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The Vascular Research Laboratory Experience

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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 cryopreservation 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 or media divisible by the split. For instance, if splitting the cells into 3 different flasks, the pellet may be re-suspended in 3 mls of media. The media should be spread all over the side growing the cells to insure equal growth throughout the flask. The flasks should be labeled appropriately with a change of passage number and placed in the incubator. The hood should be sprayed with 70% ethanol.

IMMUNOHISTOCHEMISTRY

Introduction:
Immunohistochemistry is not considered a sterile technique.
Technique:

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

PROTEIN MEASUREMENTS

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

**Protein Isolation:**

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

**Homogenizing tissue:**

1 ml of PBS is added to each specimen. 2 tubes are filled with 50 ml of isopropyl alcohol and 2 tubes are filled with de-ionized water. The homogenizing blade is washed in a tube of de-ionized water. The tissue is then homogenized, which is simply grounding the tissue so that it is well mixed. The blade is then washed with water, alcohol, and then PBS. The steps can be repeated as needed for each specimen. Once all of the specimens are homogenized, they are centrifuged at 1,000 G’s for 5 minutes. 1.5 ml microfuge tubes are labeled appropriately. 100 ul of CER I plus some extra is obtained for each vial. Protein inhibitors are added to the CER I in the correct concentration, as noted in the extraction kit. The new CER I solution is kept on ice. Before adding the CER I to the specimens, the supernatant is taken off of the centrifuged tubes. The pellet is resuspended in 0.5 ml of PBS and transferred to smaller vials. The new vials are
centrifuged at 500 G’s for 2 minutes. The supernatant is aspirated, and 100 ul of the chilled CER I is added to each pellet. The pellet is re-suspended via vortex at the highest setting for 15 seconds. The new solution is incubated on ice for 10 minutes. After incubation, 5.5 ul CER II is added to each vial. The solution is vortexed again for 5 seconds on the highest setting. It is then centrifuged for five minutes at the highest setting. The tubes are then put on ice. The supernatant, which is the cytosolic protein, is extracted and stored quickly. Care is taken not to get too close to the pellet.

**Protein Assay:**

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

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Protein concentration (ug/ul)</th>
<th>Protein Standard Solution (ul)</th>
<th>Water (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>125</td>
<td>987.5</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>25</td>
<td>975</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>62.5</td>
<td>937.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>125</td>
<td>875</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>250</td>
<td>750</td>
</tr>
<tr>
<td>7</td>
<td>200</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>
Mixtures are made with 10 uls of each sample and 990 uls of water. Starting with the least amount of water concentration needed, the water is added for the standards and then the correct amount of water is added to the samples. Next, 1 ml of Lowry's Reagant is added to every tube including the standards and the samples. Each tube is vortexed at minimum speed and then sit for 20 minutes. While vortexing for quick and immediate mixing, 0.5 ml of folin is added. The solutions sit for 30 minutes and the spectrophotometer is turned on. All the solutions are then taken to the spectrophotometer. On the spectrophotometer, the quantitative route is chosen, and parameters are changed to print the seven standards, and concentrations are entered. Then, 1 ml of water is added to each cuvet, and auto-zero is pressed. The cuvets should be placed such that the clear sides face sideways and the rippled sides face the front and back. Care should be taken not to touch the cuvet on the flat, clear sides so that the results are not skewed. The front cuvet is discarded and 1 ml of each solution is added, going through the standards first, using a new cuvet each time. The results are printed with a graph of the standard to insure there is a small deviation from the expected results, and a table reporting the unknown concentrations of the protein samples. The desired mass is typically around 30 ug. In order to obtain the desired amount of mass from each sample, the desired mass should be divided by the concentration, and the quotient is the volume needed for each sample to have that mass. Sometimes, samples must be re-concentrated because the amount is too small or large for the desired masses. Microconcentrators with filters are used. Frozen vials are thawed and 90 uls of each sample is used and centrifuged.
Project: Isolating Aortic Smooth Muscle Cells via Fluorescent Markers

Background:

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

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

Experiment:

