

## COLLEGE SCHOLARS PROJECT APPROVAL

Frances Kirkland

Scholar

Robert L. Donnell, DVM, PhD (at Vet School)

Mentor

The Vascular Research Experience

12/12/2001

Project Title and Completion Date

---

### COMMITTEE MEMBERS' SIGNATURES

(Minimum 3 Required)

John P. Pacanowski, MD

Stacy Kirkpatrick Stacy Kirkpatrick

Robert L. Donnell, DVM, PhD Robert L. Donnell

---

PLEASE ATTACH A COPY OF THE SENIOR PROJECT TO THIS SHEET AND RETURN BOTH TO THE PROGRAM DIRECTOR. THIS PAGE SHOULD BE DATED AND COMPLETED ON THE DATE THAT YOUR DEFENSE IS HELD.

DATE COMPLETED December 12, 2001

# COLLEGE SCHOLARS PROJECT PROPOSAL APPROVAL

Frances Kirkland  
Scholar

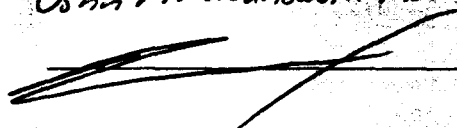
Robert L. Donnell, DVM, PhD (at Vet. School)  
Mentor

The Vascular Research Experience  
Proposed Project Title and Completion Date (semester & year)

## COMMITTEE MEMBERS' SIGNATURES

(Minimum 3 Required)

John P. Pacanowski M.D.

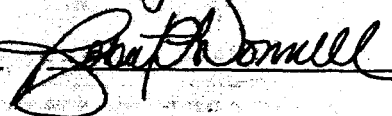


Stacy Kirkpatrick

(Lab Supervisor)

Stacy Kirkpatrick

Robert L. Donnell, DVM, PhD



PLEASE ATTACH A COPY OF THE WRITTEN PROPOSAL TO THIS SHEET, AND  
RETURN BOTH TO THE COLLEGE SCHOLARS OFFICE (1208 MCCLUNG TOWER).

DATE APPROVED 10/19/01

### 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 compromised.

### **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.

### **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 of 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<br>(ug/ul) | Protein Standard Solution<br>(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  $\mu$ l of each sample and 990  $\mu$ l 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 Reagent 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 cuvetts 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  $\mu$ g. 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  $\mu$ l 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 phosphatase 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<br>(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<br>(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, were 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<br>Levamisole                 | Amount Media<br>(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<br>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/cm<sup>2</sup> and extra-physiological stress anywhere above 60 dynes/cm<sup>2</sup>. 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 pulsatile 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 Immunohistochemistry 06/05/01
- 26-27 Product Specification Sheet 06/12/01
- 28 Notes on seeking A $\alpha$ SMC 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  $\frac{1}{3}$  Levamisol 07/22/01
- 38 HuASMC  $\rightarrow$  VR & Lev ams & Labels 07/23/01
- 39-40 Cell Cytometer  $\frac{1}{3}$  Flow 07/24/01
- 41-42 Vector Red  $\frac{1}{3}$  Levamisole Protocols 07/24/01
- 43 Vector Red emission  $\frac{1}{3}$  excitation  $\lambda$  07/27/01
- 44 Flow / FACS fluorescent  $\lambda$  07/27/01
- 45 HSP Project Flow system 07/27/01
- 46 Vector Red Fibr/SMC sorting  $\rightarrow$  tissues to test 07/27/01
- 47-48 VR Fibr/SMC sorting re-test set-up 07/27/01
- 49 To plate cells from specimen/tissues 08/10/01

SOP  
NumberVRL-SOP  
601University of Tennessee  
Medical Center  
Vascular Research Lab

Standard Operating Procedures

Page

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.

|                                     |                                                                                                         |                          |
|-------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------|
| SOP<br>Number<br><br>VRL-SOP<br>601 | University of Tennessee<br>Medical Center<br>Vascular Research Lab<br><br>Standard Operating Procedures | Page<br><br>Of<br>2 of 3 |
|-------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------|

- 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. (IMDA)  
(Get new T-75 flasks <sup>MARK</sup> type of cell, 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 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>SOP<br/>Number</b><br><br><b>VRL-SOP<br/>601</b> | <b>University of Tennessee<br/>Medical Center<br/>Vascular Research Lab</b><br><br><b>Standard Operating Procedures</b> | <b>Page</b><br><br><b>Of<br/>3 of 3</b> |
|-----------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|

- 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

|                                          |                                                                                                                  |                           |
|------------------------------------------|------------------------------------------------------------------------------------------------------------------|---------------------------|
| <p>SOP<br/>Number</p> <p>VRL-SOP-602</p> | <p>University of Tennessee<br/>Medical Center<br/>Vascular Research Lab</p> <p>Standard Operating Procedures</p> | <p>Page</p> <p>1 of 3</p> |
|------------------------------------------|------------------------------------------------------------------------------------------------------------------|---------------------------|

**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)
- e. DMSO (in hood in a foiled 15ml tube) *(a portion)*
- 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 and ~~spray with~~ *alcohol*.
- e. Place cells in hood and aspirate the media off the cells.

SOP  
Number  
  
VRL-SOP-602

University of Tennessee  
Medical Center  
Vascular Research Lab  
  
Standard Operating Procedures

Page  
  
2 of 3

- f. Place 5 ml of CMF on the cells and slide around flask to cover cells completely.
- g. Aspirate the CMF off the cells. (2) may put in incubator
- h. Place 5 ml of trypsin/EDTA on the cells. (1) may aspirate (when 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 micropipet and place 0.10 cc of DMSO into the vial, dripping it slowly over the cells. (100  $\mu$ L)
- 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

|               |                                                                    |                |
|---------------|--------------------------------------------------------------------|----------------|
| SOP<br>Number | University of Tennessee<br>Medical Center<br>Vascular Research Lab | Page<br>3 of 3 |
| VRL-SOP-602   | Standard Operating Procedures                                      |                |

4. **Trouble shooting**

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

No  
He  
Ex

anyth  
ather

(HSP) Heat

- Tryin  
a lo

- Expos  
b +

Norma  
Extra

Distribut  
Protein a

immunohis

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, 1<sup>st</sup> occurs on endothelial cells

atherosclerosis  $\rightarrow$  autoimmune injury

(HSP) Heat Shock Protein <sup>expression</sup> occurs from stress  $\rightarrow$  heat, fluid-shear stress, etc.

$\rightarrow$  possible auto-antigens

- Trying to find where stress is high & what are local conditions causing it  
a lot of fluid-shear stress  $\rightarrow$  at least initial up-regulation of HSP

- Expose cells (Ex vivo model  $\rightarrow$  dead, outside body, HSP 70)

$\rightarrow$  took carotid arteries of dogs & simultaneously exposed to different flow conditions  
put arteries in flow system

Normal physiological stress 15-30 dynes/cm<sup>2</sup> (1 vessel exposed)  
Extra-physiological stress  $\rightarrow$  anything above 60 (1 vessel exposed) <sup>compared</sup>

Blood flow, time constant for 4 hrs.

Distributive analysis  $\rightarrow$  (3 layers of vessel) [via staining] compares amt. in each layer  
<sup>intima, media, adventitia</sup> <sup>immunohistochemistry</sup>

Protein analysis  $\rightarrow$  quantification of amt. of protein in endothelial cells

immunohistochem.  $\rightarrow$  cut slides of graft, stain, etc.

(over)

cont. HSP project 5-21-01

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

static control - cells not exposed to anything

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

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

### Protein

Static w/ 0 time ← <sup>0 time</sup> immediately →

Static w/ time ← peric. dates <sup>for</sup> 4 hrs →

Flow

← 4 hr <sup>flow</sup> stress →

afterwards, frozen

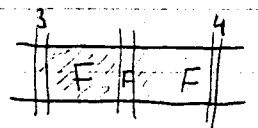
frozen

Carson's



2 sections each (from same dog)

Extra-physiological



Normal

### Distributive

Static w/ 0 time

Static w/ time

Flow

afterwards, Carson's

if had taken all out time  
vessel would be small

Statics would be same, so only 4 cuts total  
needed

### 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

## INSTRUCTIONS



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

# NE-PER™ Nuclear and Cytoplasmic Extraction Reagents

8333

0872w

### Product Description

| Number | Description                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|--------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 8333   | <b>NE-PER™ Nuclear and Cytoplasmic Extraction Reagents</b><br>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 $\mu$ l (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  $\mu$ l  
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             |
| Aprotinin                            | 2 mg/ml   | 2 $\mu$ g/ml            | N/A      | 2 $\mu$ g/ml          |
| Leupeptin                            | 2 mg/ml   | 2 $\mu$ g/ml            | N/A      | 2 $\mu$ g/ml          |
| PMSF (Phenylmethylsulfonyl Fluoride) | 0.2 M     | 0.75 mM<br>5 $\mu$ l/ml | N/A      | 2 mM<br>10 $\mu$ l/ml |

1:500

1:1000

1:1000

### Protocol

The following protocol reflects reagent volumes based on samples with a packed cell volume of approximately 20  $\mu$ l (~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 $\mu$ l (20 mg)   | 100 $\mu$ l | 5.5 $\mu$ l  | 50 $\mu$ l  |
| 20 $\mu$ l (40 mg)   | 200 $\mu$ l | 11 $\mu$ l   | 100 $\mu$ l |
| 50 $\mu$ l (100 mg)  | 500 $\mu$ l | 27.5 $\mu$ l | 250 $\mu$ l |
| 100 $\mu$ l (200 mg) | 1 ml        | 55 $\mu$ l   | 500 $\mu$ l |

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

Telephone: 800-8-PIERCE (800-874-3723) or 815-968-0747 • Fax: 815-968-7316 or 800-842-5007

Internet: <http://www.piercenet.com>

1. Isolate 200,000 x g for 5 minutes as PBS, and remove the supernatant. Add 1 ml of directly in 50 mg tissue.
2. Using a pipette, add 100  $\mu$ l of Vortex 5.
3. Add 200  $\mu$ l of Vortex 5.
4. Vortex 5 minutes.
5. Add 11  $\mu$ l of Vortex 5.
6. Vortex 5 minutes.
7. Vortex 5 minutes.
8. Immediate centrifugation.
9. Resuspend in 100  $\mu$ l of Vortex 5.
10. Vortex 5 minutes.
11. Centrifuge for 5 minutes.
12. Immediate centrifugation.
13. Store all fractions at -80°C.

### Troubleshooting

#### Observation

Low cytoplasmic

Low nuclear

Low concentration

No or low protein

Proteins not

No or low protein

fraction

### Addition

Number

23235

34080

©Pierce Chemical Company

Tel

281-820-7878  
24th 2808

**PIERCE**

1. Isolate 20  $\mu$ l packed cell volume (40 mg) of cells by centrifugation in a 1.5 ml microcentrifuge tube by centrifugation at 500  $\times$  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  $\times$  g for 2-3 minutes and remove the supernatant. Estimate the packed cell volume, add the appropriate amount of CER I according to 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  $\mu$ l CER I to 50 mg tissue). In Step 5 of the protocol, use 5.5  $\mu$ l of CER II per 100  $\mu$ l of CER I.
2. Using a pipet, carefully remove and discard the supernatant, leaving cell pellet as dry as possible.
3. Add 200  $\mu$ l 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  $\mu$ l 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  $\times$  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  $\mu$ l 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  $\times$  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                                                            |

### Additional Pierce Products of Interest

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

©Pierce Chemical Co. 10/1999. Printed in the U.S.A.

Telephone: 800-8-PIERCE (800-874-3723) or 815-968-0747 • Fax: 815-968-7316 or 800-842-5007

Internet: <http://www.piercenet.com>

K9X4 5/22/01

## Protein Isolation notes

| static 0                                                          | static 4hr                                         | normal 4hr | extraphysiological 4hr |
|-------------------------------------------------------------------|----------------------------------------------------|------------|------------------------|
| 7mm                                                               | 10mm                                               | 17mm       | 12mm                   |
| used approx. $\frac{1}{2}$ <del>etc</del><br>for protein analysis | used approx. $\frac{1}{2}$ etc<br>protein analysis |            |                        |

## What's Going On:

SER I

Putting hypotonic solution on cells (cells swell)

Add in SER II  $\rightarrow$  detergent  $\rightarrow$  pokes holes in cell  $\rightarrow$  proteins come out, ER, etc. (all but nucleus)nucleus doesn't come out  $\rightarrow$  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  $\mu$ L ~~CER 1~~ CER 1 (100  $\mu$ L for each vial + extra for easy calc. + back-up)
- ⑱ Addition of protein inhibitors to CER 1  $\rightarrow$  1 mL Benzamide,  $\frac{1}{2}$  mL Aprotinin,  
 $\frac{1}{2}$  mL Leupeptin,  $\frac{2.5 \mu$ L ~~PMSE~~ PMSE

- (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 ~~100~~  $\frac{1}{2}$  mL of PBS and transferring to smaller vials.
  - (22) Centrifuging <sup>vials</sup> 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) ~~Resuspend~~ <sup>Add</sup> CER I (100  $\mu$ L to each pellet) ~~and~~ ~~resuspend~~
  - (25) Re-suspend via Vortex (highest setting 15 sec)
  - (26) Incubate in ICE for 10 minutes
  - (27) 5.5  $\mu$ L 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  $\rightarrow$  extract supernatant & store. (do quickly)
    - $\hookrightarrow$  be careful not to get too close to pellet (really don't need that much protein anyway)
- Not extracting <sup>Nuclear proteins</sup> NER 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. Incubated for 10 minutes on ice.
12. Added 5.5 uL of CER II to each tube.
13. Vortexed for 5 sec, incubated for 1 minute, vortexed again for 5 secs
14. Centrifuged for 5 minutes at 16000Gs
15. Labeled and put four 0.5 ml microfuge tubes into ice.
16. Transferred the supernatant 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

MAY 15 1996 09:43AM SIGMA DIAG

Protein Quantitation of lysate

Chemical cabinet

**SIGMA**  
**AGNOSTICS®**

## PROTEIN ASSAY KIT

(Procedure No. P 5656)

not sterile

### INTRODUCTION

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

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

### PRINCIPLE

Cupric tartrate reagent complexes with the peptide and forms a purple-blue color when the phenol reagent is added. Absorbance is read at a suitable wavelength between 540 and 800 nm. The protein concentration is determined from a calibration curve.

### REAGENTS

(For laboratory use only.)

Not for drug, household or other uses.)

LOWRY REAGENT, MODIFIED, Catalog No. L 1013

CAUTION: CORROSIVE. Avoid contact with eyes, skin or clothing. Do not breathe dust.

LOWRY REAGENT SOLUTION is prepared by adding 40 ml water to a bottle of Lowry Reagent, Modified. Mix well and allow to completely dissolve. Do not shake so as to cause foaming. Solution is stable at room temperature. STORE IN REFRIGERATE.

DOC SOLUTION, Catalog No. D 5525

DOC solution of sodium deoxycholate, 1.5 mg/ml.

TCA SOLUTION (TCA),

Catalog No. T 4396

DOC solution of trichloroacetic acid, 72% w/v.

CAUTION: CORROSIVE. CAUSES BURNS. Avoid contact with skin and clothing.

CIOCALTEU'S PHENOL REAGENT, Catalog No. F 9252

CAUTION: CORROSIVE. CAUSES BURNS. Avoid contact with skin and 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.
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 cuvettes 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

| <u>Standard</u> |                               |                    |                    |
|-----------------|-------------------------------|--------------------|--------------------|
|                 | 400 $\mu\text{g}/\mu\text{l}$ | Standard           | H <sub>2</sub> O   |
| 1               | 0 $\mu\text{g}/\mu\text{l}$   | 0                  | 1000 $\mu\text{l}$ |
| 2               | 5                             | 125 $\mu\text{l}$  | 875                |
| 3               | 10                            | 250 $\mu\text{l}$  | 750                |
| 4               | 25                            | 625 $\mu\text{l}$  | 375                |
| 5               | 50                            | 1250 $\mu\text{l}$ | 750                |
| 6               | 100                           | 2500 $\mu\text{l}$ | 750                |
| 7               | 200                           | 5000 $\mu\text{l}$ | 500                |

Samples

10  $\mu\text{l}$  sample - 990  $\mu\text{l}$  H<sub>2</sub>O

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/ 57 & put water concentration in (7<sup>th</sup> has lowest  $H_2O$  concentration) from 6 to 1.
- ③ For samples, put 900  $\mu L$   $H_2O$  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 K9X3 Static 0)

54  $\rightarrow$  static 4hr

NO  $\rightarrow$  normal

EP  $\rightarrow$  extrapolating to logical

- ⑤ Labeled vials matched to red 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<sup>st</sup> 3 standards

1 bottle for 4-7 standards  
(did not mix bottles beforehand)  
may effect 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  $\mu L$  of <sup>extracted</sup> protein according to table

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

- ⑧ Vortex each tube @ min. speed

- ⑨ Let sit for 20 min.

- ⑩ Add 1.5 mL Polin  $\rightarrow$  quick & immediate mixing  $\rightarrow$  add while vortexing

- ⑪ Wait 30 min., Turn on spectrophotometer NOW

- ⑫ spec.  $\rightarrow$  Choose quantitative, push enter, change parameters (7 standards, print), enter concentrations

- ⑬ Add 1 mL  $H_2O$  to <sup>each</sup> ~~each~~ cuvet

- ⑭ Press auto-zero

(cont.)

5/23/01

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

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

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

- want 30  $\mu\text{g}$  mass

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

# \*\*\* QUANTITATIVE ANALYSIS \*\*\*

| W.  | ABS.  | CONC.  |                      |
|-----|-------|--------|----------------------|
| 1   | 0.147 | 12.832 | 24.93 $\mu\text{L}$  |
| ✓ 2 | 0.118 | 5.5758 | 53.80 $\mu\text{L}$  |
| ✓ 3 | 0.126 | 7.2975 | 41.109 $\mu\text{L}$ |
| ✓ 4 | 0.104 | 2.3483 | 127.75               |
| ✓ 5 | 0.118 | 3.7478 | 80.06                |
| ✓ 6 | 0.122 | 6.4368 | 46.261               |
| 7   | 0.278 | 38.929 | 7.706                |
| 8   | 0.215 | 26.879 | 11.16                |

(cont.)  
each time

(14) Change front cuvet & add 1 mL<sup>^</sup> going thru stat

(15) Print results Graph of standard, table of

- get concentration (mass / volume)

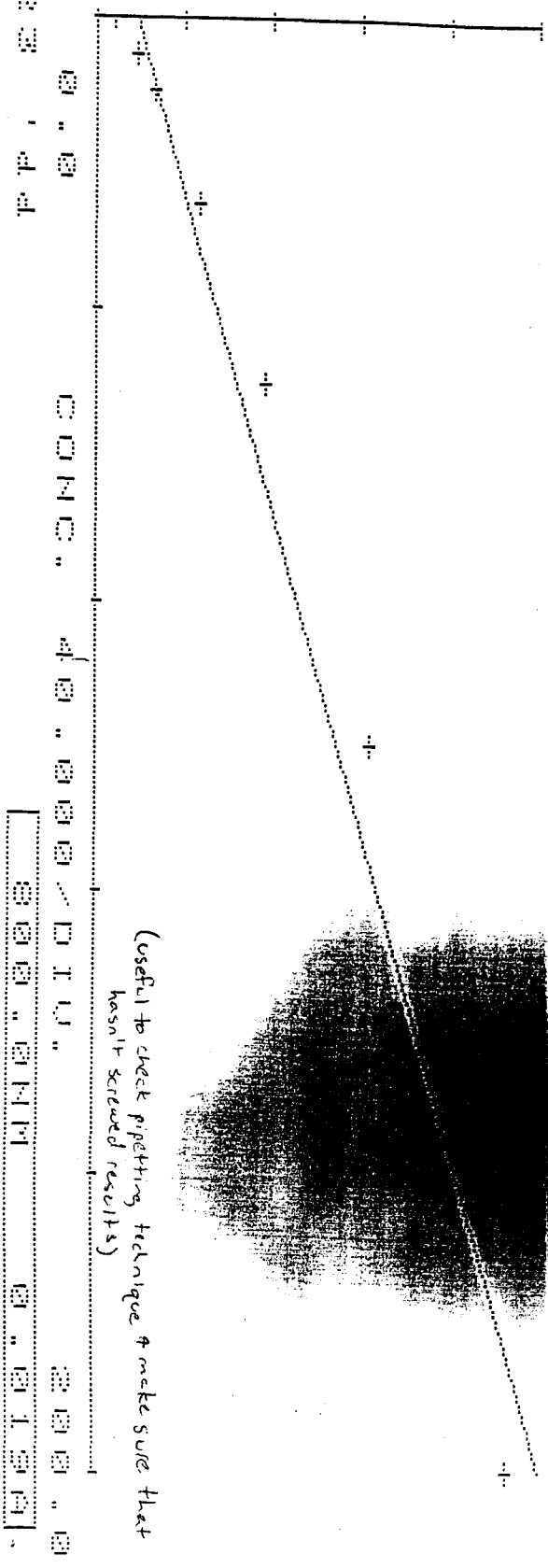
- want 30 ug mass

So divide mass by concentration (m

QUANTITATIVE ANALYSIS

| No. | ABS.  | CONC.  |           |
|-----|-------|--------|-----------|
| 1   | 0.147 | 12.032 | 24.93 uL  |
| ✓ 2 | 0.118 | 5.5768 | 53.80 uL  |
| ✓ 3 | 0.126 | 7.2975 | 46.109 uL |
| ✓ 4 | 0.104 | 2.3483 | 127.75    |
| ✓ 5 | 0.110 | 3.7470 | 80.06     |
| ✓ 6 | 0.122 | 6.4368 | 46.861    |
| 7   | 0.270 | 38.929 | 7.706     |
| 8   | 0.015 | 0.078  |           |

(useful to check pipetting technique & make sure that hasn't skewed results)



(6)

from 6 to 1

d (1-7)

ITS 3 standards  
vols  
(and)  
ough stuff.  
v bottle may  
d have to  
ter)

e. Prations

5/23/01

(16) 2, 3, 5, & 6 will have to re-concentrate to get small enough amt for gel.

Re-concentration

Use ~~can~~ microconcentrators (have filters)

rub frozen vials around fingers to thaw

Added all (90  $\mu$ L) of each to microconcentrators

Spin down

Label

↓

(4)

(9)

(10)

(11)

(12)

S.

Re

ca

L.

S.

V.

T.

