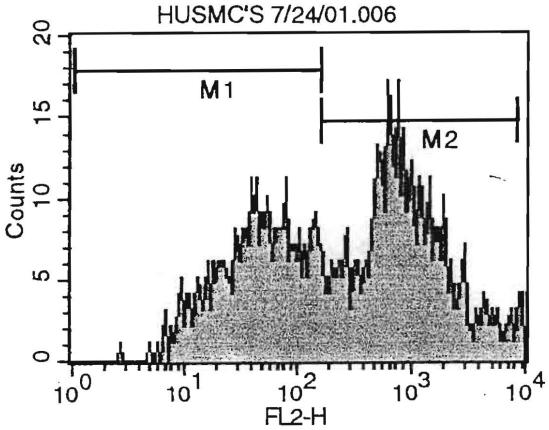




Histogram Statistics

File: HUSMC'S 7/24/01.006 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2975
Total Events: 5000

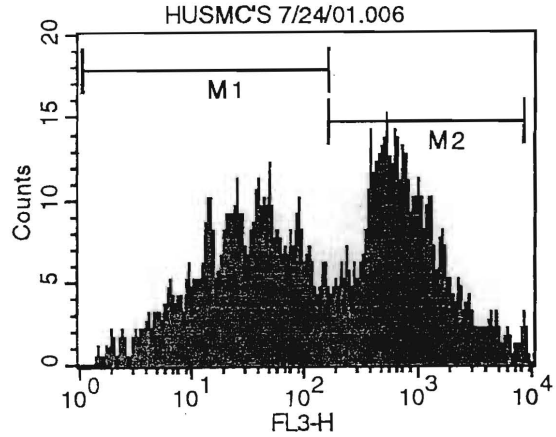
Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2975	100.00	59.50	701.59	542
M1	1, 158	1305	43.87	26.10	56.71	39
M2	158, 8429	1659	55.76	33.18	1127.21	542



Histogram Statistics

File: HUSMC'S 7/24/01.006 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2975
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2975	100.00	59.50	689.03	578
M1	1, 158	1313	44.13	26.26	57.19	36
M2	158, 8429	1664	55.93	33.28	1166.88	578

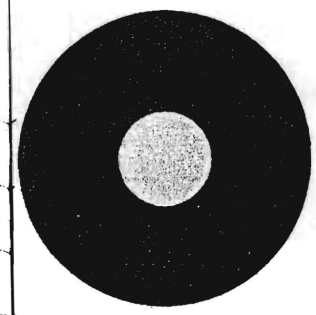


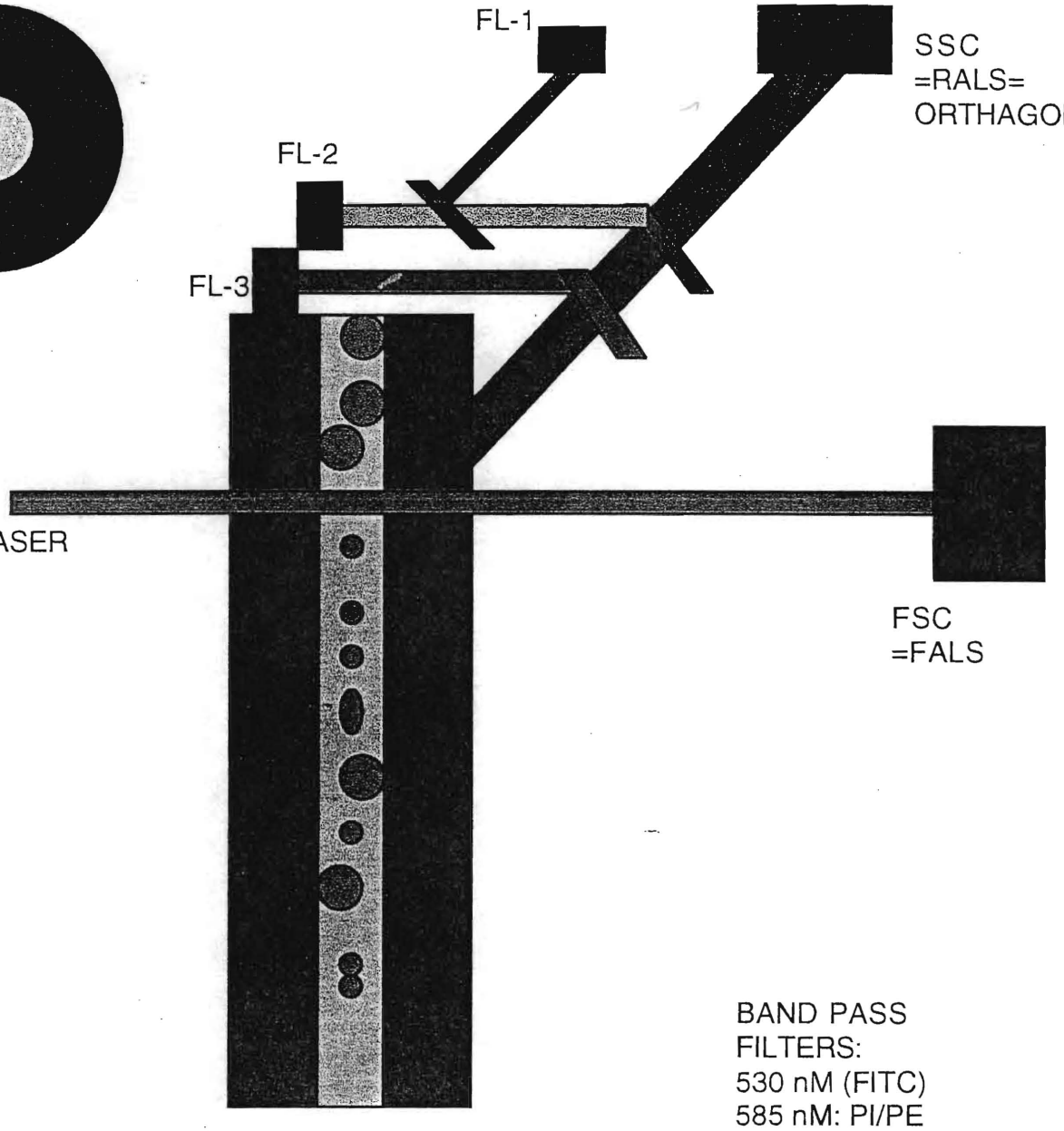
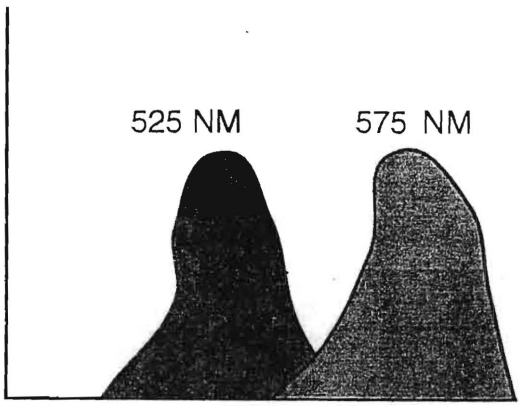
Histogram Statistics

File: HUSMC'S 7/24/01.006 Sample ID: HUMAN AORTIC SMC'S
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1
Gate: G1 Gated Events: 2975
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Peak Ch
All	1, 9910	2975	100.00	59.50	487.56	496
M1	1, 158	1474	49.55	29.48	43.73	47
M2	158, 8429	1503	50.52	30.06	922.40	496

3 Fluorescent wavelengths





BAND PASS
FILTERS:
530 nM (FITC)
585 nM: PI/PE
>650 nM: RED