Canine Fibroblasts (K9 Fibro) and Human Aortic Smooth Muscle Cells (HuAOSMC) were pulled from the storage freezer and grown separately in T-25 and T-75 flasks in Dulbecco’s Modified Eagle’s Media (DMEM) and Iscores Modified Dulbecco’s Media (IMDM), respectively. Once confluent, (and the fibroblasts were confluent much sooner), the cell lines were each plated in wells at varying concentrations (see table below). These solutions were then each tested with Vector Red and Levamisole to determine the best concentration for viability and fluorescence of the cells with the markers.
Vector Red was made using one drop of the A, B, and C alkaline phosphotase droppers for every 2.5 mL of 100mM Tris-HCL. 1 mL of this new solution was mixed with every 9 mLs of PBS. The final solution was then filter sterilized using a syringe and a .02 micron filter with acrodiscs. The Vector Red should be added first in the dark, and incubated immediately because Vector Red reacts with light and is less sensitive the longer the final solution sits.

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

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

Cells Treated with Vector Red

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Amount Vector Red</th>
<th>Amount Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>2 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>200 ul</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>20 ul</td>
<td>1.98 ml</td>
</tr>
<tr>
<td>4</td>
<td>1:500</td>
<td>4 ul</td>
<td>1.996 ml</td>
</tr>
<tr>
<td>5</td>
<td>1:1000</td>
<td>2 ul</td>
<td>1.998 ml</td>
</tr>
<tr>
<td>6</td>
<td>Control (0)</td>
<td>0 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

Cells Treated with Levamisole
<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Amount Levamisole</th>
<th>Amount Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:25</td>
<td>80 ul</td>
<td>2 ml</td>
</tr>
<tr>
<td>2</td>
<td>1:50</td>
<td>40 ul</td>
<td>2 ml</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>20 ul</td>
<td>2 ml</td>
</tr>
<tr>
<td>4</td>
<td>1:200</td>
<td>10 ul</td>
<td>2 ml</td>
</tr>
<tr>
<td>5</td>
<td>1:400</td>
<td>5 ul</td>
<td>2 ml</td>
</tr>
<tr>
<td>6</td>
<td>2:25</td>
<td>160 ul</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration of solution</th>
<th>Approximate Percent dead (floating)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 %</td>
<td>90%</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>
It should also be noted that Wells 5 and 6 appeared to have more dead cells because of over-confluency.

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration of solution</th>
<th>Approximate Percent dead (floating)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1:500</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>5</td>
<td>1:1000</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>6</td>
<td>Control (0 %)</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

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

The cells from each well were then counted with a Hemocytometer using protocol VRL-SOP-607 (Vascular Research Lab-Standard Operating Procedure-607). Each of the 6 wells were trypsinized, i.e. sloughed from the plate to allow mobility of cells. They were then centrifuged
for five minutes at 1500 rpm’s. They were each re-suspended in 1 ml of media (DMEM). Using Trypan Blue the cells were counted.

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

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

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Amount Vector Red</th>
<th>Amount Media (SMGM2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:10</td>
<td>200 ul</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>2</td>
<td>1:40</td>
<td>50 ul</td>
<td>1.95 ml</td>
</tr>
<tr>
<td>3</td>
<td>1:200</td>
<td>10 ul</td>
<td>1.99 ml</td>
</tr>
<tr>
<td>4</td>
<td>1:400</td>
<td>5 ul</td>
<td>1.995 ml</td>
</tr>
<tr>
<td>5</td>
<td>1:1000</td>
<td>2 ul</td>
<td>1.998 ml</td>
</tr>
</tbody>
</table>
Cells Treated with Levamisole

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Amount Levamisole</th>
<th>Amount Media (SMGM2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:40</td>
<td>50 ul</td>
<td>1.95 ml</td>
</tr>
<tr>
<td>2</td>
<td>1:200</td>
<td>10 ul</td>
<td>1.99 ml</td>
</tr>
<tr>
<td>3</td>
<td>1:400</td>
<td>5 ul</td>
<td>1.995 ml</td>
</tr>
<tr>
<td>4</td>
<td>1:1000</td>
<td>2 ul</td>
<td>1.998 ml</td>
</tr>
<tr>
<td>5</td>
<td>1:1:100</td>
<td>10 ul Vector Red</td>
<td>1.98 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ul Levamisole</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Control (0)</td>
<td>0 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Viable</th>
<th>Non-viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:10</td>
<td>0</td>
<td>30,000</td>
</tr>
<tr>
<td>2</td>
<td>1:40</td>
<td>20,000</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1:200</td>
<td>10,000</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1:400</td>
<td>10,000</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1:1000</td>
<td>30,000</td>
<td>10,000</td>
</tr>
<tr>
<td>6</td>
<td>Control (0)</td>
<td>20,000</td>
<td>20,000</td>
</tr>
</tbody>
</table>