(13)

(14)

(15)

05/24/01 protein extraction

labeled rna 4

05/24/01

Notes 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

1 mL of CER I made

Supernatant discarded

Vortex (15s)

Incubate in ice (10 min)

5/24/01

(13) H10S

(14) H14NO

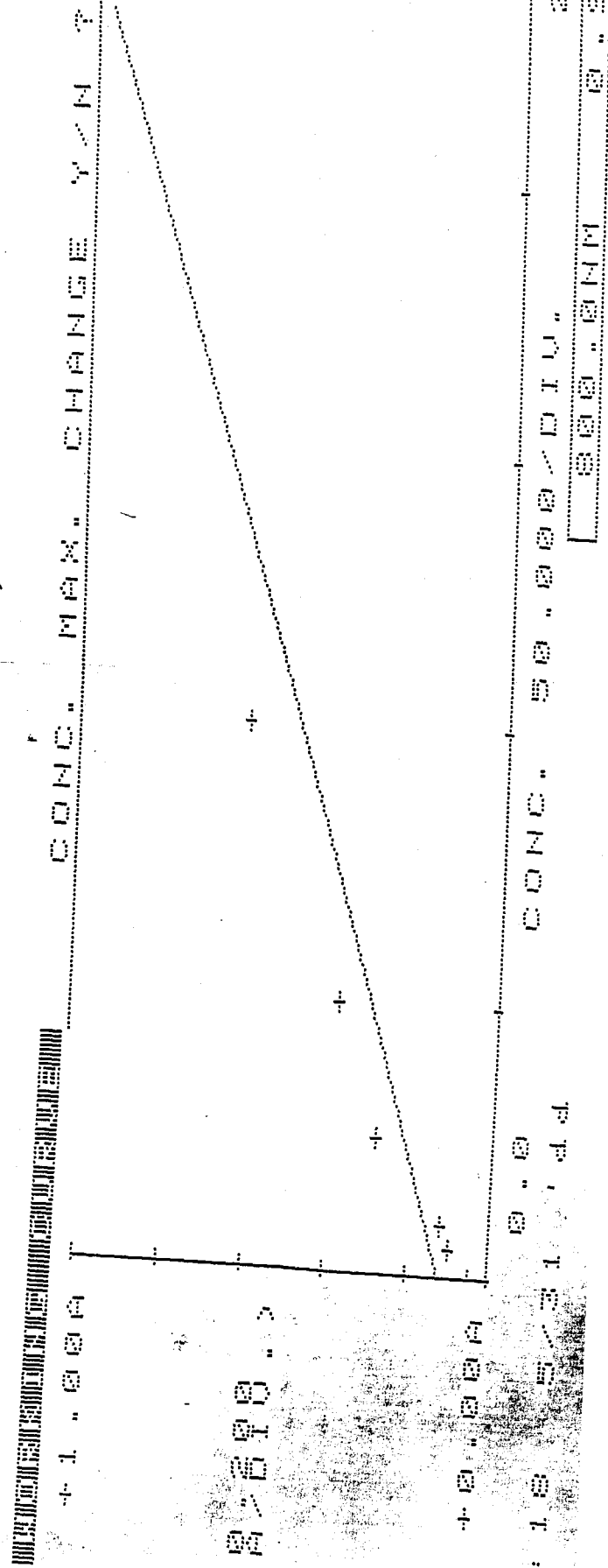
(15) H14EP

5/31/01 Protein Assay

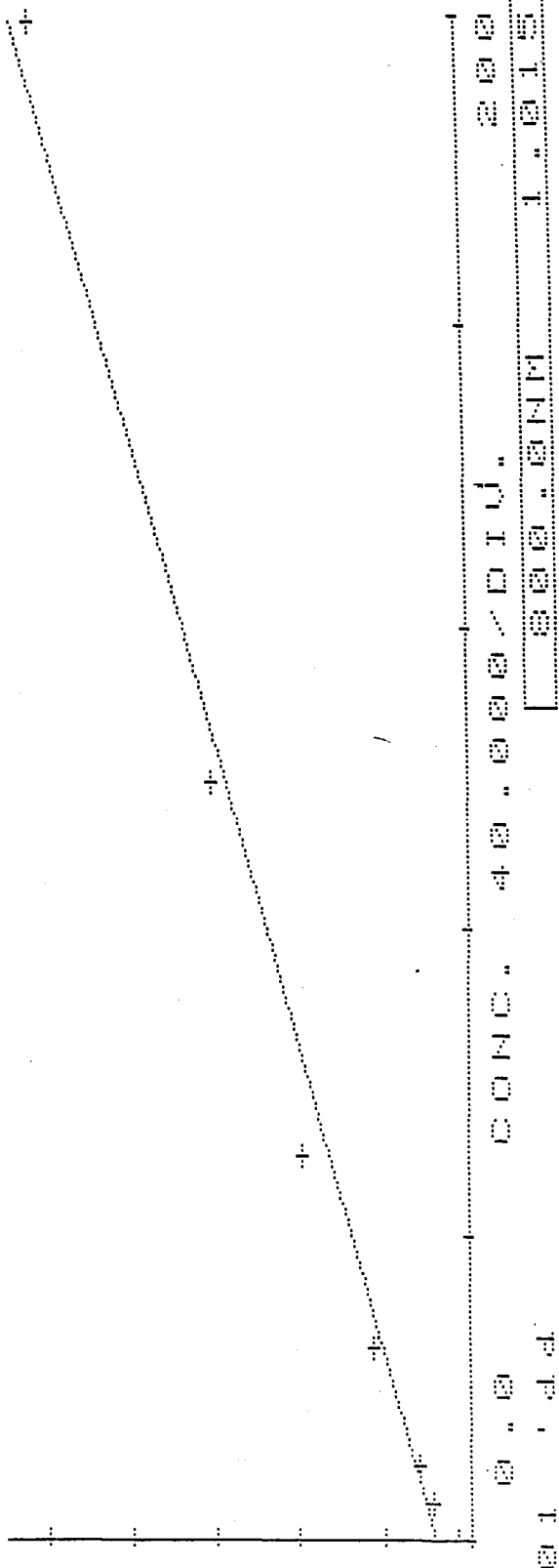
QUANTITATIVE ANALYSIS

| No. | ABS.  | CONC.   |
|-----|-------|---------|
| 1   | 0.101 | -4.7948 |
| 2   | 0.095 | -6.3286 |
| 3   | 0.117 | -0.5768 |
| 4   | 0.239 | 31.505  |
| 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 | -4.0279 |
| 14  | 0.133 | 3.7690  |
| 15  | 0.143 | 6.3253  |

Protein Assay (5/31/01)



C = KKKKSS + B K = 204.47 B = 16.371

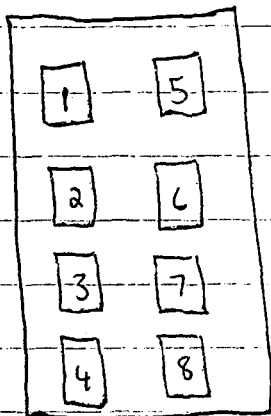


# QUANTITATIVE ANALYSIS

| No. | ABS.  | $\left( \frac{30 \text{ or } 20 \mu\text{g}}{\text{CONC.} \times 100} \right) \times 10^3$ |        |       |
|-----|-------|--------------------------------------------------------------------------------------------|--------|-------|
| 1   | 0.107 | 20.49                                                                                      | 4.6958 | 63.89 |
| 3   | 0.112 | 29.48                                                                                      | 5.6926 | 44.83 |
| 4   | 0.246 | 5.89                                                                                       | 33.949 | 9.84  |
| 6   | 0.106 | 36.40                                                                                      | 5.4946 | 54.60 |
| 7   | 0.199 | 8.28                                                                                       | 24.165 | 12.41 |
| 8   | 0.168 | 11.00                                                                                      | 18.174 | 16.51 |
| 9   | 0.129 | 19.63                                                                                      | 18.187 | 29.45 |
| 10  | 0.149 | 14.00                                                                                      | 14.281 | 21.01 |
| 11  | 0.127 | 20.64                                                                                      | 9.6879 | 30.97 |
| 12  | 0.138 | 16.83                                                                                      | 11.884 | 25.24 |
| 13  | 0.098 | 70.92                                                                                      | 2.1998 | 36.38 |
| 14  | 0.155 | 12.92                                                                                      | 15.479 | 19.38 |
| 15  | 0.132 | 18.72                                                                                      | 10.686 | 28.07 |

## Immunohistochemistry

06-05-01

Protocol

(1-4 wells)

wash PBS (15 min)

④ add protein block (10 min)

⑤ add 1° HSP Ab 1hr

add 1:90 to well 1

1:180 to well 2

1:360 to well 3

(-) control well 4

⑥ wash PBS 5 min

⑦ Anti mouse 2° Ab (Link)  
30 min.

(5-8 wells)

① Wash PBS 5 min

② add protease antigen retrieval 10 min

③ add PBS wash 5 min.

④ add protein block (10 min)

⑤ add 1° HSP Ab for 1hr

1:90 to well 5

1:180 to well 6

1:360 to well 7

(-) control well 8

⑥ wash PBS 5 min

⑦ Anti mouse 2° Ab Link 30 min.

06-12-01

Aniket

Page 1 of 2

## 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.<sup>1</sup> Other domains of fibronectin are also involved with this adhesion process and may involve interaction with collagen, heparin and other cell surface glycosaminoglycans.<sup>1</sup> The conformation and orientation of adsorbed fibronectin is also important and has an effect on cell spreading and strength of adhesion of endothelial cells.<sup>2</sup> Fibronectin addition to serum free medium promotes cell adhesion.<sup>3</sup> More significant effects are observed with BHK, CHO and other cell lines by coating of cultureware with fibronectin at 1-5 ug/cm<sup>2</sup>. 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.<sup>4,5</sup>

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 (HBsAg) 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 &amp; PHYSICAL FORM: 1 milligram per vial, lyophilized.

FORMULATION: 100 mM CAPS, 0.15M NaCl, 1 mM calcium chloride, pH 11.0.

RECONSTITUTION &amp; 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<sup>2</sup> 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

(cont.) 06-12-01

Page 2 of 2

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  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  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  $\mu\text{g}/\text{cm}^2$ , dilute the material to 50  $\mu\text{g}/\text{ml}$  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  $\text{Ca}^{++}$  and/or  $\text{Mg}^{++}$  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  $\text{dH}_2\text{O}$  - 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.

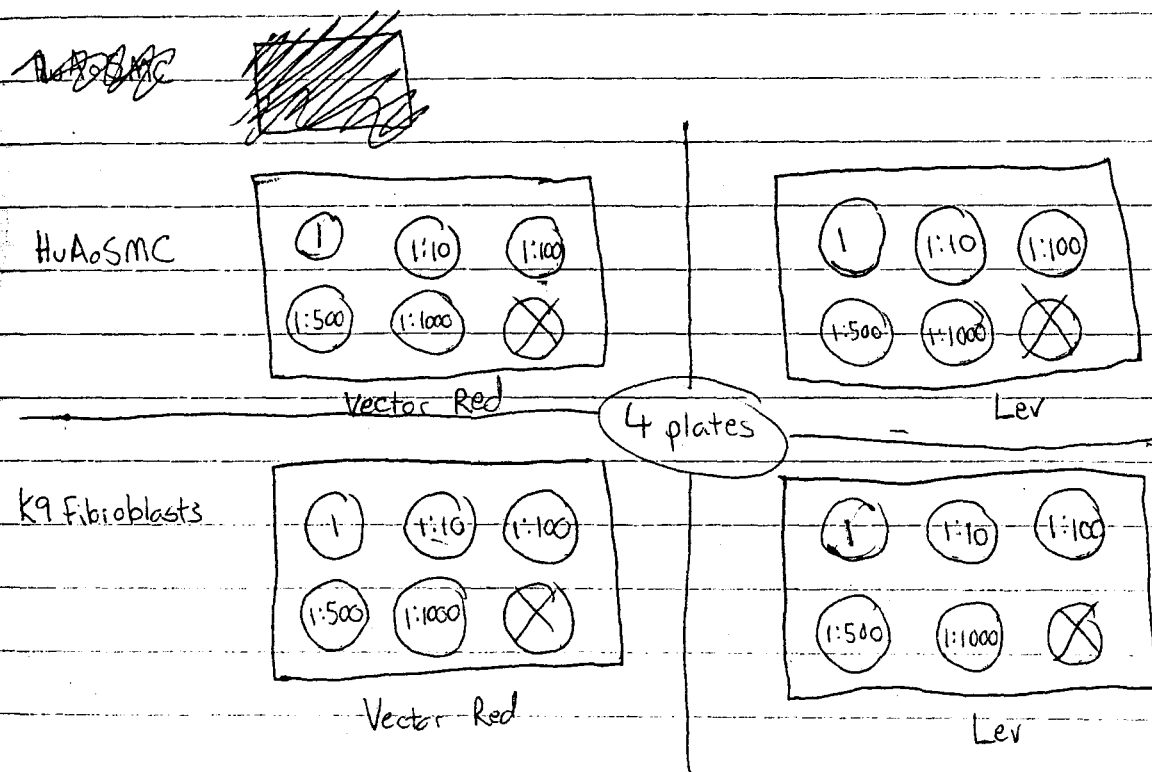
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

## 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

H&E staining of tissue slides  
after cut rabbit kidney (paraffin embedded)

06-15-01

|               |                                                                    |        |
|---------------|--------------------------------------------------------------------|--------|
| SOP<br>Number | University of Tennessee<br>Medical Center<br>Vascular Research Lab | Page   |
| VRL-SOP.307   | Standard Operating Procedures                                      | 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**

- 1% acid alcohol
- Ammonia water
- Working eosin solution
- Hematoxylin stain
- 100% ethanol
- 95% ethanol
- Xylene (or equivalent)

**2. Equipment required**

- Solution containers (staining boats).
- Multi-well slide staining station.
- Exhaust fume hood.

**3. Protocol Guidelines**

- Deparaffinize and hydrate tissue to deionized H<sub>2</sub>O (VRL.SOP.)
- 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)
- Rinse in tap water until glass is clear.
- Differentiate by dipping 5-10 times in 1% acid alcohol.
- Place in ammonia water to blue. (<10 sec)
- Rinse in warm running water for 30 sec.
- Place in 80 % Alcohol for 1 min.
- Place in 1 % working eosin for 30 sec.
- Serially place in 3 changes of 95 % Alcohol for 30 sec each change.
- Place in absolute alcohol for 1 min.

under Stacy's supervision on rabbit kidney

(cont)

06-15-01

|                                          |                                                                                                                         |                           |
|------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|---------------------------|
| <b>SOP<br/>Number</b><br><br>VRL-SOP.307 | <b>University of Tennessee<br/>Medical Center<br/>Vascular Research Lab</b><br><br><b>Standard Operating Procedures</b> | <b>Page</b><br><br>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 H<sub>2</sub>O
  - 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<sup>R</sup> 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

(cont) 00-15-01

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

## IV. References

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

## V. Attachments

NA.

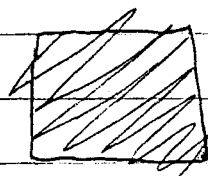
VRL.SOP.307 2000 / RLD

Vector Red for sorting project

06-20-01

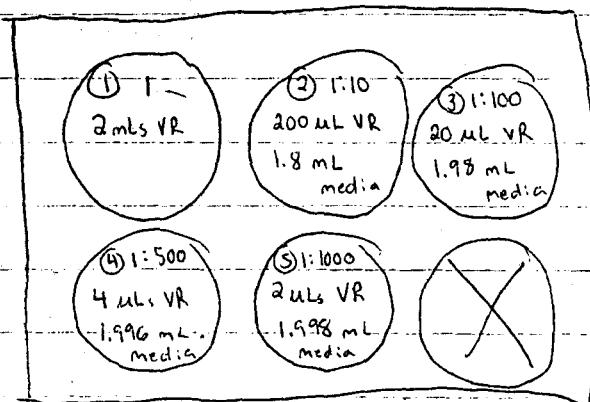
checking for toxicity &amp; flow

Fibroblasts in Vector Red



A

Make 3 mLs of Vector Red

Well ① → ~~2~~<sup>1.8</sup> mLs Vector Red1:10 ② → 200  $\mu$ Ls VR + 1.8 mL media1:100 ③ → 20  $\mu$ Ls VR + 1.98 mL media1:500 ④ → 4  $\mu$ Ls VR + 1.996 mL media1:1000 ⑤ → 2  $\mu$ Ls VR + 1.998 mL media

B

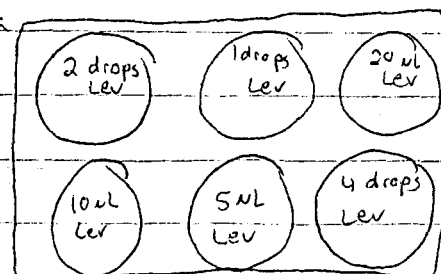
Levamisole 1 drop = 1 mL

How many  $\mu$ Ls  
is one drop?38  $\mu$ Lsforget old  
ratios for  
Lev

1:1 ① → 2 drops Lev + 2 mLs media

~~1:10~~ ② → 1 drop Lev + 2 mLs③ → 20  $\mu$ Ls Lev + 2 mLs④ → 10  $\mu$ Ls Lev + 2 mLs⑤ → 5  $\mu$ Ls Lev + 2 mLs

⑥ → 4 drops Lev + 2 mLs



Jun 21, 01

Vector Red + Levamisol on fibroblasts

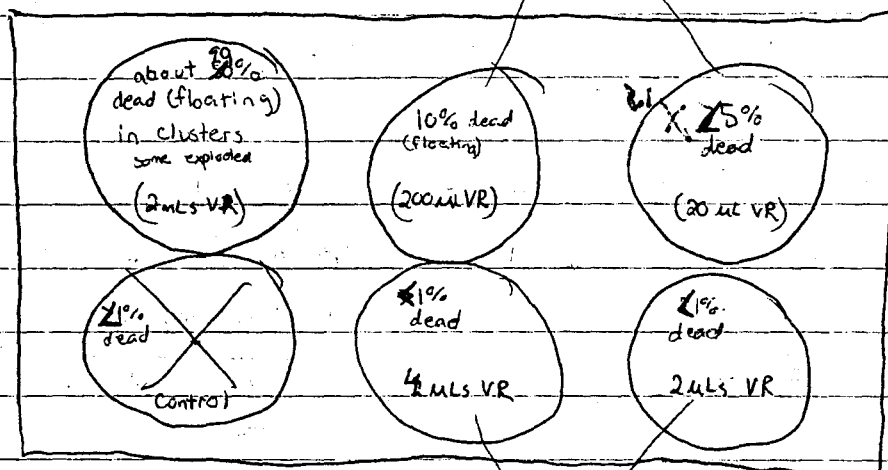
After 16 hrs. (8:30 a.m. Jun 21)

under microscope

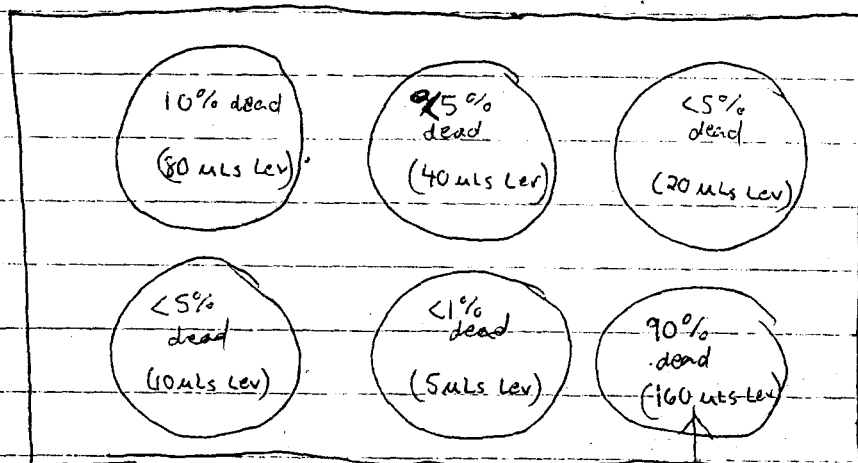
A Vector Red (fibro)

send to flow  
→

+ B#5

Lw/ least amt  
Lev b/ccontrol had  
so few cells  
to begin with)

B Levamisol

had most Lev  
in it. (about 160 uLs)

June 21, 01

separately 4 at a time  
 trypsinize each well (1 mL trypsin)  
 spin-down  
 re-suspend in 1 mL of media

Cell Counting w/ Hemocytometer

|               |                                                                    |        |
|---------------|--------------------------------------------------------------------|--------|
| SOP<br>Number | University of Tennessee<br>Medical Center<br>Vascular Research Lab | Page   |
| VRL-SOP-607   | Standard Operating Procedures                                      | 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:  $\text{cells/ml} = (n) \times 10^4$ ,  
 where n = the average cell count per square of the four corner squares counted. For example:  $\text{cells/ml} = (n) \times 10^4$  or  $\text{cells/ml} = 30 \times 10,000 = 300,000 \text{ cells/ml}$ .
    - 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/ Hemacytometer

| SOP<br>Number | University of Tennessee<br>Medical Center<br>Vascular Research Lab | Page   |
|---------------|--------------------------------------------------------------------|--------|
| VRL-SOP-607   | Standard Operating Procedures                                      | 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

VRL.SOP.607 / 2000 / AS

10,520

9308

Sandus  
Barton

VR - Fluorescent -  
SS  
FS

VR treated  
VR control  
#5 Chlorisol

} FACS

Making Vector Red

7-22-01

need ~~275~~  $\mu$ Ls  
285

100 mM Tris - HCL

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

~~(100)~~

10 mL 1M + 90 PBS

acrodiges

syringe filter @ sterilized (using .02 micron filter)

Making Levamisol2 drops of Levamisol per 2.5 mL of <sup>100 mM</sup> Tris-HCL80  $\mu$ Ls

## Smooth Muscle Cells

7-23-01

16 hrs.