Cells Treated with Levamisole

<table>
<thead>
<tr>
<th>Well</th>
<th>Concentration</th>
<th>Viable</th>
<th>Non-viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:40</td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>2</td>
<td>1:200</td>
<td>20,000</td>
<td>30,000</td>
</tr>
<tr>
<td>3</td>
<td>1:400</td>
<td>10,000</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1:1000</td>
<td>10,000</td>
<td>20,000</td>
</tr>
<tr>
<td>5</td>
<td>1:1:100</td>
<td>0</td>
<td>30,000</td>
</tr>
</tbody>
</table>
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 “arteriovenosclerosis” or post-implantation sclerosis if exposed to arterial hemodynamic conditions, which is relevant to the use of vein grafts.

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

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

**The Flow System:**

A 2000 ml Erlenmeyer flask was filled with media and a stop cork was used with holes for the gas-permeable tubing. A pulse dampener and 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.
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Title: Protocol for splitting cells

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

Purpose: To describe the procedures for proper splitting of cells

Procedures:

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

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

3. **Operation Guidelines**
   a. Turn the hood on at least 10 minutes prior to beginning.
   b. Place the Clonetics trypsin/EDTA, CMF, and media with serum in the hood and spray with 70% Ethanol.
   c. Place the cells in the hood.
   d. Aspirate the media off the cells.
   e. Place 5 ml of CMF on the cells and slide around flask to cover cells completely.
   f. Aspirate the CMF off the cells.
g. Place 5 ml of trypsin/EDTA on the cells.

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

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

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

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

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.

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.

Trouble shooting

a. If at any point during the procedure, the sterility of the cells are compromised, the cells are to be disposed off or labeled as being
b. contaminated until further instructions for course of action from the lab supervisor.

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

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

5. Calibration Instructions
   NA

6. Routine Maintenance Instructions and Schedule
   NA

7. Non-routine maintenance instructions
   NA

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

III. References
   None

IV. Attachments
   None

VRL.SOP.601 / 2000 / AS
Freezing Back Cells

Wednesday, May 16, 2001
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)
   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)
   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 syringe
   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.
   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.
f. Place 5 ml of CMF on the cells and slide around flask to cover cells completely.

g. Aspirate the CMF off the cells.

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

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.

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

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.

o. Obtain a 1 cc syringe and place 0.10 cc of DMSO into the vial, dripping it slowly over the cells.

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

V. Calibration Instructions
   NA

VI. 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.

VII. Non-routine maintenance instructions
   NA

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

IV. References
   None

V. Attachments
   None
5/21/01

Notes on project w/ Aniket
"Heat Shock Protein Project"

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

anything that happens, 1st occurs on endothelial cells

atherosclerosis \rightarrow autoimmunologic injury

(HSP) Heat Shock Protein 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 \n dead, outside body \n HSP 70)
  b. took carotid arteries of dogs \n simultaneously exposed to different flow conditions

put arteries in flow system

Normal physiological stress 15 - 30 dynes/cm² (1 vessel exposed)

Extra-physiological stress \rightarrow anything above 60 (1 vessel exposed)

Blood flow, time constant for 4 hrs.

entroma, media, adventitia

Distributive analysis \rightarrow (3 layers of vessel) [via staining] compares amt. in each layer

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

- Static control - cells not exposed to anything
- Static w/o time → Static for 4 hrs. before frozen
- Static w/o time → immedi. After vessel cut, Set, fixed, 1 section frozen

Protein

Static w/o time → immediately → Static w/o time

Flow → Flow

Protein analysis

Extract protein, protein away, Western blotting, Immunoblotting

1% tubes to do

Big picture: exposing venous vessels to arterial stress
Tuesday, May 22, 2001

Protein Isolation

PRODUCT DESCRIPTION

NE-PERM™ Nuclear and Cytoplasmic Extraction Reagents

This kit supplies a complete set of lysis reagents that enable the separation of nuclear extract and cytoplasmic fractions from cultured cells and tissue. The optimized reagents and protocol allow non-denatured, active proteins to be purified in less than two hours. This kit contains sufficient cytoplasmic and nuclear extraction reagents for extracting 50 cell pellet fractions with a packed cell volume of 10 ml (a total of -2.0 g of cell paste).