Tagging Markers

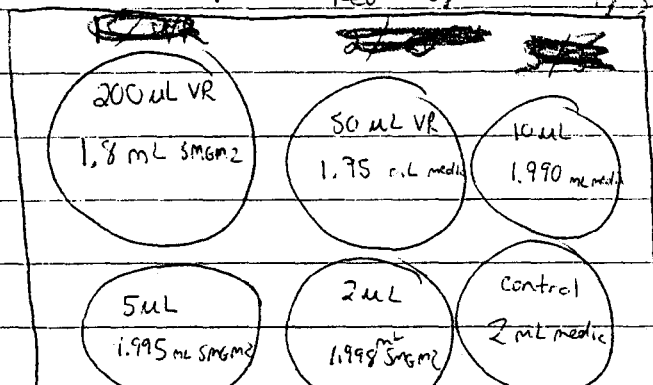
7/1VR

Vector

Red 8/2VR

9/3VR

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



FACS

Trypan blue

10/4 VR

11/5 VR

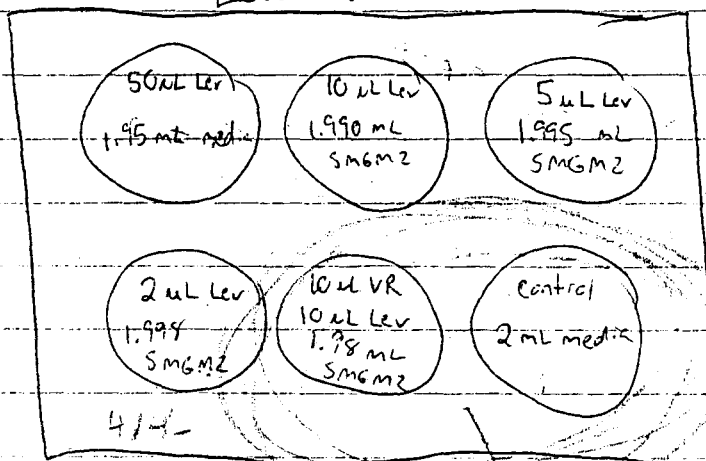
12/6 VR

1/1L

2/2L

3/3L

Levamisole



PI

FACS

Trypan Blue

4/4L

5/5L

12/12L

mixed  
accidentally

Cell Cytometer  
 ↑  
 Trypan Blue  $\frac{1}{3}$  Flow

7-24-01

~~Trypsinized~~

~~Re-suspend in media~~

~~aspirate~~

~~re-suspend in paraformaldehyde~~

Trypsinize

Dilute in media  $\rightarrow$  SMGM2

Take sample for trypan blue - 25  $\mu$ L sample

spin, fix  
 ↓ 1500 rpm      ↓ para formaldehyde

Aspirate media, wash w/ CMF, <sup>↑ aspirate</sup> strip w/ 1 mL trypsin, dilute in SMGM2

- take sample for trypan blue 25  $\mu$ L  $\rightarrow$  Results
- Spin remaining in centrifuge 1500 rpm for 5 min.
- Aspirate
- Bring up pellet in 1 mL <sup>para</sup> formaldehyde
- ~~Q22~~ Transfer to flow tubes
- Take to flow

FACS

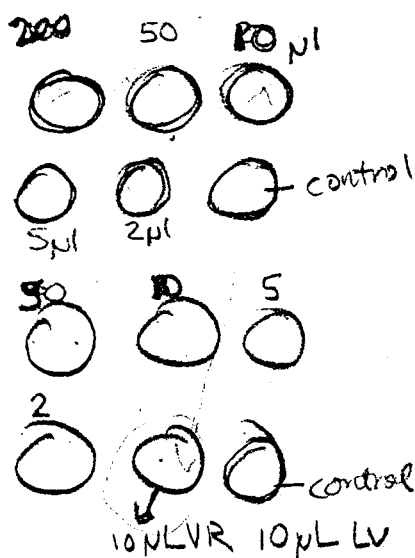
Fluorescent Activated Cell Sorter/Scanner

7-24-01

## Cell Cytometer Results (Trypan Blue)

| Cell # | Concentration  | Viable | Non-viable |                 |
|--------|----------------|--------|------------|-----------------|
| VR 1   | 200 $\mu$ L VR | 0      | 3          | $\times 10,000$ |
| 2      | 50             | 2      | 0          |                 |
| 3      | 10             | 1      | 0          |                 |
| 4      | 5              | 1      | 3          |                 |
| 5      | 2              | 3      | 1          |                 |
| 6      | control        | 2      | 2          |                 |

|       |                               |   |   |
|-------|-------------------------------|---|---|
| Lev 1 | 50 $\mu$ L Lev                | 1 | 1 |
| 2     | 10                            | 2 | 3 |
| 3     | 5                             | 1 | 0 |
| 4     | 2                             | 1 | 2 |
| 5     | 10 $\mu$ L VR, 10 $\mu$ L Lev | 0 | 3 |
| 6     | control                       | 1 | 0 |



VR  
FACS  
Trypan

Lev  
PI FACS  
Trypan



## 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 volume 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 situ.

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 different 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.



## 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.

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

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.

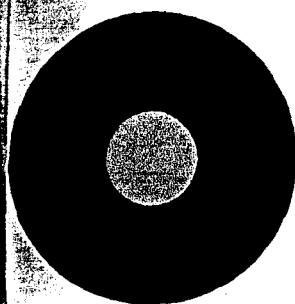
Sincerely,

Technical Service  
Vector Labs.

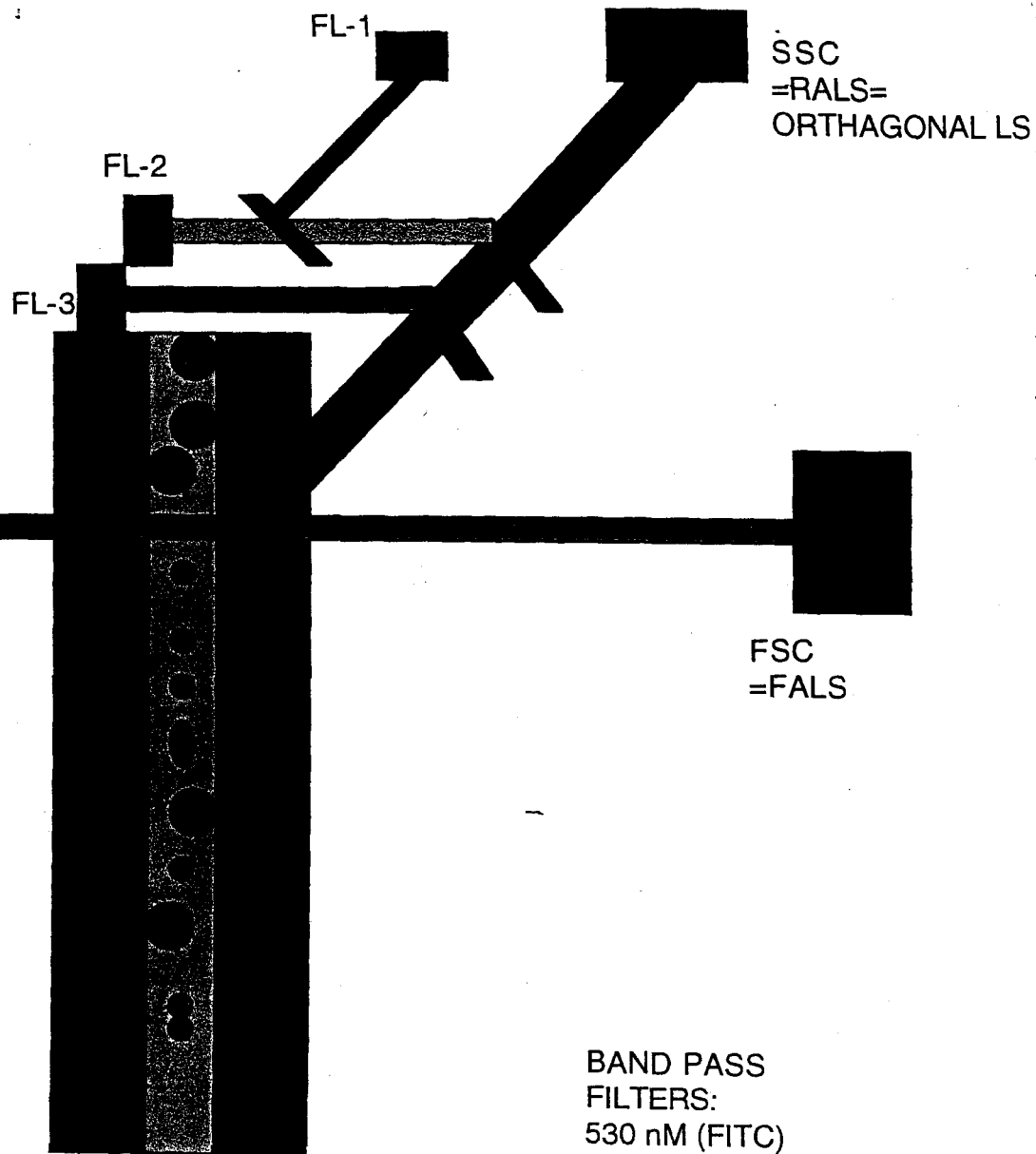
----- Original Message -----

**From:** "Donnell, Robert L." <RDonnell@mc.utmck.edu>  
**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  
> rdonnell@mc.utmck.edu  
> (865) 544-9774



488 NM  
ARGON LASER

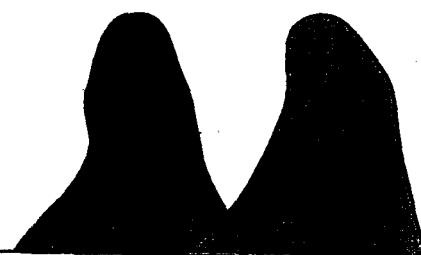


BAND PASS  
FILTERS:  
530 nM (FITC)  
585 nM: PI/PE  
>650 nM: RED



525 NM

575 NM



HSP Project → Flow System

07/27/01

2000 mL Erlenmeyer flask

gas permeable tubing

2000 mL media

pump → pulsatile 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°

6/27/01

Want from ATLS K9

SMC  
fibroblasts

> from Aorta  
Carotid  
Iliac

} endothelial

↓  
may be mixed or  
sorted.

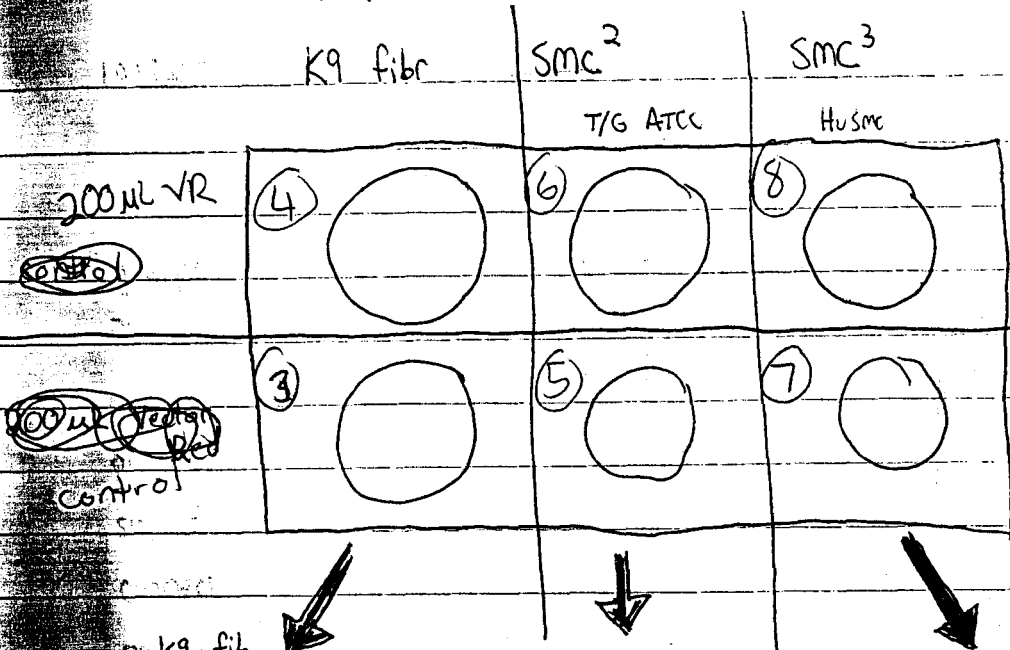
↓  
Try FACS/Flow test using Vector Red

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

possibly coming from contaminated flasks  
↓

possibly not SMC may be fibroblasts  
↓

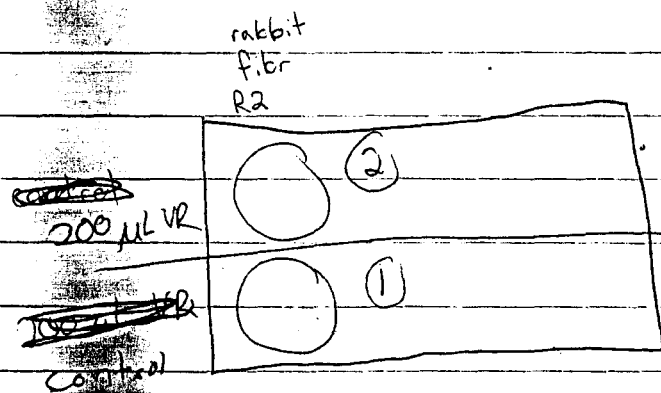
07/27/01



K9 fibr  
currently in T-25  
↓  
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  
↓  
T-75 change media  
↓  
T-75 spin  
↓  
3 mLs  
↓  
1 mL T-75  
1 mL T-75  
2X .5 mL in each well

SMC<sup>3</sup>  
↓  
1 well already done  
↓  
trypsin quickly  
some will ball up  
add media immediately  
↓  
pull out  
↓  
centrifuge  
↓  
re-suspend  
5 mLs media  
↓  
1 well



concentration

cells

media

07/27/01

|   |                |                           |                                                |         |
|---|----------------|---------------------------|------------------------------------------------|---------|
| 1 | control        | rabbit fibro              | }<br>did not grow well<br>↓<br>no FBS in media | } DMEM  |
| 2 | 200 $\mu$ L VR | rabbit fibro              |                                                |         |
| 3 | control        | K9 fibro                  |                                                |         |
| 4 | 200 $\mu$ L VR | K9 fibro                  |                                                |         |
| 5 | control        | smc <sup>2</sup> T/G ATCC | }                                              | } SMGM2 |
| 6 | 200 $\mu$ L VR | smc <sup>2</sup> T/G ATCC |                                                |         |
| 7 | control        | smc <sup>3</sup> HuSMC    | } possibly not<br>smc, may<br>be fibro         | } IMDM  |
| 8 | 200 $\mu$ L VR | smc <sup>3</sup> HuSMC    |                                                |         |

media  
in  
task

8-10-01

1 To plate cells from specimen/tissues:

2 Get about a  $1\text{ cm}^2$  block.

3 Mince 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, ~~and~~ forceps) in other side of petri dish

d - use sterile gloves

4 Put pieces of minced tissue in 6-well plates

5 Place 1 drop of FBS on each piece of tissue

★ Typically, use

6 Turn over (Invert) well-plate

IMDM for media

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

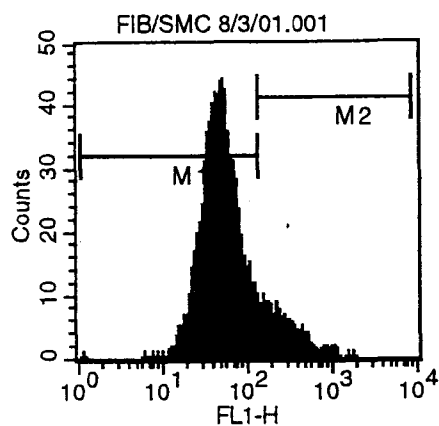
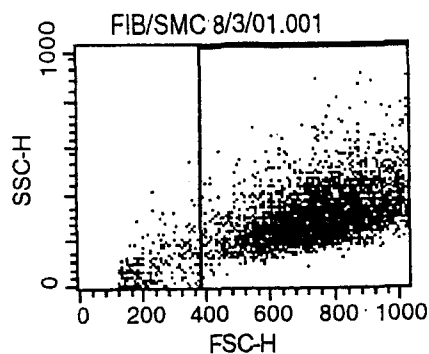
11 Let sit overnight in incubator

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

W. M. O.  
rabbit fibroblasts

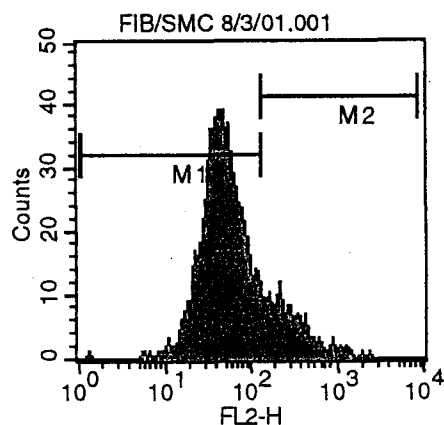


#### Histogram Statistics

File: FIB/SMC 8/3/01.001  
Patient ID: TAG=TUBE  
Gate: G1  
Total Events: 5000

Sample ID: FIBROBLAST VECTOR RED  
Acquisition Date: 3-Aug-1  
Gated Events: 4730

| 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     |

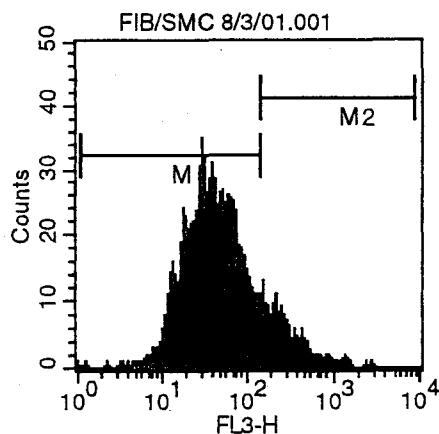


#### Histogram Statistics

File: FIB/SMC 8/3/01.001  
Patient ID: TAG=TUBE  
Gate: G1  
Total Events: 5000

Sample ID: FIBROBLAST VECTOR RED  
Acquisition Date: 3-Aug-1  
Gated Events: 4730

| 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     |



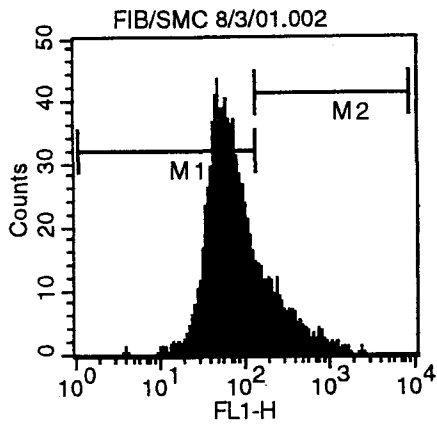
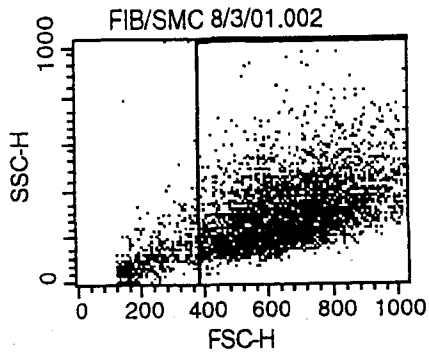
#### Histogram Statistics

File: FIB/SMC 8/3/01.001  
Patient ID: TAG=TUBE  
Gate: G1  
Total Events: 5000

Sample ID: FIBROBLAST VECTOR RED  
Acquisition Date: 3-Aug-1  
Gated Events: 4730

| 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     |

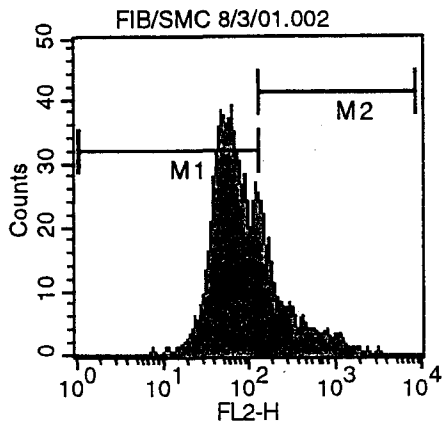
small vector red  
rabbit fibroblasts



#### 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

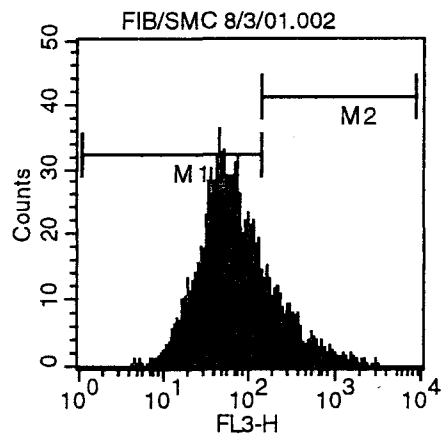
| Marker | Left, Right | Events | % Gated | % Total | Mean   | Peak Ch |
|--------|-------------|--------|---------|---------|--------|---------|
| All    | 1, 9910     | 4548   | 100.00  | 90.96   | 97.73  | 47      |
| M1     | 1, 132      | 3782   | 83.16   | 75.64   | 61.34  | 47      |
| M2     | 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     |

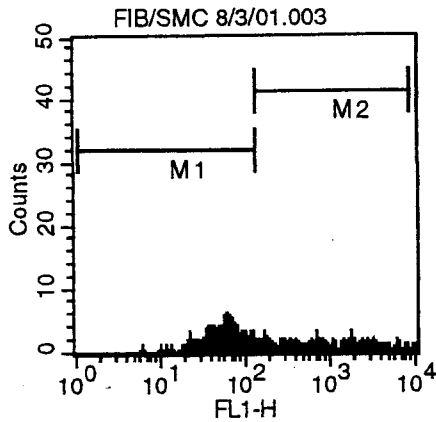
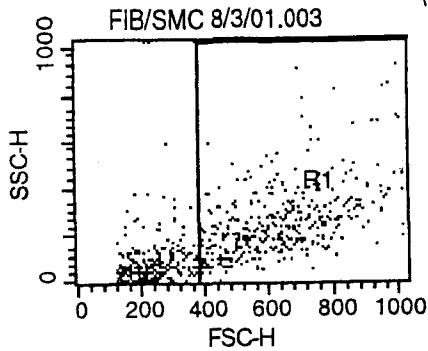


#### Histogram Statistics

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     |

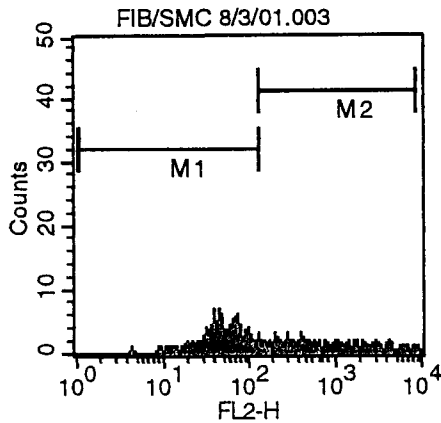
control K1 fibroblasts  
(did not grow well - no FBS  
in media)



#### Histogram Statistics

File: FIB/SMC 8/3/01.003 Sample ID: FIBROBLAST VECTOR RED  
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1  
Gate: G1 Gated Events: 462  
Total Events: 750

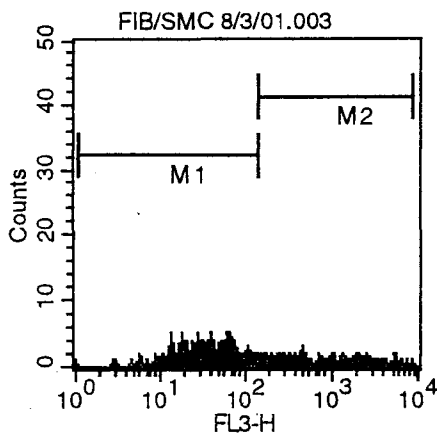
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 462    | 100.00  | 61.60   | 473.44  | 58      |
| M1     | 1, 132      | 313    | 67.75   | 41.73   | 57.05   | 58      |
| M2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



#### Histogram Statistics

File: FIB/SMC 8/3/01.003 Sample ID: FIBROBLAST VECTOR RED  
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1  
Gate: G1 Gated Events: 462  
Total Events: 750

| 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 Sample ID: FIBROBLAST VECTOR RED  
Patient ID: TAG=TUBE Acquisition Date: 3-Aug-1  
Gate: G1 Gated Events: 462  
Total Events: 750

| 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     |

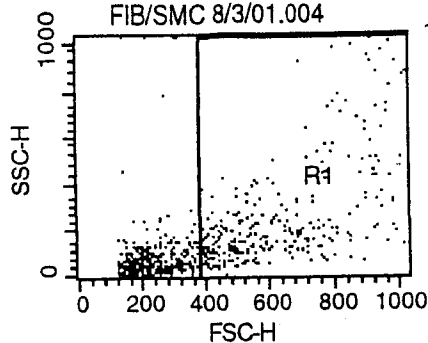
200 uL Vector Red

K9 fibroblasts

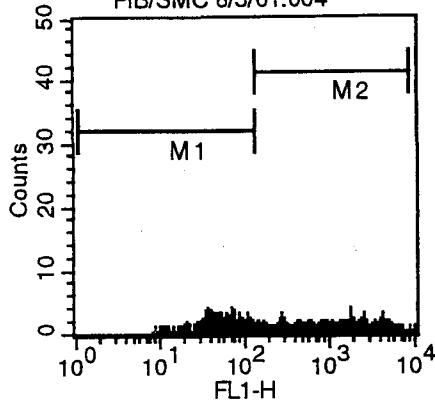
did not grow well

- no FBS in media

FIB/SMC 8/3/01.004



FIB/SMC 8/3/01.004



#### Histogram Statistics

File: FIB/SMC 8/3/01.004

Sample ID: FIBROBLAST VECTOR RED

Patient ID: TAG=TUBE

Acquisition Date: 3-Aug-1

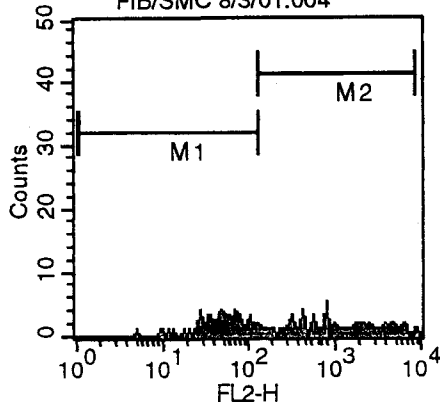
Gate: G1

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    |

FIB/SMC 8/3/01.004



#### Histogram Statistics

File: FIB/SMC 8/3/01.004

Sample ID: FIBROBLAST VECTOR RED

Patient ID: TAG=TUBE

Acquisition Date: 3-Aug-1

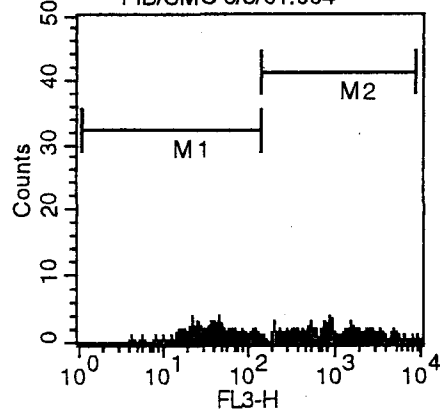
Gate: G1

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     |

FIB/SMC 8/3/01.004



#### 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

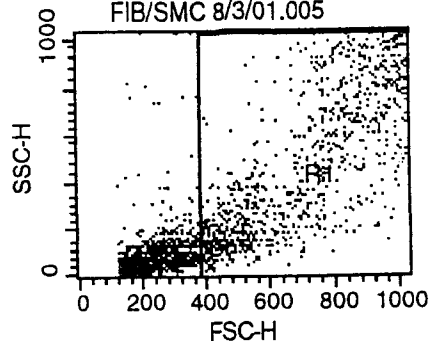
Gated Events: 360

Total Events: 720

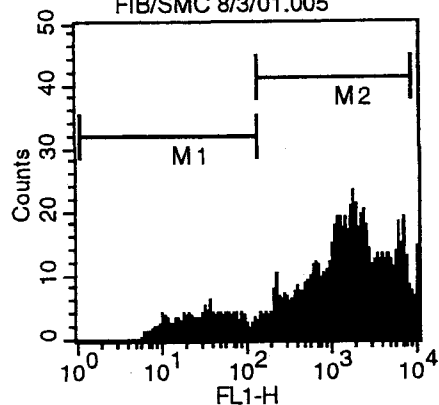
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 360    | 100.00  | 50.00   | 690.35  | 21      |
| M1     | 1, 132      | 187    | 51.94   | 25.97   | 43.71   | 21      |
| M2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

SMC<sup>2</sup> T/G ATCC

FIB/SMC 8/3/01.005



FIB/SMC 8/3/01.005

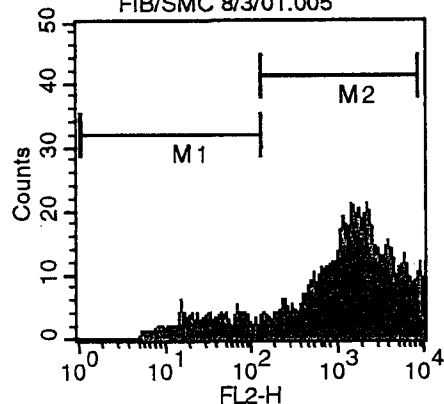


#### Histogram Statistics

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    |

FIB/SMC 8/3/01.005

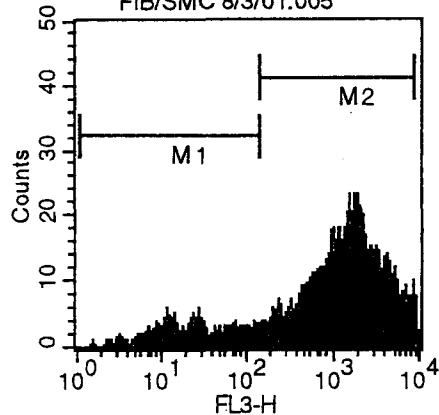


#### Histogram Statistics

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    |

FIB/SMC 8/3/01.005

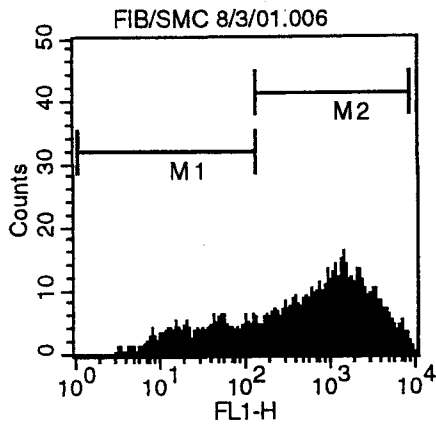
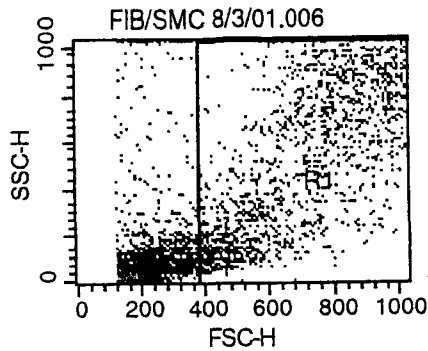


#### Histogram Statistics

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    |

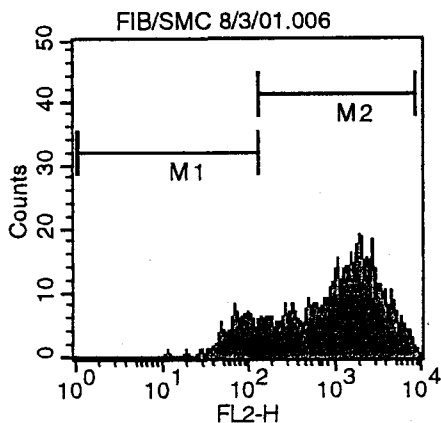
200 uL VECTOR RED  
SMC<sup>2</sup> T/G ATCC



#### 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

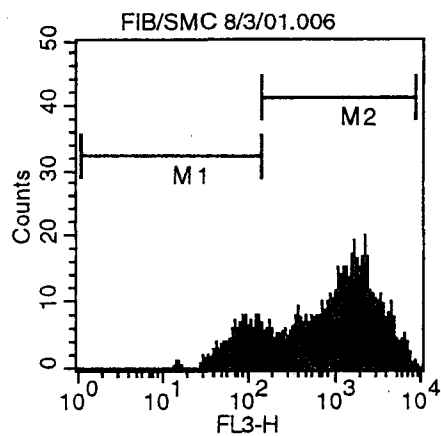
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 2785   | 100.00  | 55.70   | 1129.67 | 1241    |
| M1     | 1, 132      | 599    | 21.51   | 11.98   | 48.53   | 41      |
| M2     | 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    |

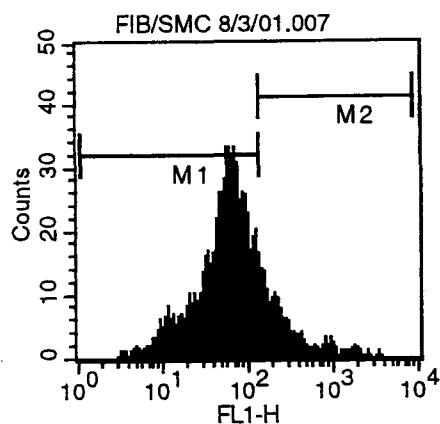
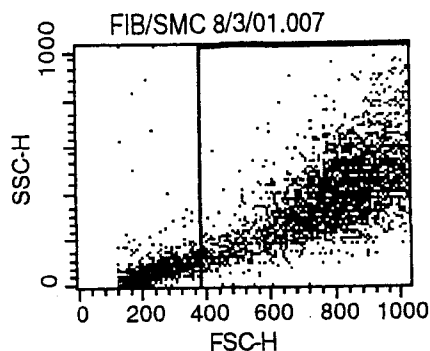


#### Histogram Statistics

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    |

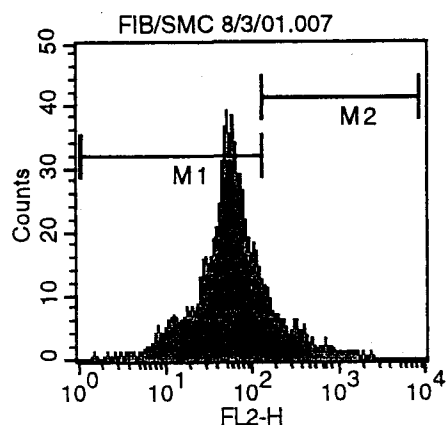
W01101  
SMC<sup>3</sup> HUSMC?



#### 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

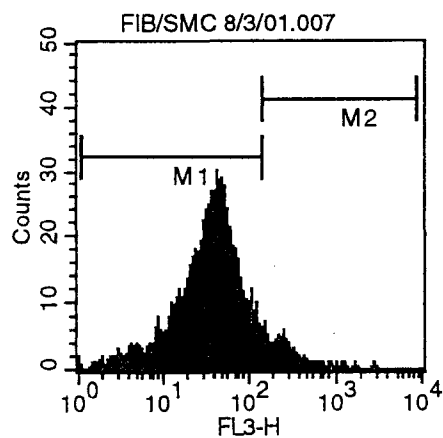
| Marker | Left, Right | Events | % Gated | % Total | Mean   | Peak Ch |
|--------|-------------|--------|---------|---------|--------|---------|
| All    | 1, 9910     | 3766   | 100.00  | 75.32   | 90.53  | 54      |
| M1     | 1, 132      | 3218   | 85.45   | 64.36   | 57.86  | 54      |
| M2     | 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     |

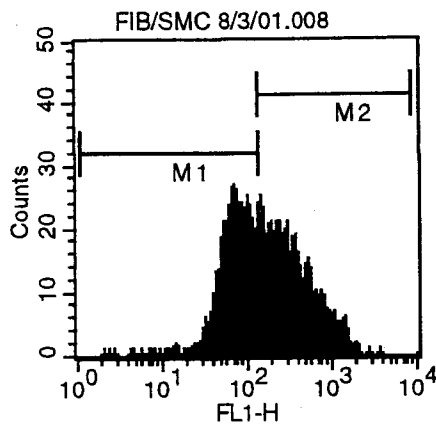
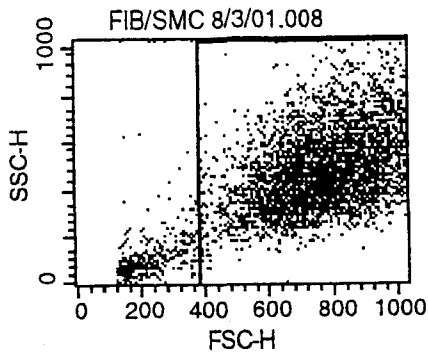


#### Histogram Statistics

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     |

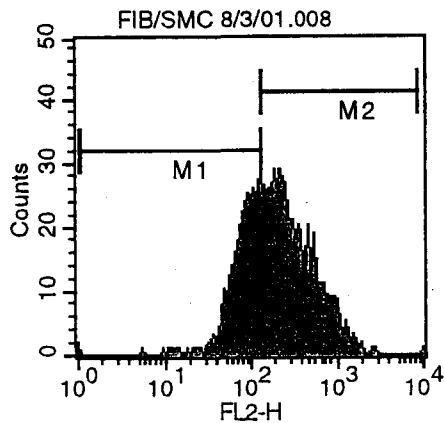
SMC<sup>3</sup> HuSMC?



#### 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

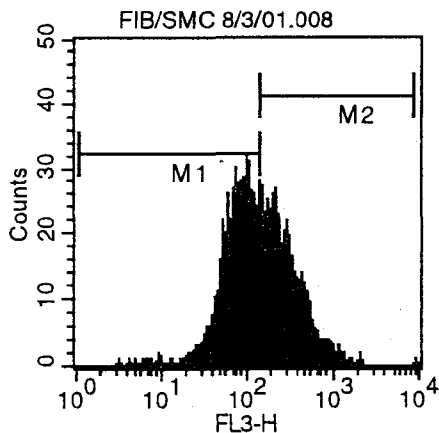
| Marker | Left, Right | Events | % Gated | % Total | Mean   | Peak Ch |
|--------|-------------|--------|---------|---------|--------|---------|
| All    | 1, 9910     | 4648   | 100.00  | 92.96   | 228.75 | 66      |
| M1     | 1, 132      | 2295   | 49.38   | 45.90   | 76.34  | 66      |
| M2     | 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     |

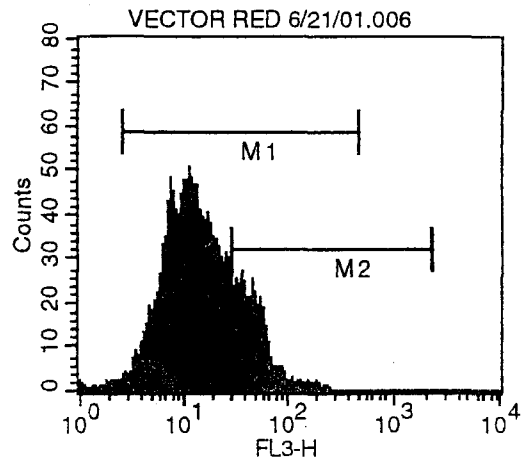
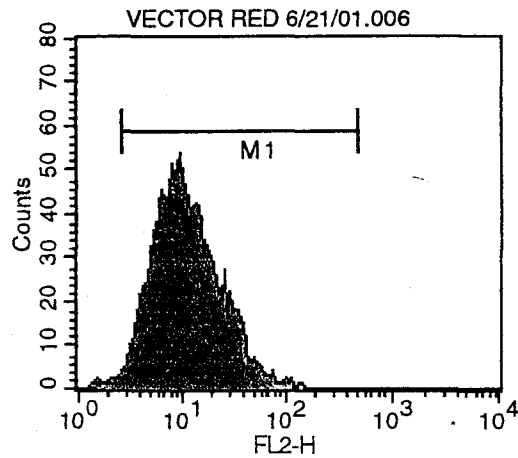
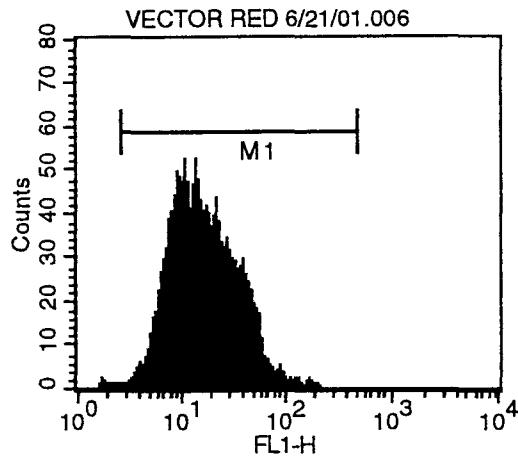
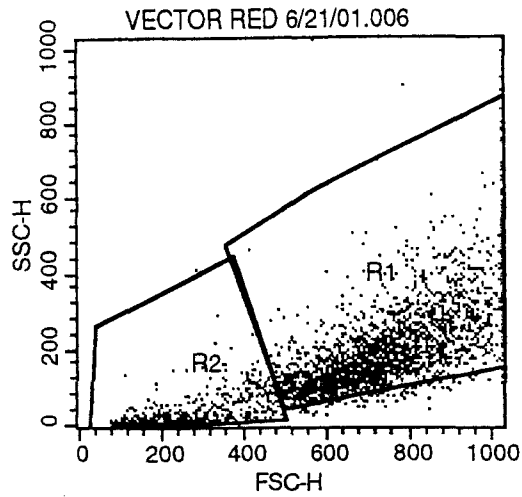


#### Histogram Statistics

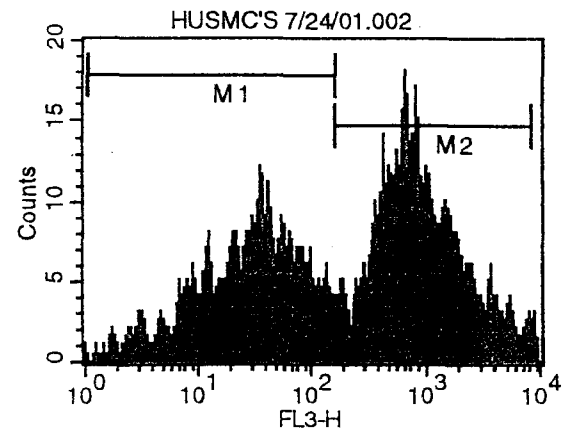
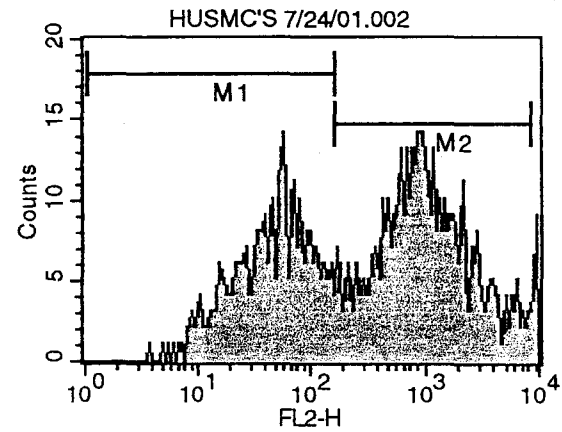
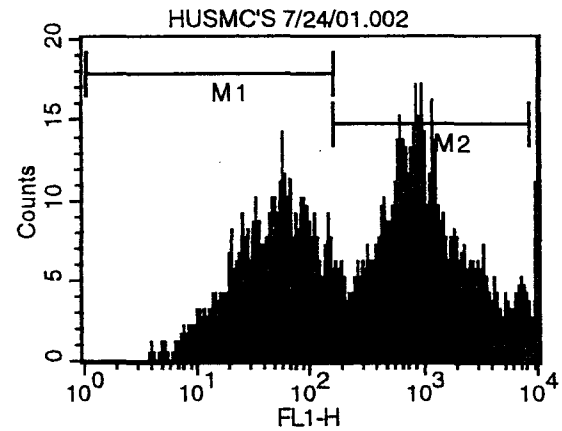
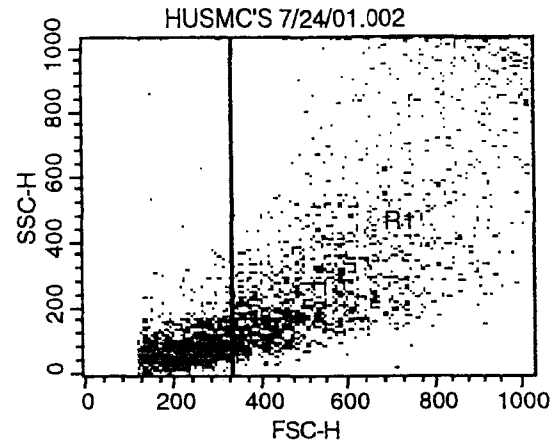
File: FIB/SMC 8/3/01.008      Sample ID: FIBROBLAST VECTOR RED  
 Patient ID: TAG=TUBE      Acquisition Date: 3-Aug-1  
 Gate: G1      Gated Events: 4648  
 Total Events: 5000

| 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'S  
Patient ID: T  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 15 |

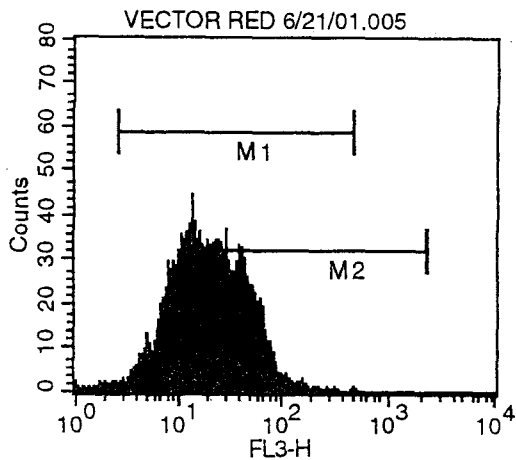
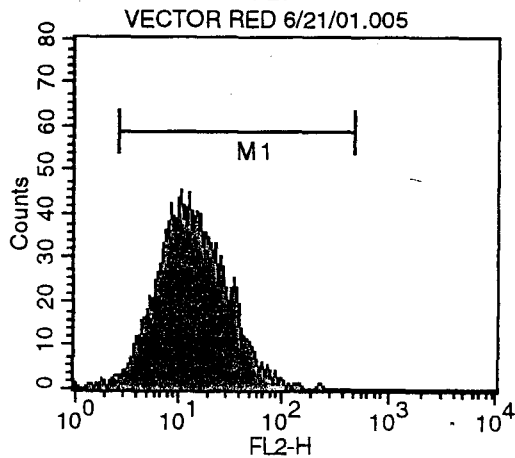
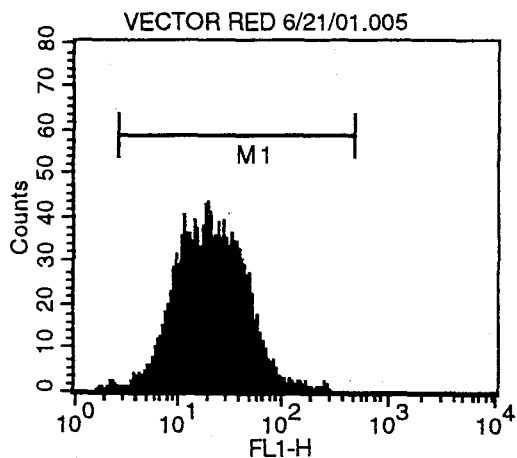
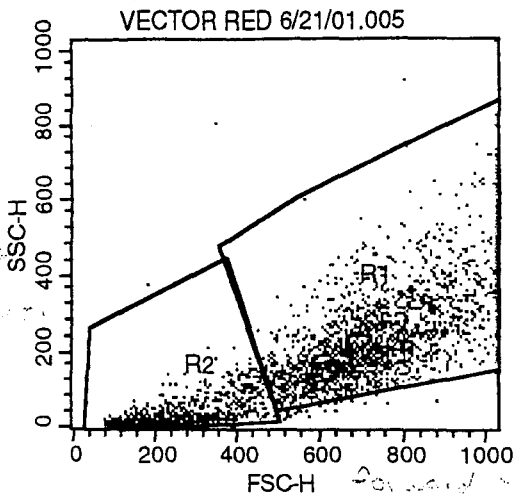
File: HUSMC'S  
Patient ID: T  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 15 |