Product Description

Number

Description

833

NE-PERM™ Nuclear and Cytoplasmic Extraction Reagents

This kit supplies a complete set of lysis reagents that enable the separation of nuclear extract and cytoplasmic fractions from cultured cells and tissue. The optimized reagents and protocol allow non-denatured, active proteins to be purified in less than two hours. This kit contains sufficient cytoplasmic and nuclear extraction reagents for extracting 50 cell pellet fractions with a packed cell volume of 20 ml (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), 5 ml

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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[Stock]</th>
<th>[CER I]</th>
<th>[CER II]</th>
<th>[NER]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzamidine</td>
<td>250 mg/ml</td>
<td>0.5 mg/ml</td>
<td>N/A</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>2 mg/ml</td>
<td>2 µg/ml</td>
<td>N/A</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>2 mg/ml</td>
<td>2 µg/ml</td>
<td>N/A</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>PMSF (Phenylmethylsulfonyl)</td>
<td>0.2 M</td>
<td>0.75 mM</td>
<td>N/A</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Protocol

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

<table>
<thead>
<tr>
<th>Packed Cell Volume</th>
<th>CER I</th>
<th>CER II</th>
<th>NER</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µl (20 mg)</td>
<td>100 µl</td>
<td>3.5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>20 µl (40 mg)</td>
<td>200 µl</td>
<td>11 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>50 µl (100 mg)</td>
<td>500 µl</td>
<td>27.5 µl</td>
<td>250 µl</td>
</tr>
<tr>
<td>100 µl (200 mg)</td>
<td>1 ml</td>
<td>55 µl</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

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

Telephone: 800-3-PIERCE (800-374-3723) or 815-968-9747 • Fax: 815-968-7316 or 800-842-5007

Internet: http://www.piercenet.com
1. Isolate 20 µl packed cell volume (40 mg) of cells by centrifugation in a 1.5 ml microcentrifuge tube by centrifugation at 500 x g for 2-3 minutes. **Note:** If using tissue samples, cut the tissue into small pieces, add an appropriate buffer such as PBS, and homogenize in a tissue homogenizer. Pellet the cells by centrifugation at 300 x 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 proceed with Step 4 below. Alternatively, weigh the tissue, cut it into small pieces, dounce homogenize directly in CER I, and proceed to Step 4. Use a 10-fold excess of CER I over the weight of tissue (e.g., 500 µl CER I to 50 mg tissue). In Step 5 of the protocol, use 5.5 µl of CER II per 100 µl of CER I.

2. Using a pipet, carefully remove and discard the supernatant, leaving cell pellet as dry as possible.

3. Add 200 µl of ice-cold CER I to the cell pellet.

4. Vortex vigorously on the highest setting for 15 seconds to fully resuspend the cell pellet. Incubate the tube on ice for 10 minutes.

5. Add 11 µl of ice-cold CER II to the tube.

6. Vortex 5 seconds on the highest setting. Incubate on ice for 1 minute.

7. Vortex 5 seconds on the highest setting. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge (16,000 x g).

8. Immediately transfer the supernatant (cytoplasmic extract) fraction to a clean pre-chilled tube. Place this tube on ice.

9. Resuspend the insoluble (pellet) fraction produced in Step 7, which contains nuclei, in 100 µl of ice-cold NER.

10. Vortex on the highest setting for 15 seconds. Return the sample to ice and continue vortexing for 15 seconds every 10 minutes, for a total of 40 minutes.

11. Centrifuge the tube at full speed (16,000 x g) in microcentrifuge for 10 minutes.

12. Immediately transfer the supernatant (nuclear extract) fraction to a clean pre-chilled tube. Place on ice.

13. Store all extracts at -80°C until use.

**Troubleshooting Guide**

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

**Additional Pierce Products of Interest**

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>23235</td>
<td>Micro BCA™ Protein Assay Reagent Kit</td>
</tr>
<tr>
<td>34080</td>
<td>SuperSignal® West Pico Chemiluminescent Substrate</td>
</tr>
</tbody>
</table>

©Pierce Chemical Co. 10/1999. Printed in the U.S.A.
<table>
<thead>
<tr>
<th>Static 0</th>
<th>Static 4hr</th>
<th>Normal 4hr</th>
<th>Extra-physiological 4hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>7mm</td>
<td>10 mm</td>
<td>17 mm</td>
<td>12 mm</td>
</tr>
<tr>
<td>used approx 1/3 used approx 1/6 for protein analysis</td>
<td>protein analysis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**What's Going On:**

- **CER I**
  - Putting hypotonic solution on cells (cell swell)
  - Add in CER II + detergent + pierces holes in cell + protein come out, ER, etc. (blebs)
  - Nucleus doesn't come out → NER added
  - Causing cell to shrink causing nucleus to come out.