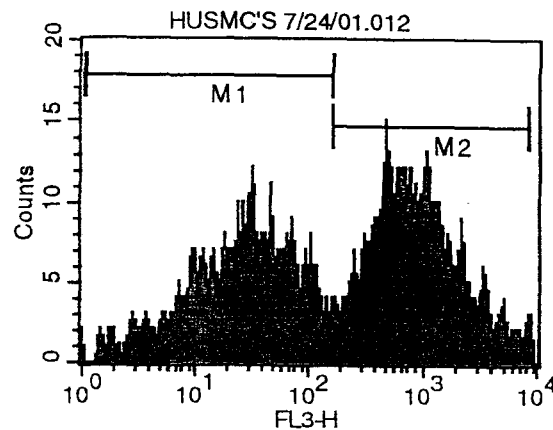
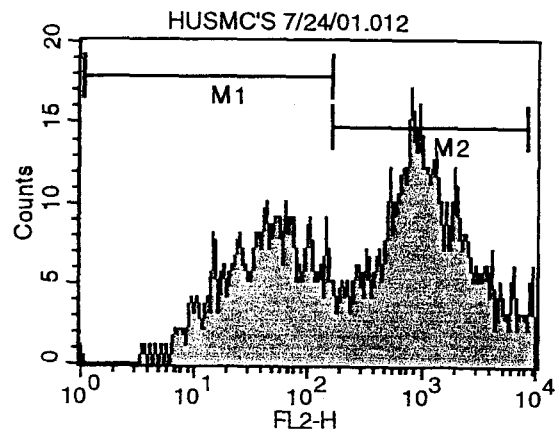
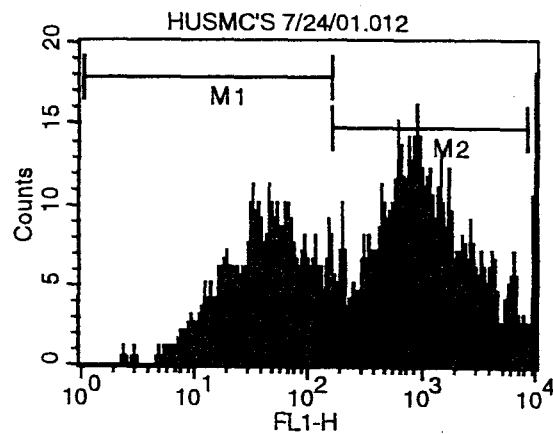
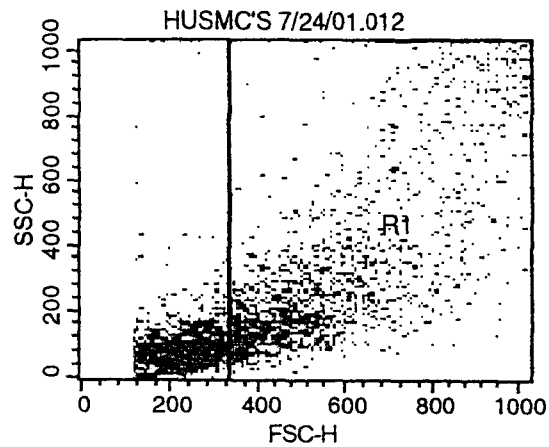
File: HUSMC'S  
Patient ID: T  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 1  |

K9 fibroblasts  
2uLs Vector Red



HuAoSMC  
2uLs Vector Red



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

| Marker | Left |
|--------|------|
| All    |      |
| M1     |      |
| M2     | 15   |

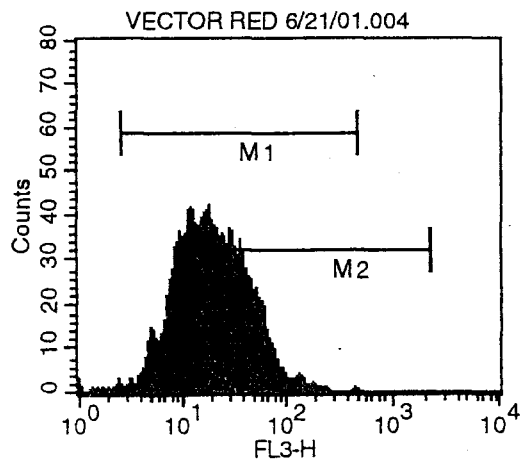
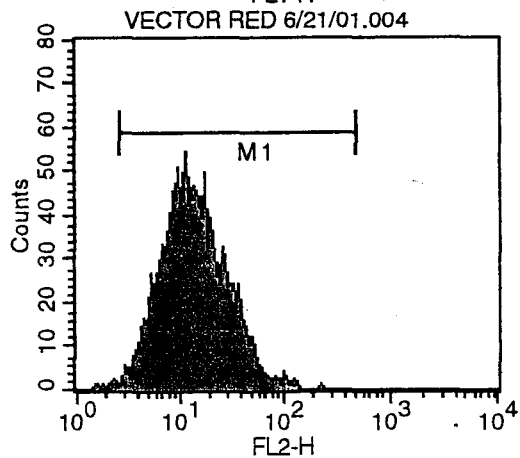
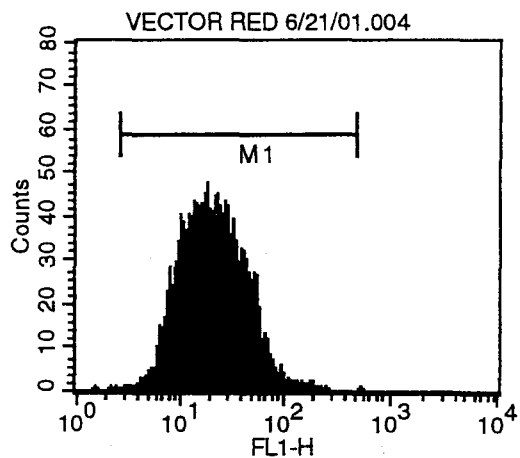
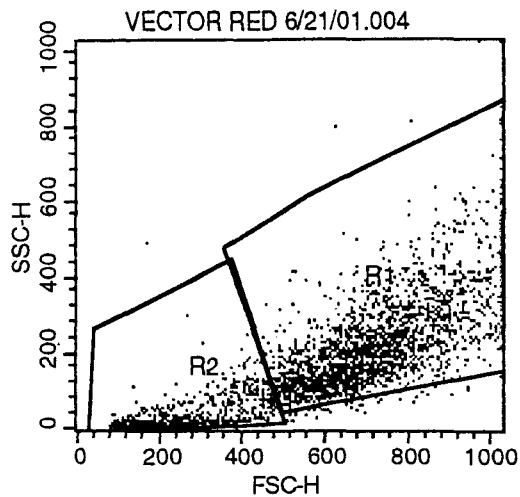
File: HUSMC  
Patient ID: T  
Gate: G1  
Total Events

| Marker | Left |
|--------|------|
| All    |      |
| M1     |      |
| M2     | 15   |

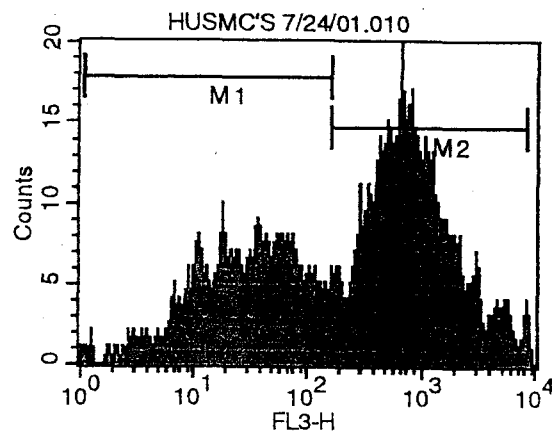
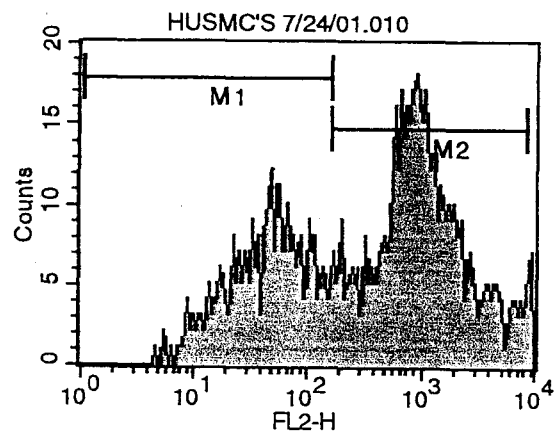
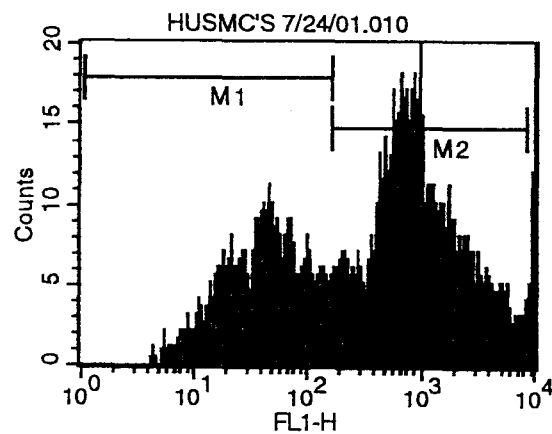
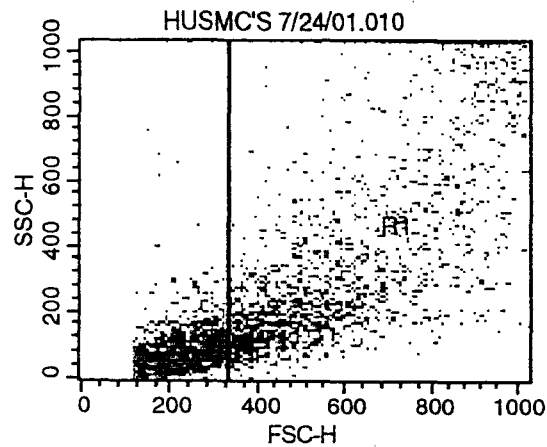
File: HUSMC  
Patient ID: T  
Gate: G1  
Total Event

| Marker | Left |
|--------|------|
| All    |      |
| M1     |      |
| M2     | 15   |

KY fibroblasts  
4 uLs Vector Red



HuAoS MC  
5 uLs VR



File: HUSM  
Patient ID:  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 1  |

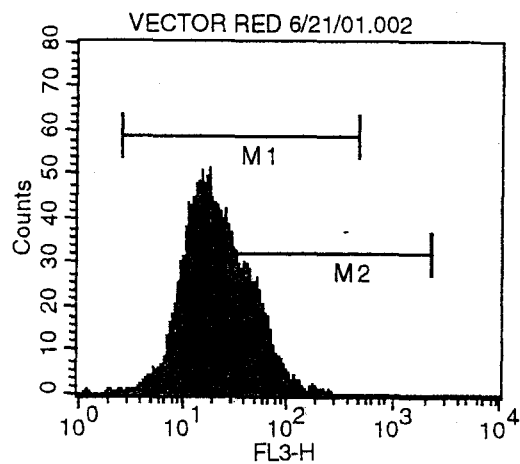
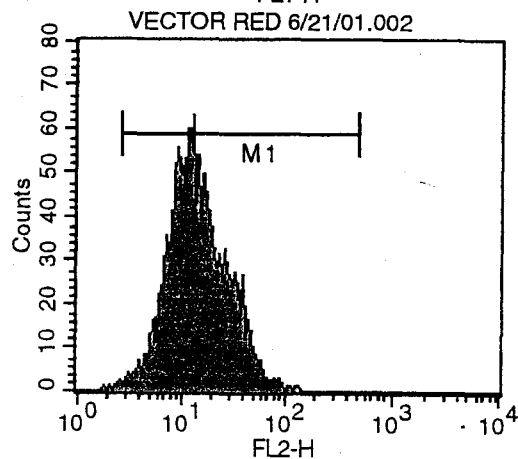
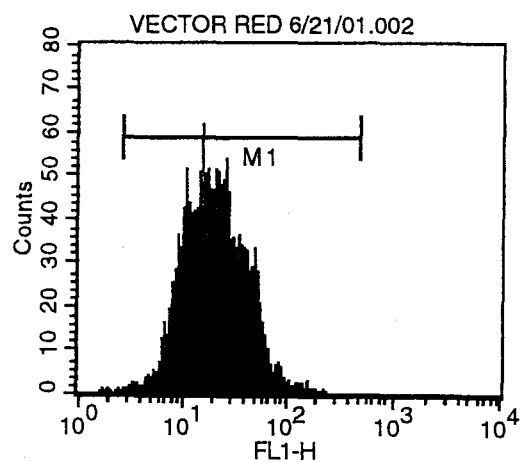
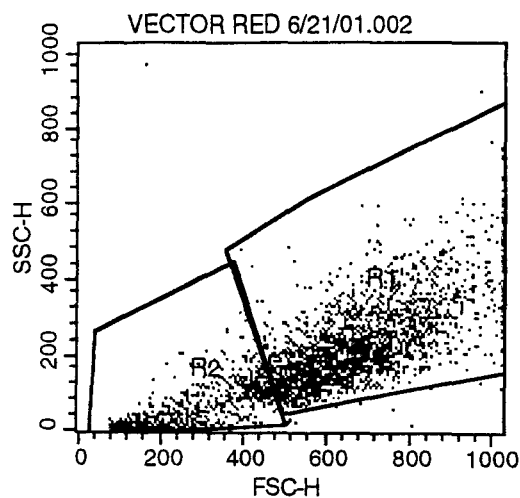
File: HUSM  
Patient ID:  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 15 |

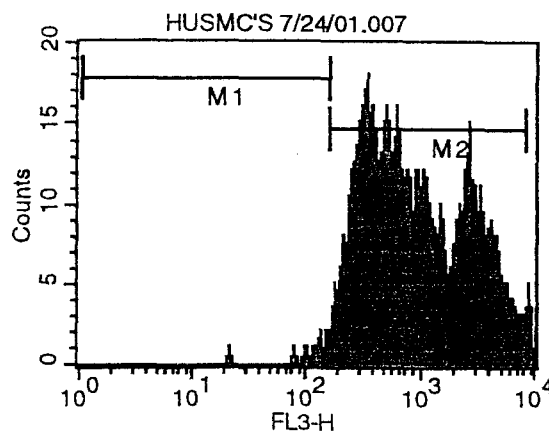
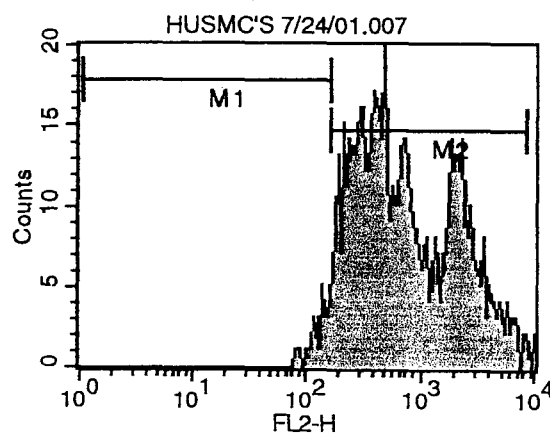
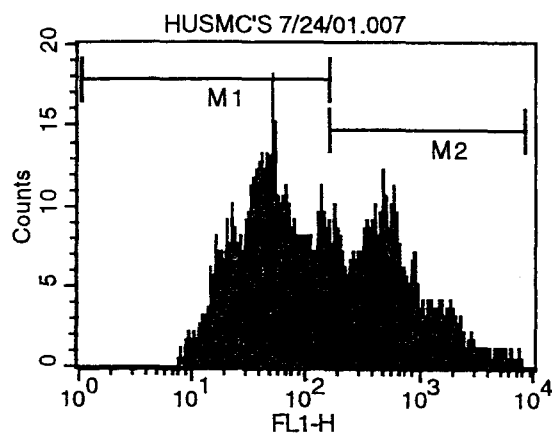
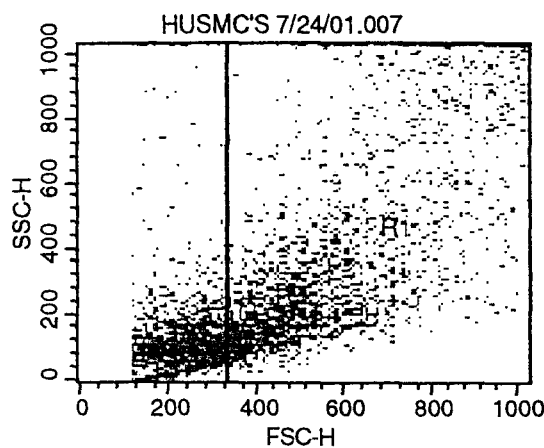
File: HUSM  
Patient ID:  
Gate: G1  
Total Event

| Marker | Le |
|--------|----|
| All    |    |
| M1     |    |
| M2     | 1  |

K9 Fibroblasts  
200 uLs Vector Red



HuAoS(MC)  
200 uLs Vector Red



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

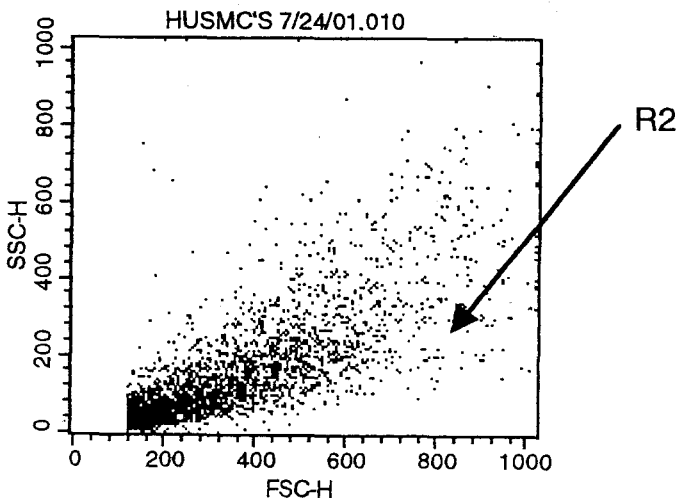
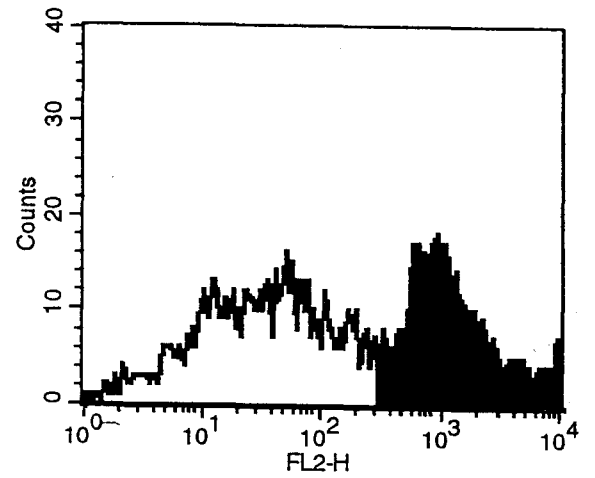
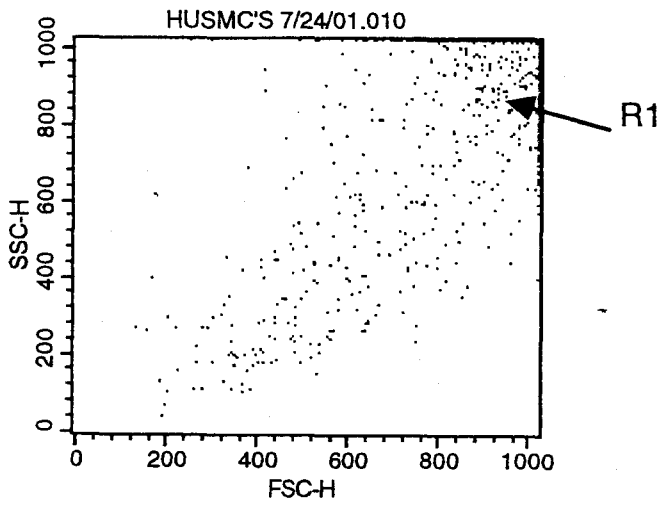
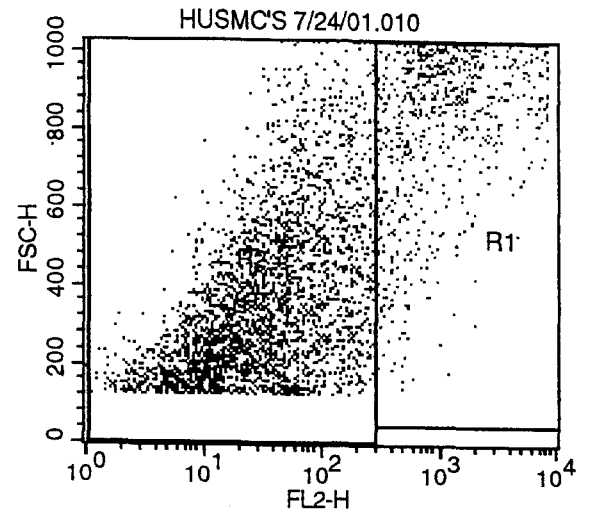
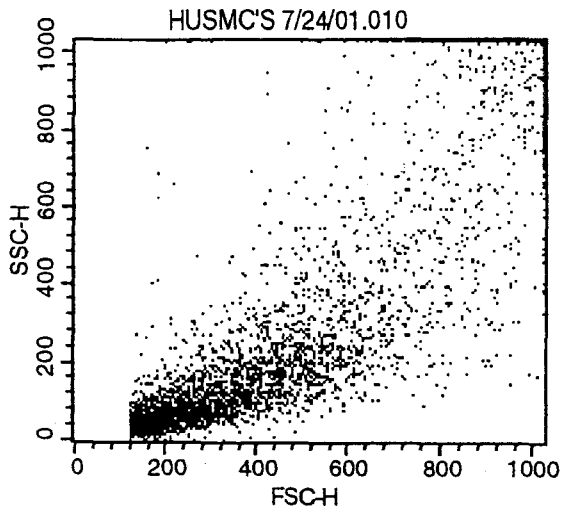
| Marker | Left, R |
|--------|---------|
| All    | 1, 9    |
| M1     | 1,      |
| M2     | 158, 8  |

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

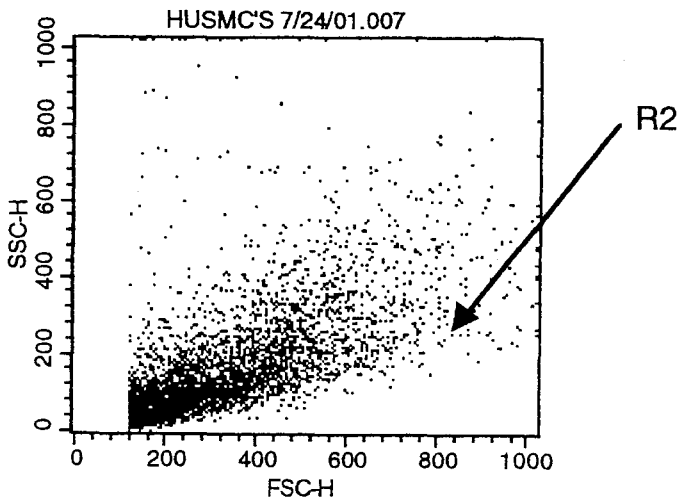
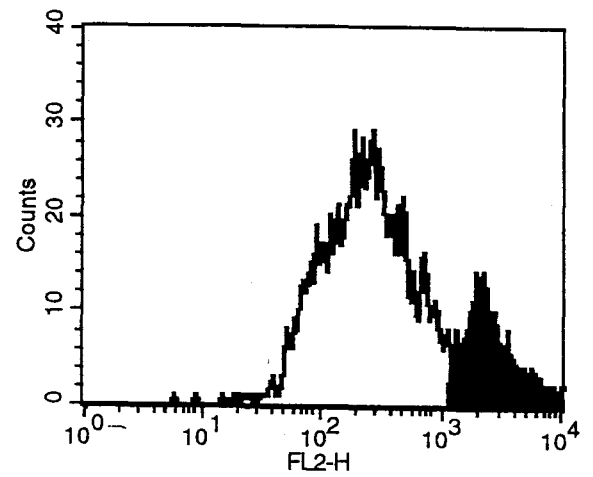
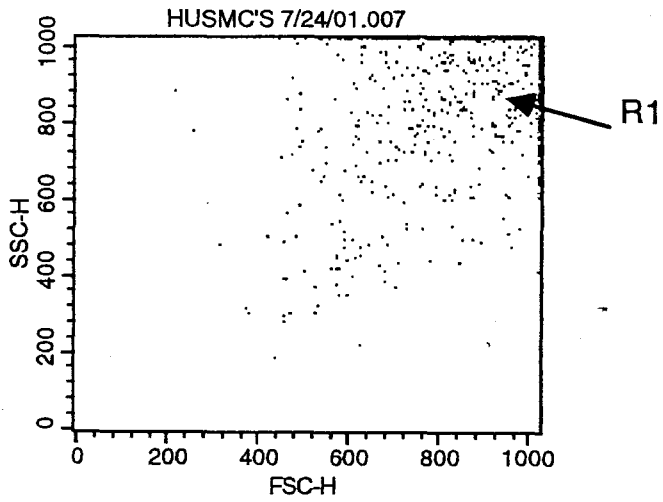
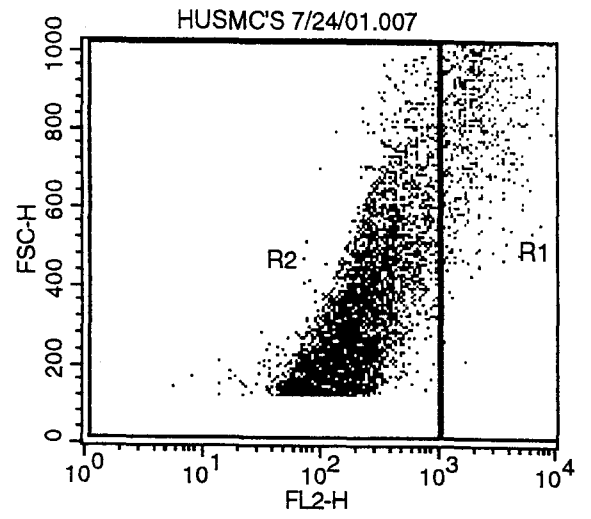
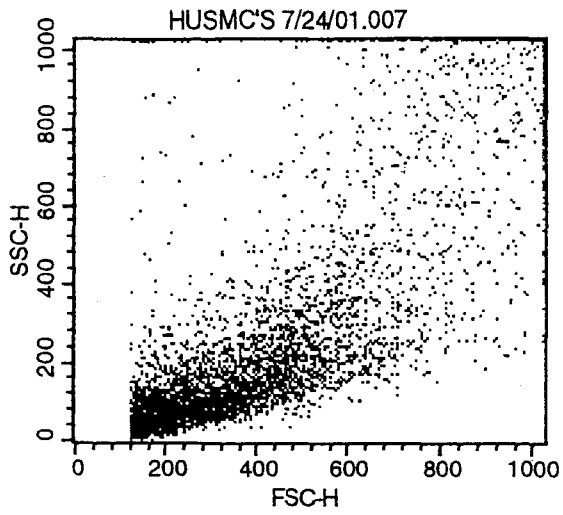
| Marker | Left, R |
|--------|---------|
| All    | 1, 9    |
| M1     | 1,      |
| M2     | 158, 8  |

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

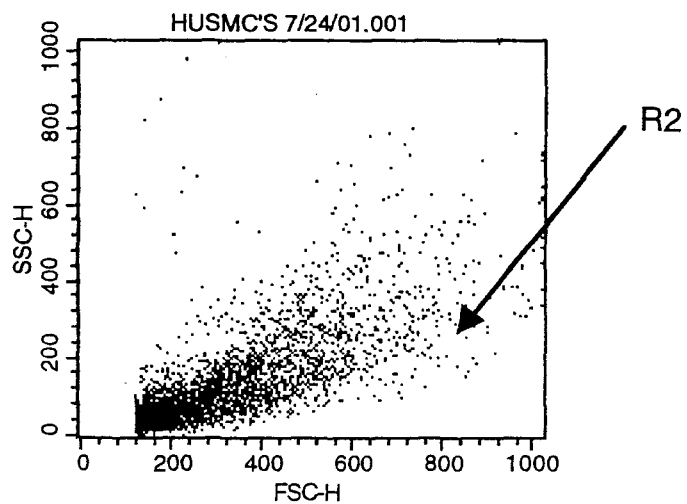
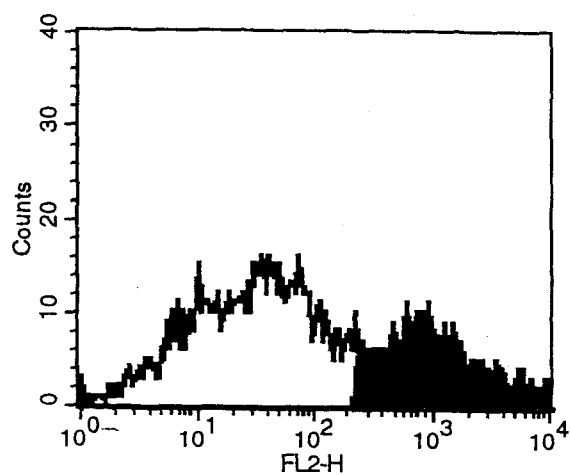
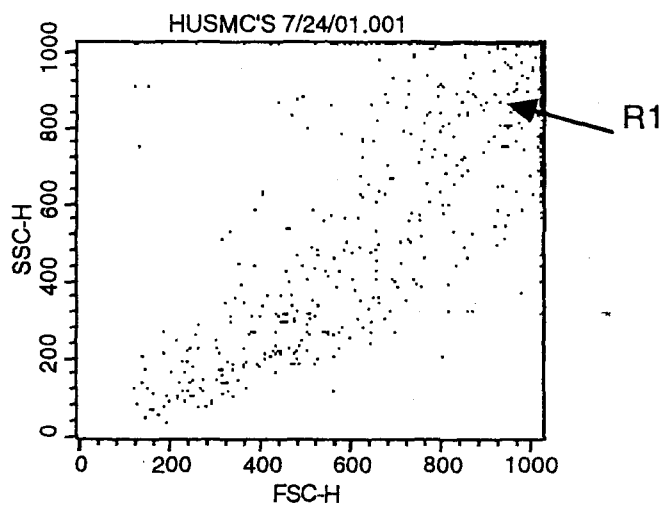
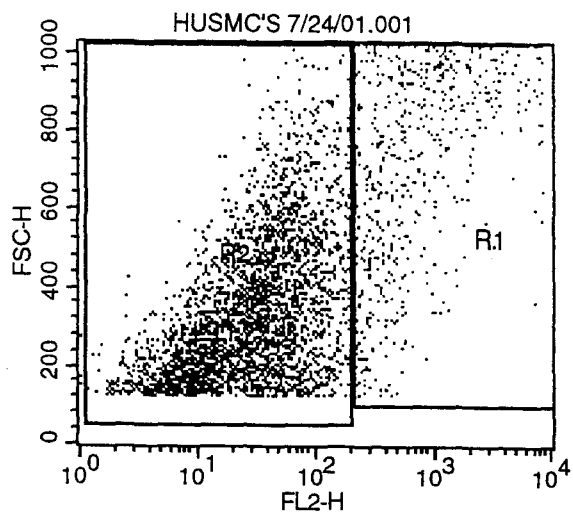
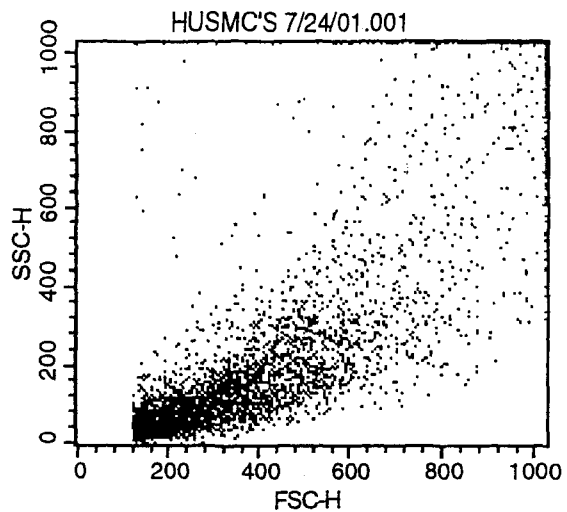
| Marker | Left, F |
|--------|---------|
| All    | 1,      |
| M1     | 1,      |
| M2     | 158,    |



HuAosMC  
5uls Vector Red



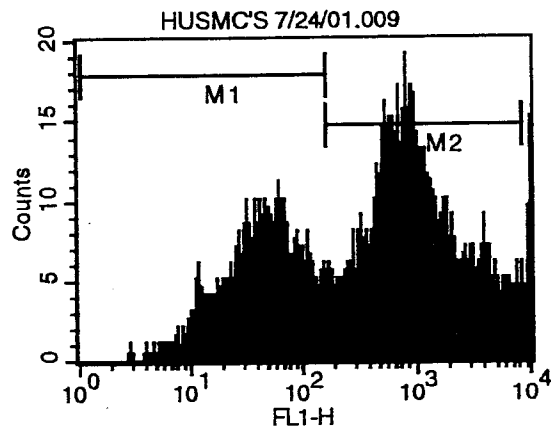
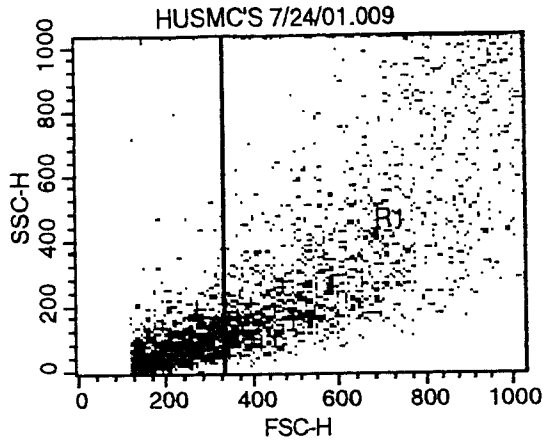
HuA6SMC  
200 uLs Vector Red



HuAoSMC (L1)  
50  $\mu$ ls Levamisol

Hu Ao SMC  
10 uLs VR

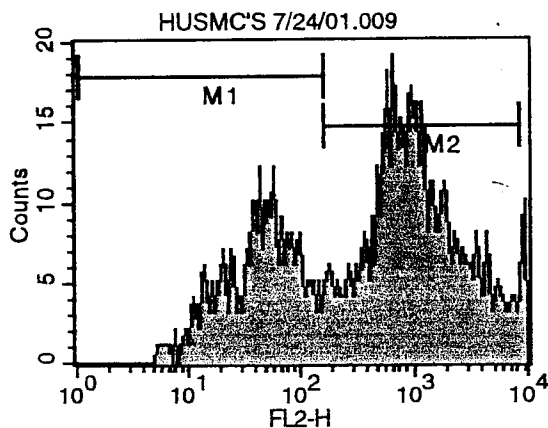
10 uLs VR



#### 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

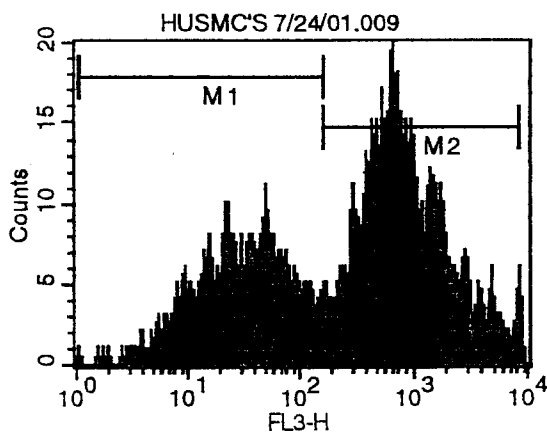
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 3423   | 100.00  | 68.46   | 1076.34 | 743     |
| M1     | 1, 158      | 1110   | 32.43   | 22.20   | 55.12   | 58      |
| M2     | 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     |



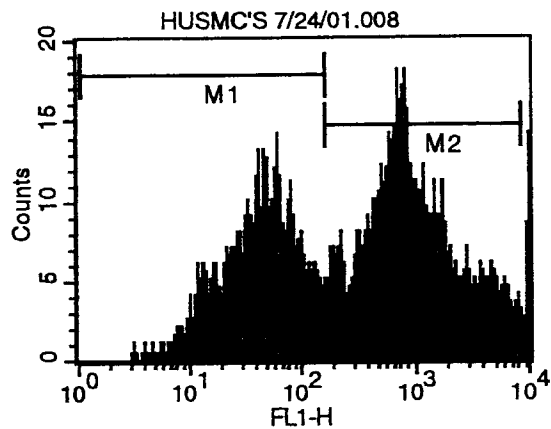
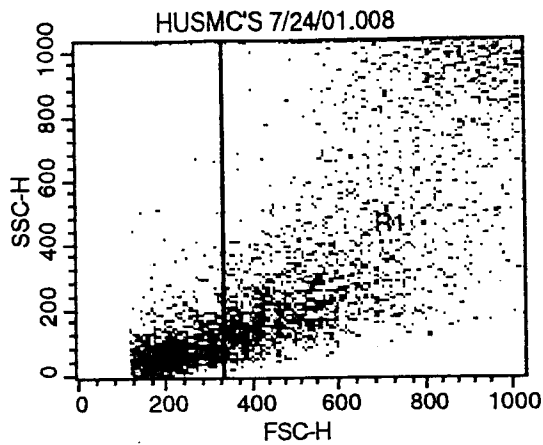
#### Histogram Statistics

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     |

HUSMC  
50 uLs VR

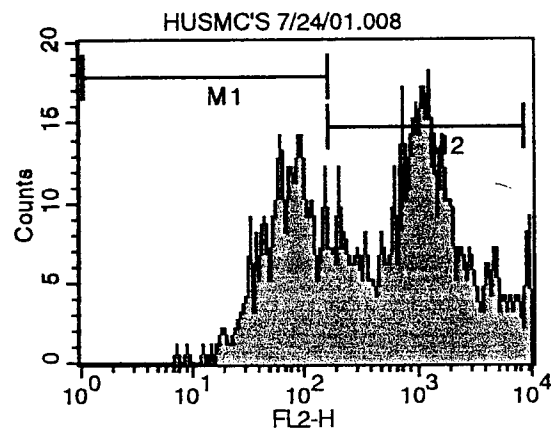
50 uLs VR



#### Histogram Statistics

File: HUSMC'S 7/24/01.008 Sample ID: HUMAN AORTIC SMC'S  
 Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1  
 Gate: G1 Gated Events: 3393  
 Total Events: 5000

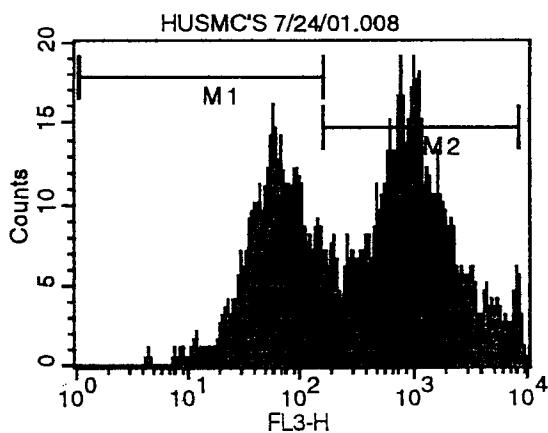
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 3393   | 100.00  | 67.86   | 901.02  | 632     |
| M1     | 1, 158      | 1302   | 38.37   | 26.04   | 53.17   | 57      |
| M2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



#### Histogram Statistics

File: HUSMC'S 7/24/01.008 Sample ID: HUMAN AORTIC SMC'S  
 Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1  
 Gate: G1 Gated Events: 3393  
 Total Events: 5000

| 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    |



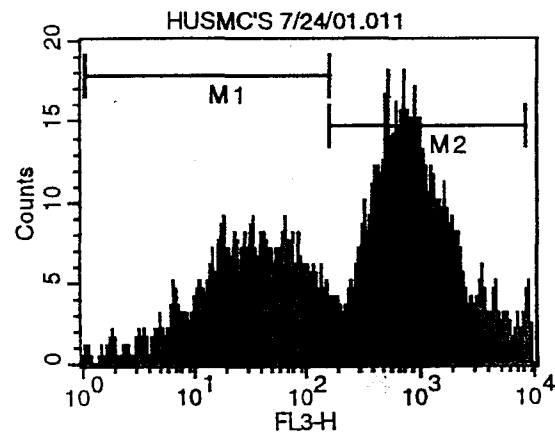
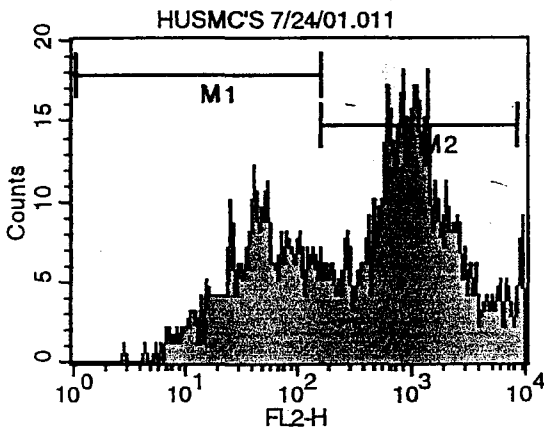
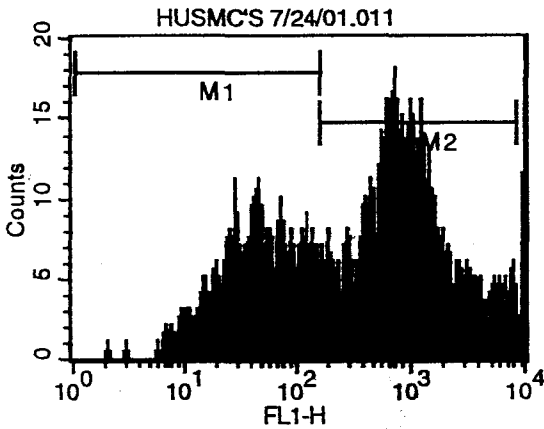
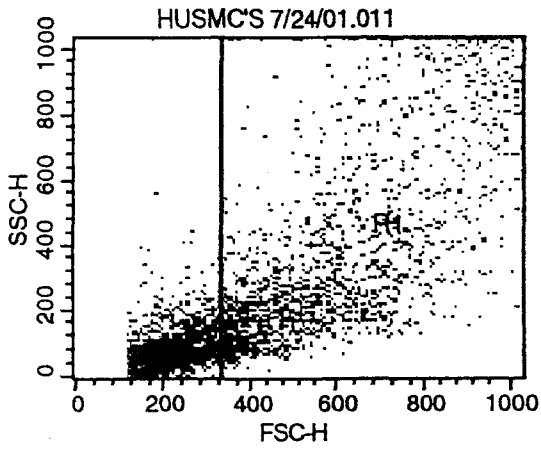
#### Histogram Statistics

File: HUSMC'S 7/24/01.008 Sample ID: HUMAN AORTIC SMC'S  
 Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1  
 Gate: G1 Gated Events: 3393  
 Total Events: 5000

| 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     |

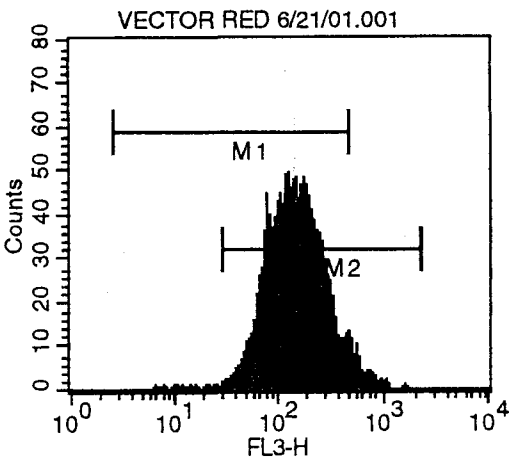
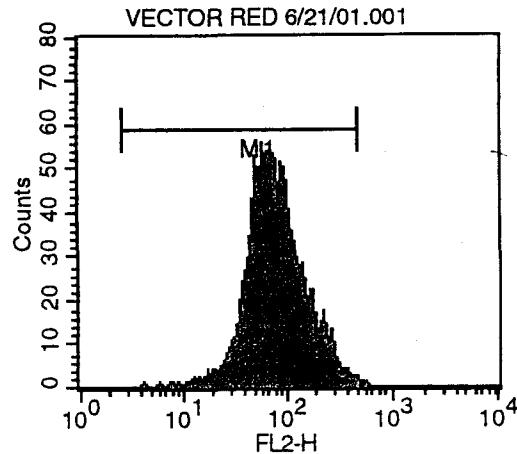
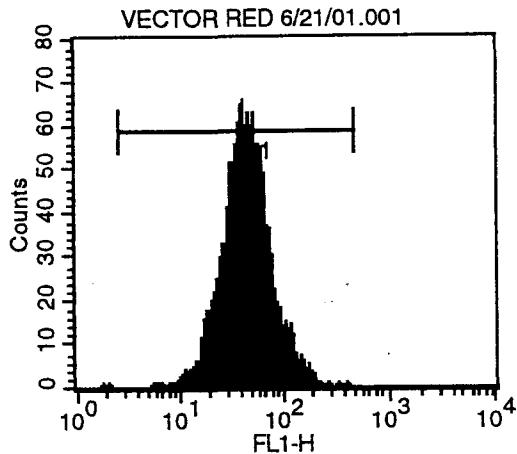
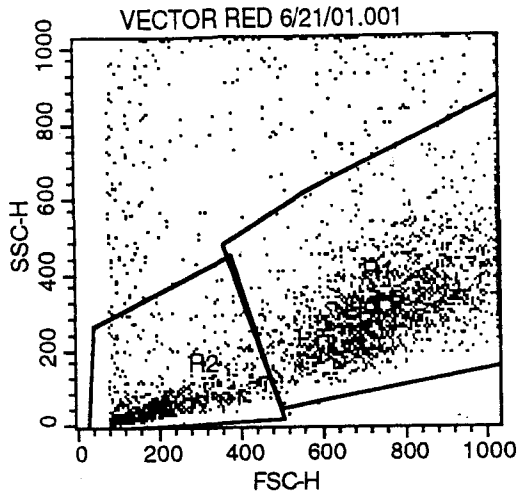
#### Histogram Statistics