- **Steps:**
  1. 1.0 mL of PBS in each
  2. 2 tubes (50 mLs) of isopropyl alcohol
  3. 2 tubes (50 mLs) of de-ionized H₂O
  4. Homogenizing blade washed w/ H₂O
  5. Static O homogenized (attempt)
  6. blade washed w/ H₂O, alcohol, then PBS
  7. PBS added to static O and homogenized
  8. blade washed w/ H₂O, alcohol, then PBS
  9. Static 4 hr homogenized
  10. blade washed w/ H₂O, alcohol, then PBS
  11. Normal 4 hr homogenized
  12. Extra-physiological 4 hr homogenized
  13. blade washed w/ H₂O, alcohol, then PBS
  14. Follow instructions on previous 2 pg (11-12)
  15. Put tubes in centrifuge at 1,000 G's for 5 min.
  16. Big microfuge tubes (1.5 mL) labeled
  17. 500 µL CER 1 (200 µL for each vial + extra for aspiration + backup)
  18. Addition of protein inhibitors to CER 1: 1 mL Benamidine, 1/2 µL Aprotinin, 2.5 µL Leupeptin, 1/2 µL PMSF
(16) Put CER I on ice (does not have to be sterile)

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

(21) Resuspend pellet in ~ 500 μL of PBS and transferring to smaller vials.

(23) Centrifuge at 500 Gs for 2 min. (last few steps trying to get down to smaller volume to do big steps)

(23) Take out supernatant

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

(25) Resuspend via vortex (highest setting 15 sec)

(26) Incubate on ice for 10 minutes

(27) 5.5 μL CER I added to each vial.

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

(29) Incubate on ice for 1 min.

(30) Vortex again for 5 sec.

(31) Centrifuge for 5 min. @ highest setting

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

(33) Supernatant is protein → extract supernatant 1/2 store. (do quickly) be careful not to get too close to pellet (really, don’t need that much protein anyway)

Not extracting NER so only went through step 8 on pg. 11-12
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
**INTRODUCTION**

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

Any protein, the Lowry reaction can be run directly in a solution. However, interference in the direct Lowry is caused by commonly used chemicals, such as Tris, succinate, EDTA, sucrose, dextran, amino acid, and peptide and phenolic compounds. The procedure with protein precipitation, uses DCC (dechoxylate) and TCA (trichloroacetic acid), to remove or minimize the interference with the exception of phenolic. The amount of various proteins recovered through the step may vary depending on the particular protein.

**PRINCIPLE**

Amine in the tissue reagent complexes with the peptide to form a purple-blue color when the phenol reagent is added. The absorbance is read at a suitable wavelength between 595 and 600 nm. The protein concentration is determined by a calibration curve.

**REAGENTS**

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

1. **REAGENT, MODIFIED, Catalog No. 1013**
   - CORROSIVE. Avoid contact with eyes, skin, and clothing. Do not breathe dust.
   - VRY REAGENT SOLUTION is prepared by adding 40 ml of reagent to a bottle of Lowry Reagent. Mix well until it completely dissolves. Do not shake so as to form a foaming. Solution is stable at room temperature. Store REFRIGERATE.

2. **ULTRASON, Catalog No. 05525**
   - USA solution of sodium dechoxylate, 1.5 mg/ml.
   - 2.4% ACETIC ACID SOLUTION (TCA), 1 ml 4396
   - USA solution of trichloroacetic acid, 72% w/v.
   - CORROSIVE. CAUSES BURNS. Avoid contact with eyes, skin, and clothing.
   - FAULIN & CICOALTIEU'S PHENOL REAGENT, No. 7656
   - CORROSIVE. CAUSES BURNS. Avoid contact with eyes, skin, and clothing. Do not breathe vapor.

**DIRECT PROCEDURE**

*Without Protein Precipitation*

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

<table>
<thead>
<tr>
<th>Protein Standard Solution (ml)</th>
<th>Water (ml)</th>
<th>Protein Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.750</td>
<td>1.000</td>
<td>30</td>
</tr>
<tr>
<td>5.000</td>
<td>1.250</td>
<td>10</td>
</tr>
<tr>
<td>0.750</td>
<td>1.250</td>
<td>300</td>
</tr>
<tr>
<td>0.250</td>
<td>1.250</td>
<td>300</td>
</tr>
<tr>
<td>0.125</td>
<td>1.250</td>
<td>300</td>
</tr>
</tbody>
</table>

2. Label one test tube BLANK and add 1.0 ml water.
3. Add sample to approximately 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 & Cicoaltieu'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 content in the original sample.
Concentrations for standard in Protein Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Standard</th>
<th>Standard</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µl</td>
<td>0 µg/ml</td>
<td>0</td>
<td>1000 µl</td>
</tr>
<tr>
<td>0 µl</td>
<td>5 µg/ml</td>
<td>0</td>
<td>125 µl</td>
</tr>
<tr>
<td>10 µl</td>
<td>10 µg/ml</td>
<td>25 µl</td>
<td>125 µl</td>
</tr>
<tr>
<td>50 µl</td>
<td>25 µg/ml</td>
<td>125 µl</td>
<td>937.5 µl</td>
</tr>
<tr>
<td>100 µl</td>
<td>50 µg/ml</td>
<td>500 µl</td>
<td>937.5 µl</td>
</tr>
<tr>
<td>200 µl</td>
<td>100 µg/ml</td>
<td>250 µl</td>
<td>937.5 µl</td>
</tr>
</tbody>
</table>

10 µl sample + 990 µl H2O

OD at 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/ 574 (Put water concentration in 7th has lowest H2O concentration) from 0 to 1
3. For samples, put 900 mL H2O in all of them
4. Put standard 1 (protein standard) ... from lowest volume to highest of standard (1-7)
   - can make it from kit
   - Just making concentrations H330 (HSP K9X3 Static 0) S4 STATIC HFR
   NO = normal
   EP = extraphysiological
5. Label vials matched to red tubes
   1. H330
   2. H354
   3. H34NO
   4. H34EP
   5. H450
   6. H45H
   7. H44NO
   8. H44EP

   Today:
   - Lowry's Reagent change 3 bottles
   - I worry for all HSP, a 153 standard
   - bottle in 47 standards
   - add 5% H2O before adding
   - may affect results
   - check sure you have enough stuff.
   - add 100L Lowry Reagent to every tube (standards & HSPs)
   - vortex each tube @ min. speed
   - let sit for 20min.

6. Adding 10 mL of protein according to table
7. Add 100L Lowry Reagent to every tube (standards & HSPs)
   - vortex each tube @ min. speed
   - let sit for 20min.
8. Add 15 mL form → quick immediate mixing → add while vortexing
9. Wait 30 min. Turn on Spectrophotometer, now
10. Check quantitative, push back, change parameters (7 standards, print), enter concentrations
11. Add 1 mL H2O to each cuvet
12. Press auto-zero
**Quantitative Analysis**

<table>
<thead>
<tr>
<th>No.</th>
<th>Abs</th>
<th>Conc</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.147</td>
<td>12.932</td>
<td>24.93 mL</td>
</tr>
<tr>
<td>2</td>
<td>0.113</td>
<td>5.5769</td>
<td>53.80 mL</td>
</tr>
<tr>
<td>3</td>
<td>0.126</td>
<td>7.2975</td>
<td>41.109 mL</td>
</tr>
<tr>
<td>4</td>
<td>0.184</td>
<td>2.3483</td>
<td>127.75 mL</td>
</tr>
<tr>
<td>5</td>
<td>0.119</td>
<td>3.7470</td>
<td>80.0 mL</td>
</tr>
<tr>
<td>6</td>
<td>0.122</td>
<td>6.4368</td>
<td>46.561 mL</td>
</tr>
<tr>
<td>7</td>
<td>0.276</td>
<td>38.929</td>
<td>7.706 mL</td>
</tr>
<tr>
<td>8</td>
<td>0.215</td>
<td>26.979</td>
<td>11.16 mL</td>
</tr>
</tbody>
</table>
**Quantitative Analysis**