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 Sample ID: VECTOR RED  
Patient ID: A1 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 6389  
Total Events: 10000 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 Sample ID: VECTOR RED  
Patient ID: A1 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 6389  
Total Events: 10000 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      |

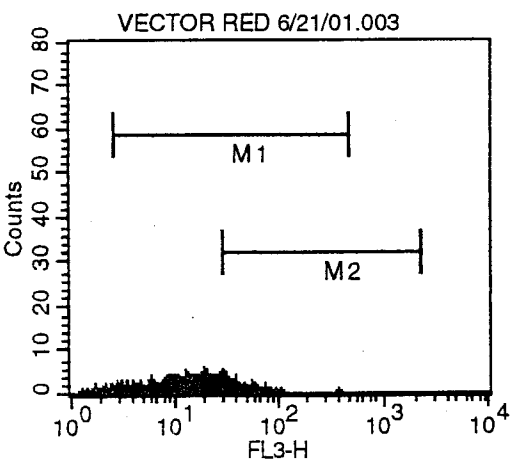
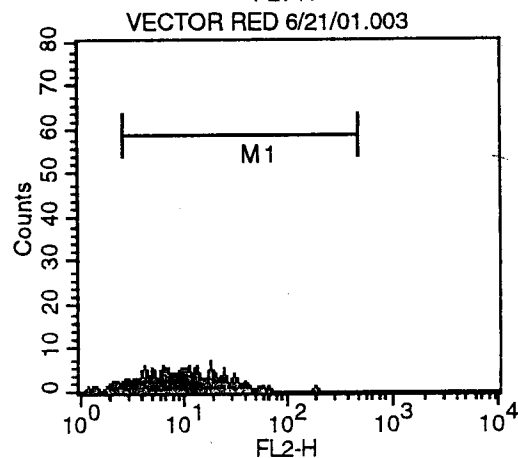
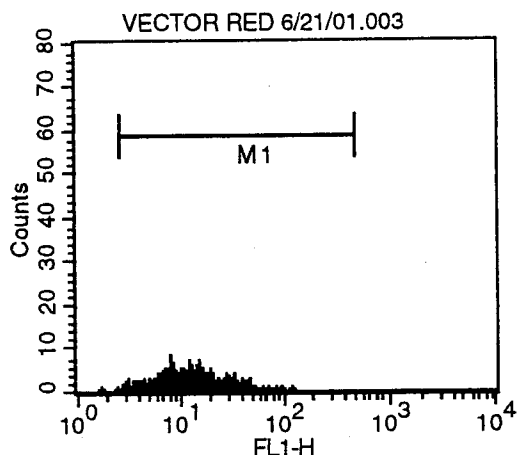
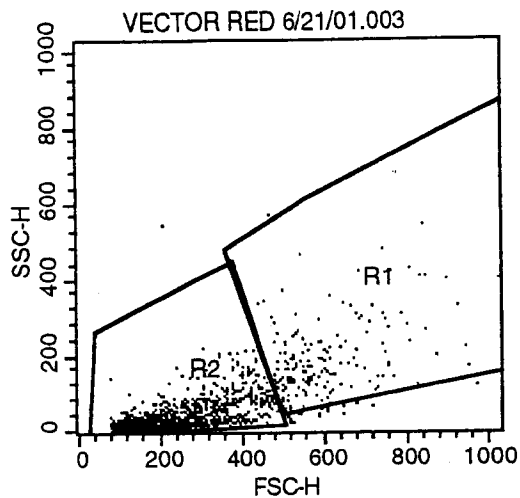
#### Histogram Statistics

File: VECTOR RED 6/21/01.001 Sample ID: VECTOR RED  
Patient ID: A1 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 6389  
Total Events: 10000 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  $\mu$ Ls VR

20  $\mu$ Ls VR



#### Histogram Statistics

File: VECTOR RED 6/21/01.003 Sample ID: VECTOR RED  
Patient ID: A3 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 517  
Total Events: 5190 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

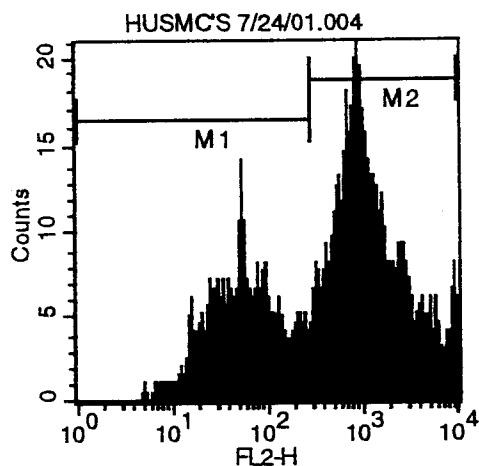
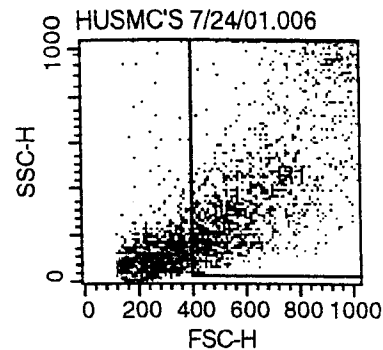
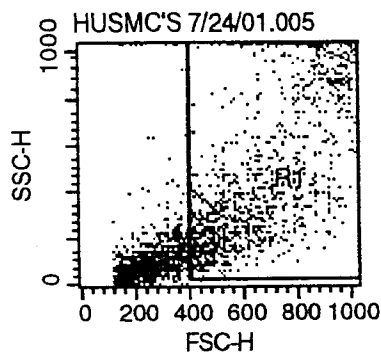
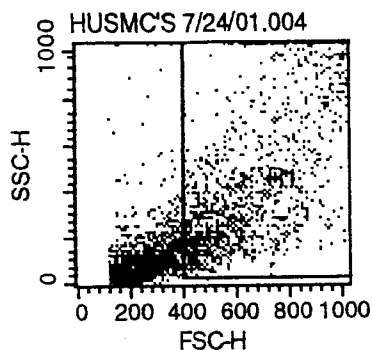
File: VECTOR RED 6/21/01.003 Sample ID: VECTOR RED  
Patient ID: A3 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 517  
Total Events: 5190 X Parameter: FL2-H (Log)

| 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 Sample ID: VECTOR RED  
Patient ID: A3 Acquisition Date: 21-Jun-1  
Gate: G1 Gated Events: 517  
Total Events: 5190 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

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

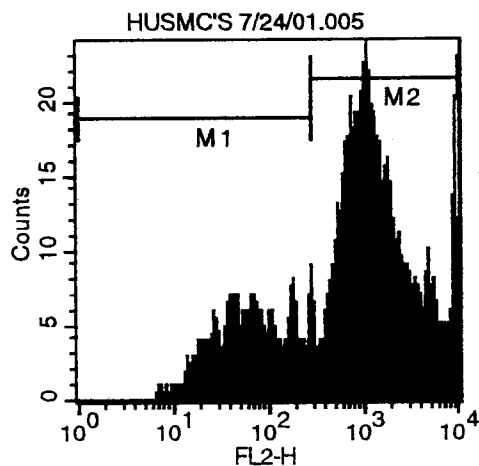
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 3002

Total Events: 5000

| 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

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

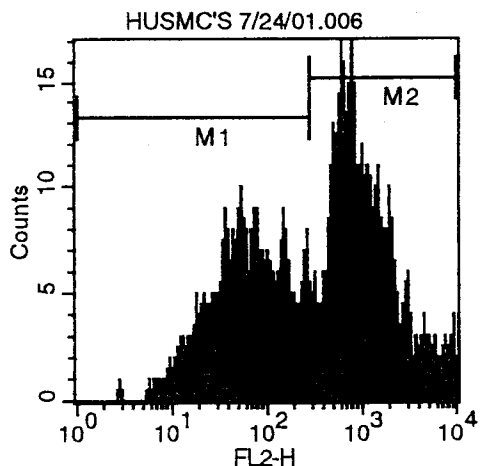
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 3479

Total Events: 5000

| 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    |



#### Histogram Statistics

File: HUSMC'S 7/24/01.006

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

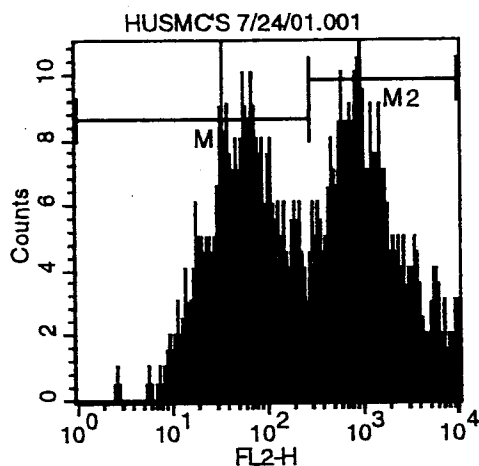
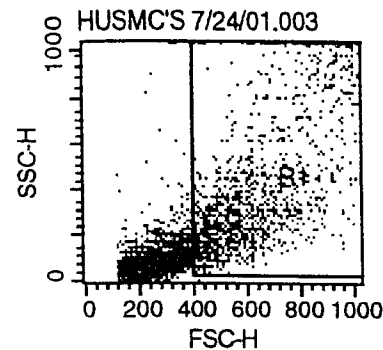
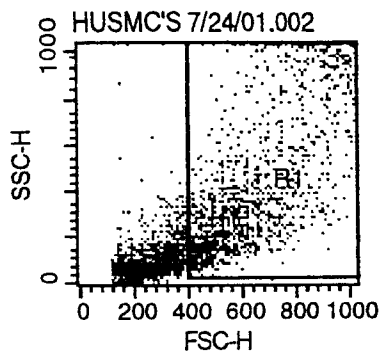
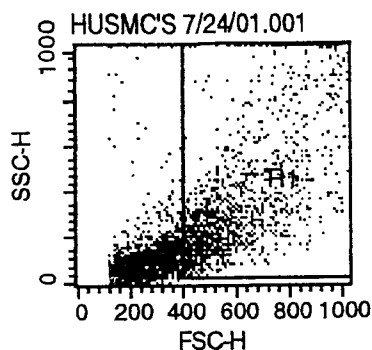
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 2591

Total Events: 5000

| 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

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

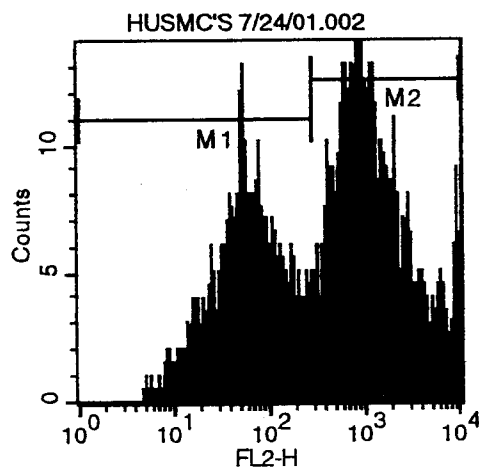
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 2133

Total Events: 4425

| 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

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

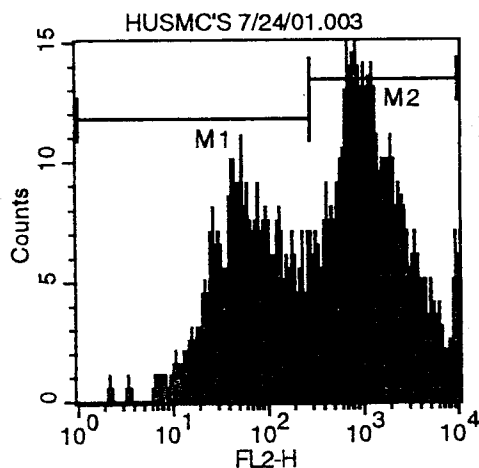
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 2757

Total Events: 4785

| 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

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

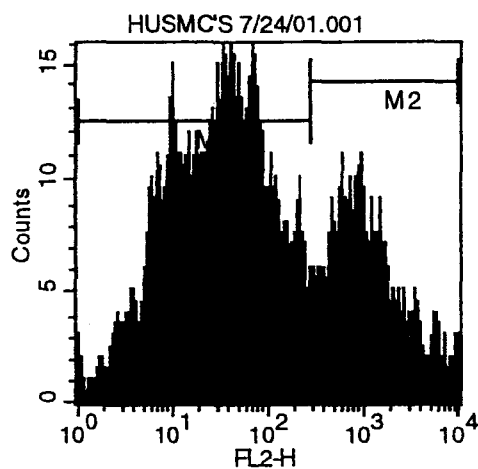
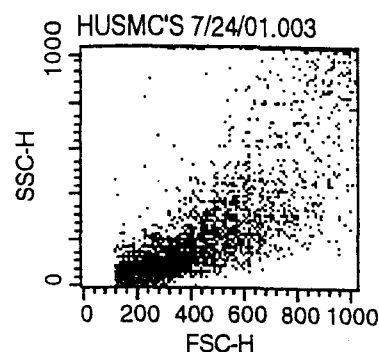
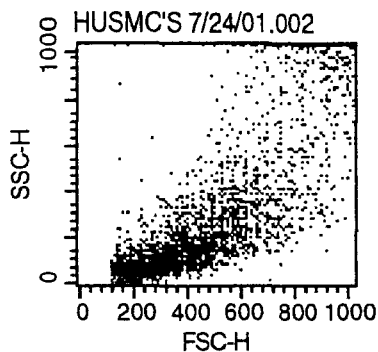
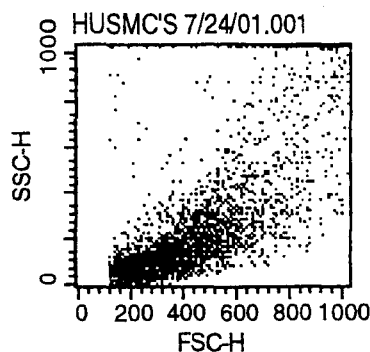
Acquisition Date: 24-Jul-1

Gate: G1

Gated Events: 2905

Total Events: 5235

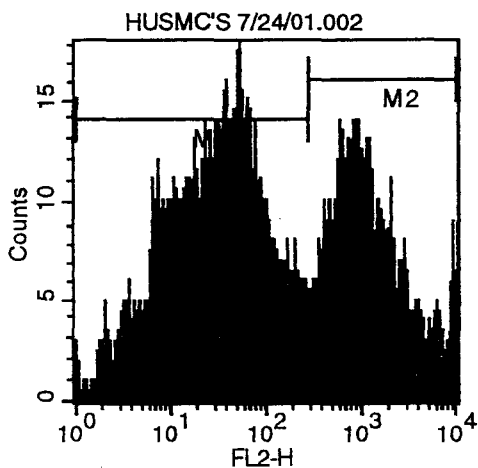
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 2905   | 100.00  | 55.49   | 1046.99 | 667     |
| M1     | 1, 289      | 1094   | 37.66   | 20.90   | 88.79   | 50      |
| M2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



#### 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

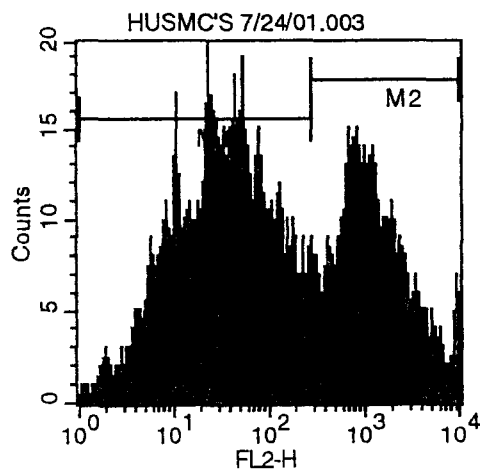
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 4425   | 100.00  | 100.00  | 402.17  | 34      |
| M1     | 1, 289      | 3296   | 74.49   | 74.49   | 52.57   | 34      |
| M2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



#### 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

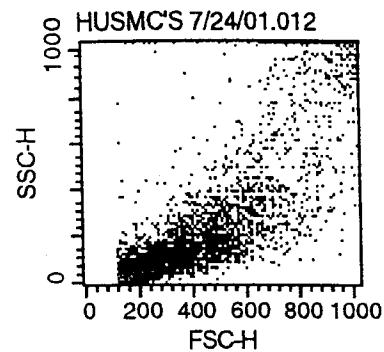
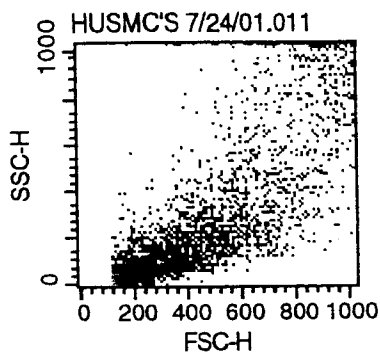
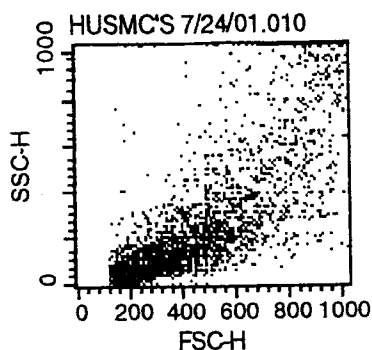
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 4785   | 100.00  | 100.00  | 584.11  | 53      |
| M1     | 1, 289      | 3060   | 63.95   | 63.95   | 53.41   | 53      |
| M2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



#### 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     |



#### Histogram Statistics

File: HUSMC'S 7/24/01.010

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

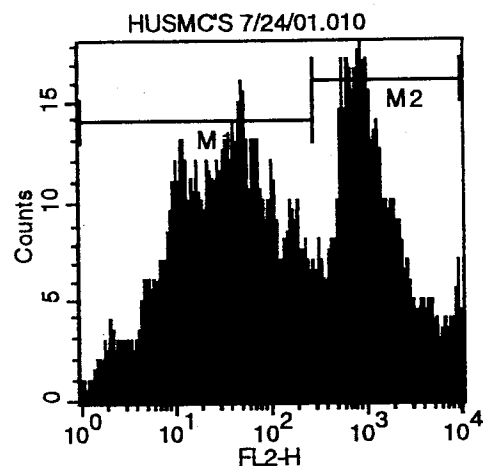
Acquisition Date: 24-Jul-1

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     |



500 VR

#### Histogram Statistics

File: HUSMC'S 7/24/01.011

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

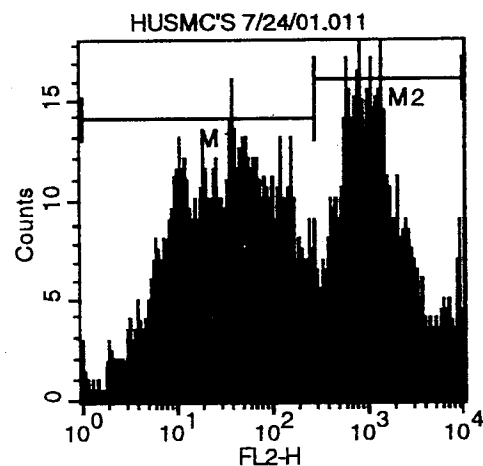
Acquisition Date: 24-Jul-1

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     |



500 VR

#### Histogram Statistics

File: HUSMC'S 7/24/01.012

Sample ID: HUMAN AORTIC SMC'S

Patient ID: TAG=TUBE

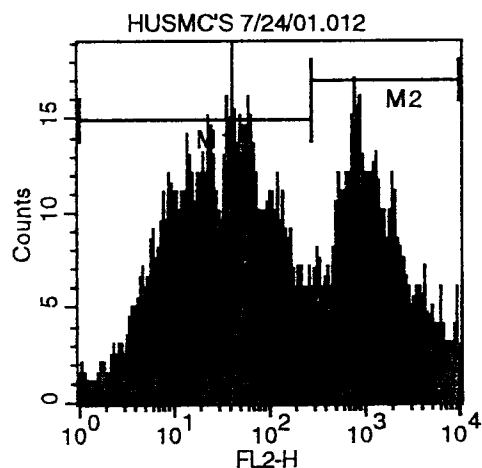
Acquisition Date: 24-Jul-1

Gate: No Gate

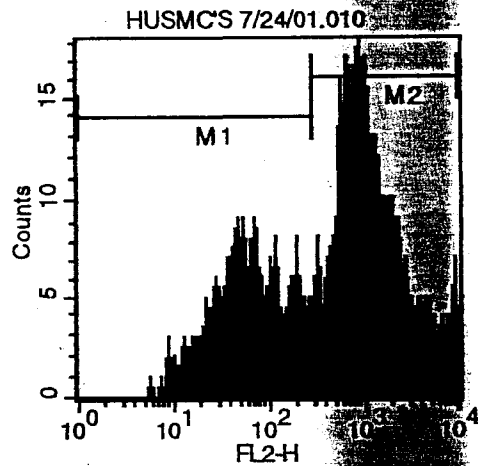
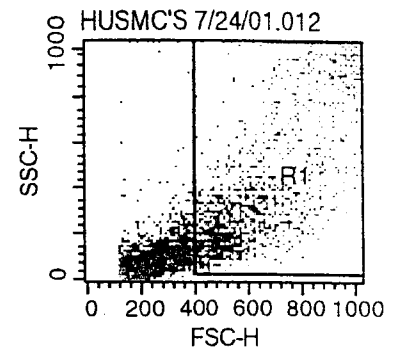
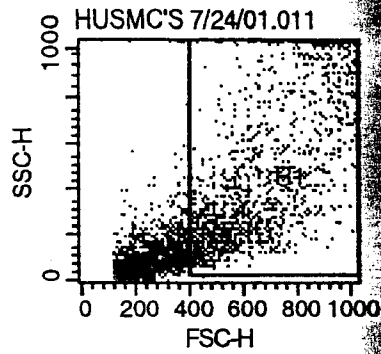
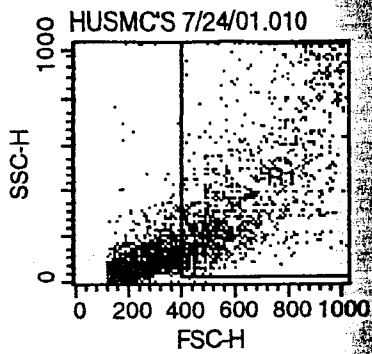
Gated Events: 5000

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     |



500 VR



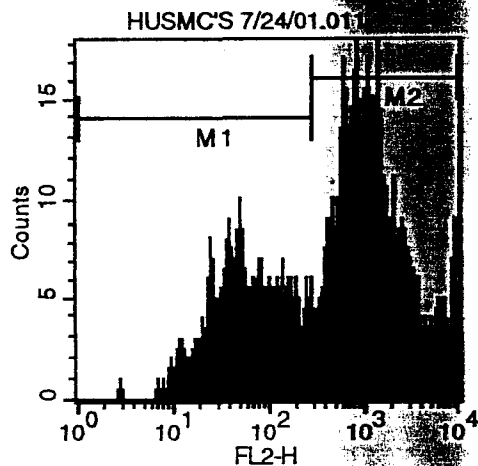
5 uL VR

#### 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     |



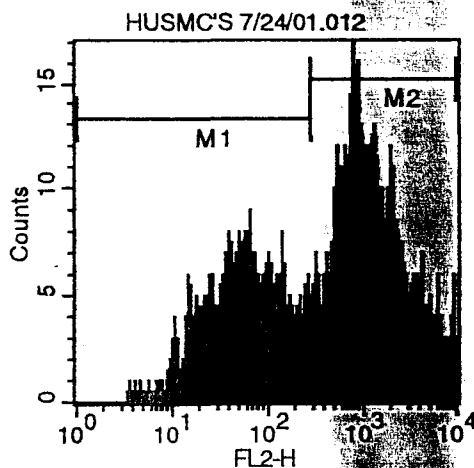
2 uL VR

#### 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     |



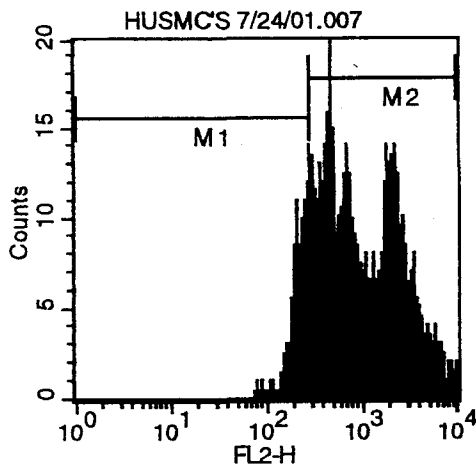
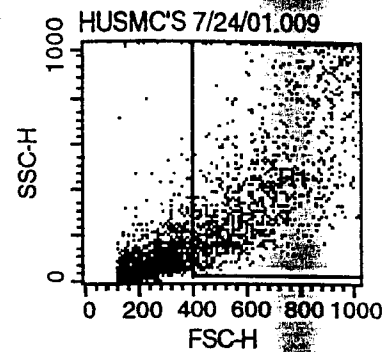
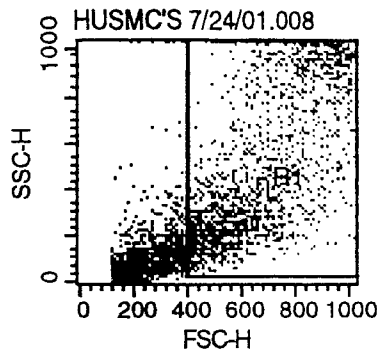
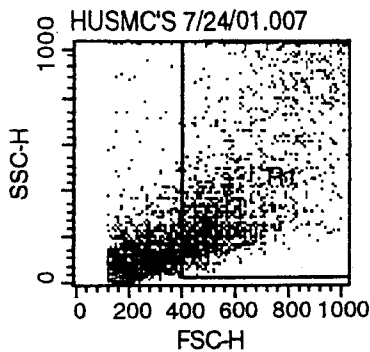
control

#### 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     |



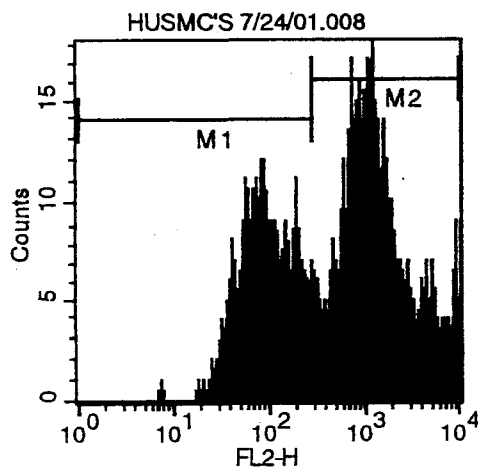
200 uL VR

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

#### Histogram Statistics

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     |



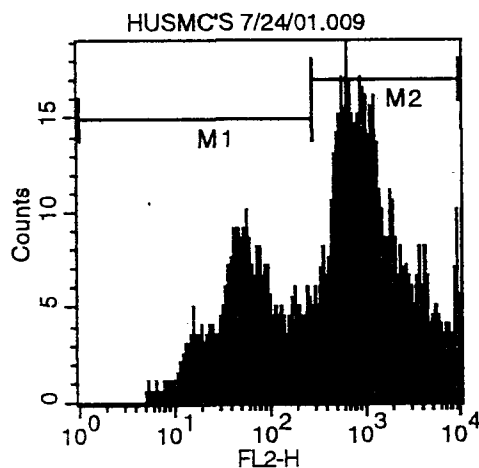
50 uL VR

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

#### Histogram Statistics

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    |



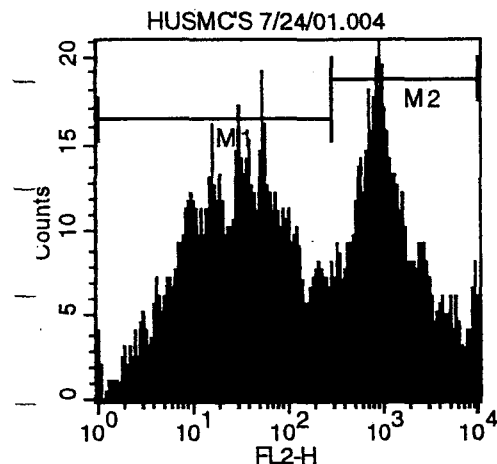
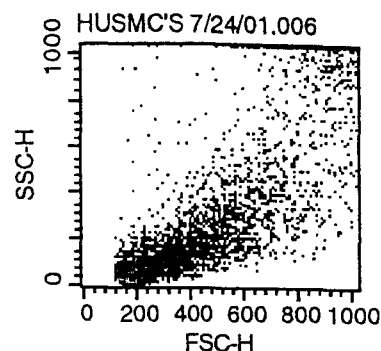
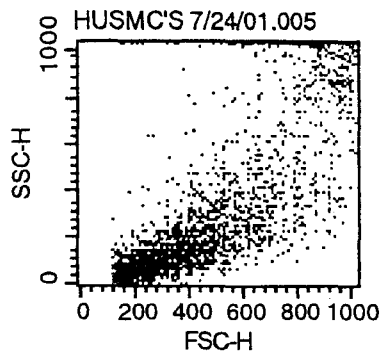
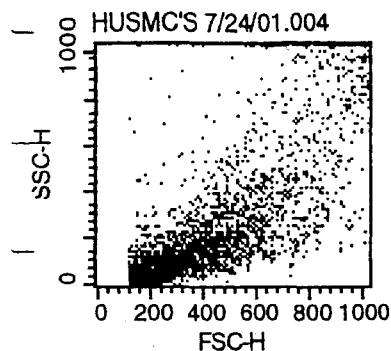
10 uL VR

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

#### Histogram Statistics

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     |

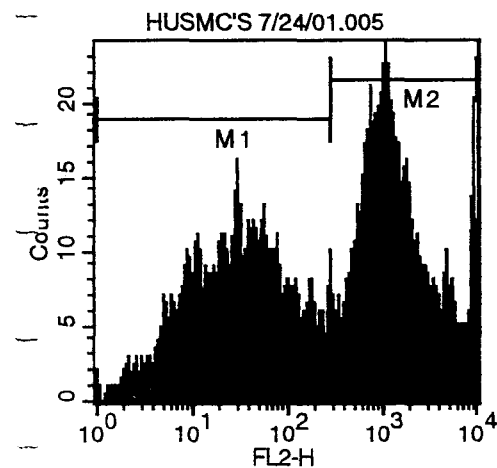


#### Histogram Statistics

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     |

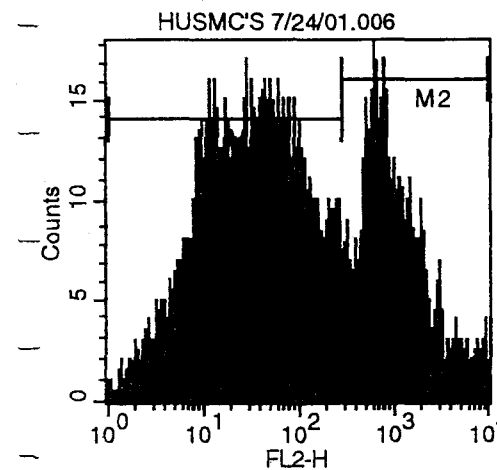


#### Histogram Statistics

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    |



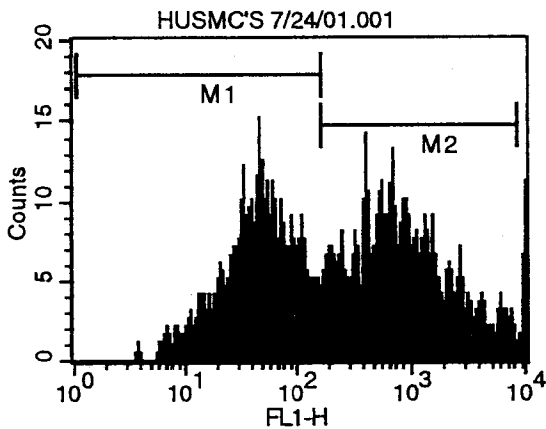
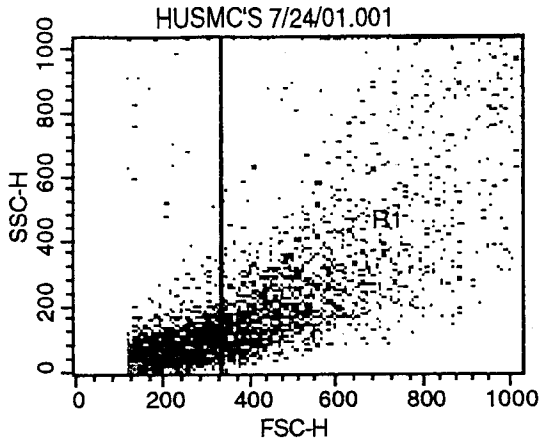
#### Histogram Statistics

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     |

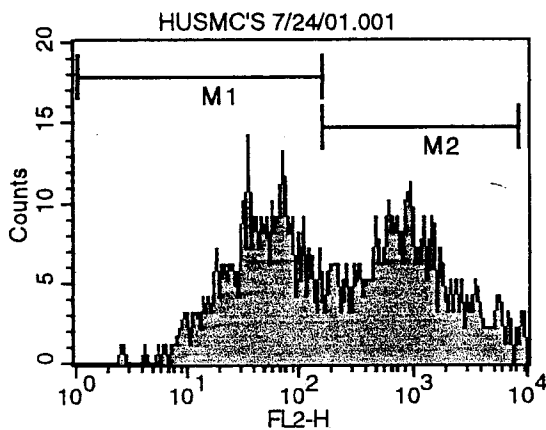
HuAoSMC  
50 uLs Lev



#### 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

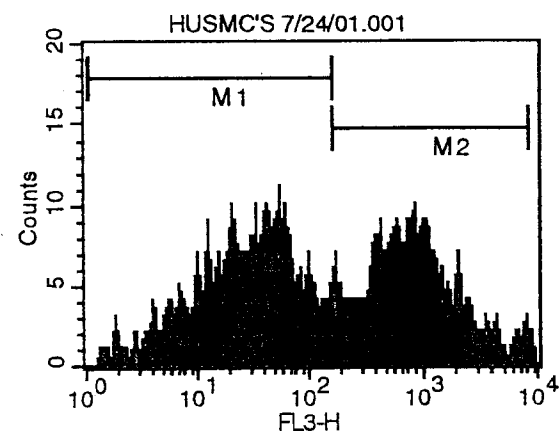
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 2494   | 100.00  | 56.36   | 709.29  | 43      |
| M1     | 1, 158      | 1212   | 48.60   | 27.39   | 55.80   | 43      |
| M2     | 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     |

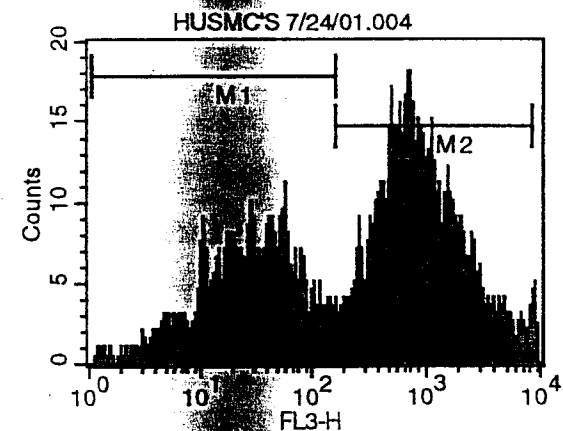
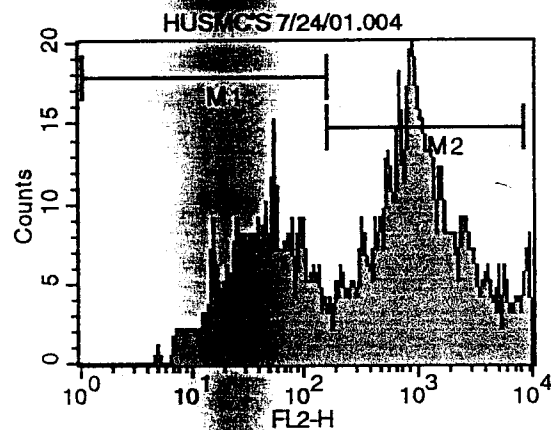
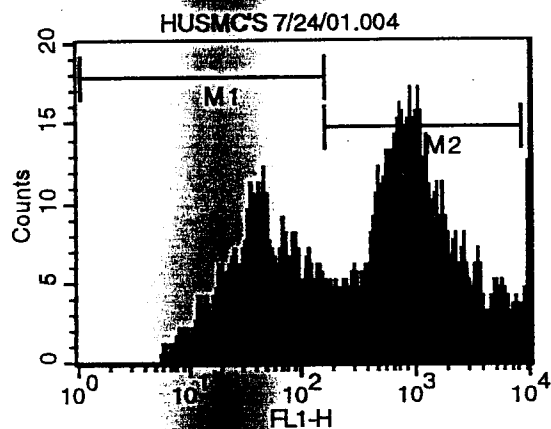
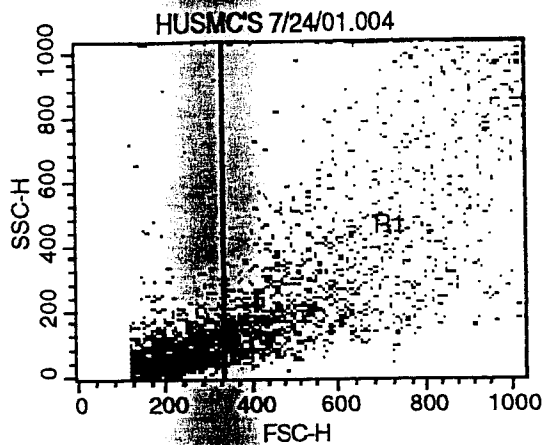


#### Histogram Statistics

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     |

HuAoS MC  
2 ML 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

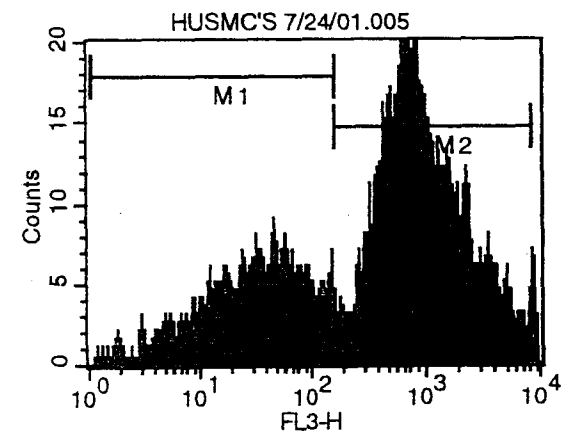
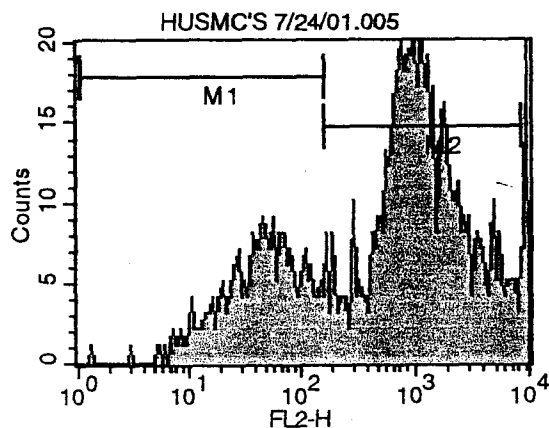
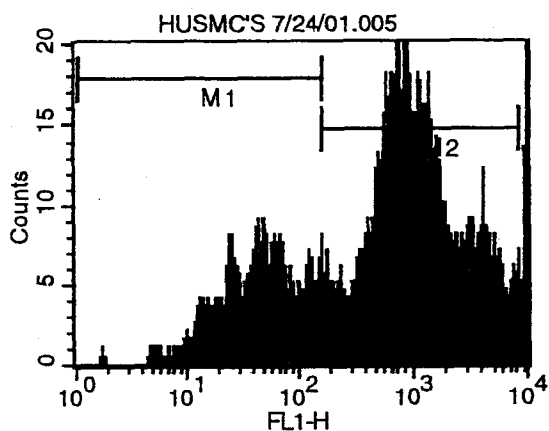
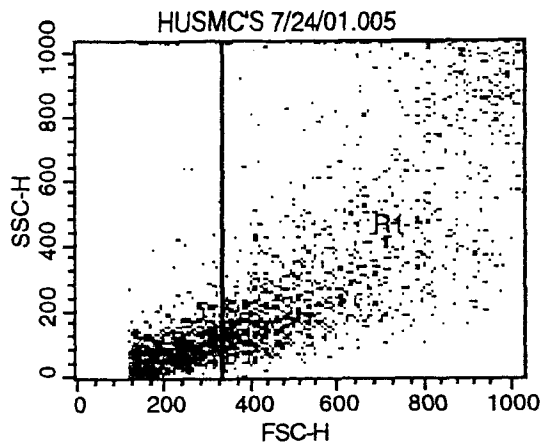
File: HUSMC'S 7/24/01.004 Sample ID: HUMAN AORTIC SMC'S  
Patient ID: TAG=TUBE Acquisition Date: 24-Jul-1  
Gate: G1 Gated Events: 3294  
Total Events: 5000

| 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

~~200000~~

10 uL VR 3 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

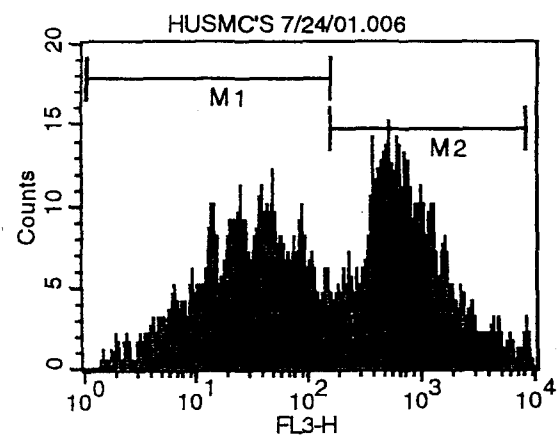
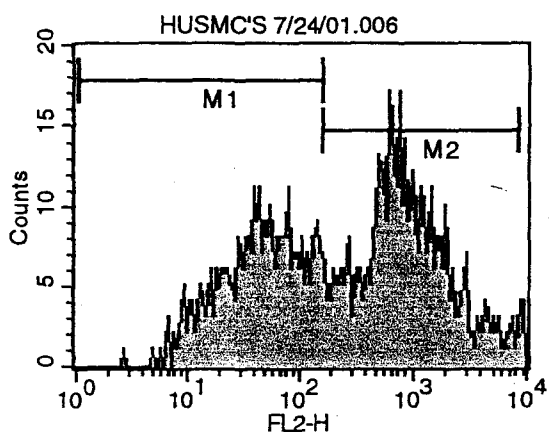
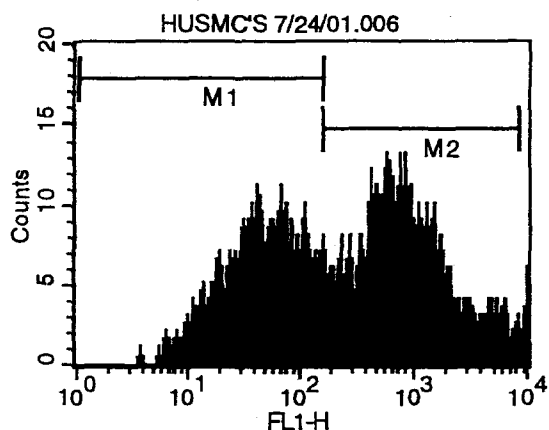
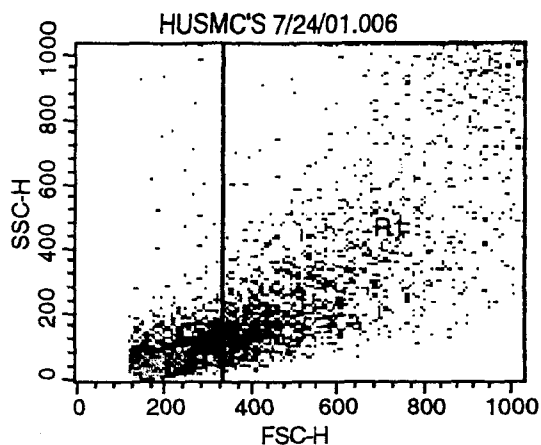
| Marker | Left, Right | Events | % Gated | % Total | Mean    | Peak Ch |
|--------|-------------|--------|---------|---------|---------|---------|
| All    | 1, 9910     | 3739   | 100.00  | 74.78   | 935.87  | 661     |
| M1     | 1, 158      | 1027   | 27.47   | 20.54   | 43.42   | 43      |
| M2     | 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     |