<table>
<thead>
<tr>
<th>No.</th>
<th>Abs.</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.147</td>
<td>12.832</td>
</tr>
<tr>
<td>2</td>
<td>0.118</td>
<td>5.5769</td>
</tr>
<tr>
<td>3</td>
<td>0.126</td>
<td>7.2975</td>
</tr>
<tr>
<td>4</td>
<td>0.184</td>
<td>2.7483</td>
</tr>
<tr>
<td>5</td>
<td>0.118</td>
<td>3.7478</td>
</tr>
<tr>
<td>6</td>
<td>0.122</td>
<td>6.4368</td>
</tr>
<tr>
<td>7</td>
<td>0.279</td>
<td>38.929</td>
</tr>
</tbody>
</table>

(11/19) Change front curet & add 1mL going thru each
(9/15) Point results (graph of standard, table of
- get concentration (mass/volume)
- want 30 mg mass
So divide mass by concentration (m

1/3 standards)
rough stuff, may have to

(used to check filtration technique & make sure that
2, 3, 5, 46 will have to re-concentrate to get small enough amount for gel.

Re-concentration

Use microconcentrators (have filters)

Rub frozen vials around fingers to thaw

Added all (90 ul) of each to microconcentrators

Spin down
05/24/01  Nuts Protein extraction (again)  (like protein isolation pg. 11)

(4) H34EP  - protein extracted again  
(9) G2H20S  
(10) H24S  
(11) H24NO  
(12) H24EP  

Supernatant Extracted.
Resuspended in 0.5 mL PBS  
Centrifuged  
1 mL of OtR I made  
Supernatant discarded  
Vortex (15 s)  
Incubate in ice (10 min.)  
5/24/01  
(13) H10S  
(14) H14NO  
(15) H14EP
\[ C = k \cdot \text{ABS} + B \]
\[ k = 2.84 - 0.371 \]

---

<table>
<thead>
<tr>
<th>No.</th>
<th>ABS</th>
<th>CONC ( \times 10^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.186</td>
<td>42.544.6958 43.59</td>
</tr>
<tr>
<td>2</td>
<td>0.112</td>
<td>46.6926 44.83</td>
</tr>
<tr>
<td>3</td>
<td>0.245</td>
<td>8.5933.949 7.74</td>
</tr>
<tr>
<td>4</td>
<td>0.186</td>
<td>36.405.4946 54.60</td>
</tr>
<tr>
<td>5</td>
<td>0.198</td>
<td>7.2324.155 12.41</td>
</tr>
<tr>
<td>6</td>
<td>0.168</td>
<td>11.0018.174 16.51</td>
</tr>
<tr>
<td>7</td>
<td>0.129</td>
<td>19.4318.197 29.95</td>
</tr>
<tr>
<td>8</td>
<td>0.149</td>
<td>14.0014.281 21.01</td>
</tr>
<tr>
<td>9</td>
<td>0.127</td>
<td>20.499.6979 30.17</td>
</tr>
<tr>
<td>10</td>
<td>0.138</td>
<td>16.8211.884 25.27</td>
</tr>
<tr>
<td>11</td>
<td>0.099</td>
<td>70.922.1999 13.38</td>
</tr>
<tr>
<td>12</td>
<td>0.155</td>
<td>12.9215.479 17.38</td>
</tr>
<tr>
<td>13</td>
<td>0.172</td>
<td>12.7210.686 38.07</td>
</tr>
<tr>
<td>Protocol</td>
<td>(1-4 wells)</td>
<td>(5-8 wells)</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>(1-4 wells)</td>
<td>(5-8 wells)</td>
</tr>
<tr>
<td></td>
<td>wash PBS (15 min)</td>
<td>1. Wash PBS 5 min</td>
</tr>
<tr>
<td></td>
<td>2. add protease antigen retrieval 10 min</td>
<td>2. add protease antigen retrieval 10 min</td>
</tr>
<tr>
<td></td>
<td>3. add PBS wash 5 min</td>
<td>3. add PBS wash 5 min</td>
</tr>
<tr>
<td></td>
<td>4. add protein block (10 min)</td>
<td>4. add protein block (10 min)</td>
</tr>
<tr>
<td></td>
<td>5. add 1° HSP Ab 1 hr</td>
<td>5. add 1° HSP Ab for 1 hr</td>
</tr>
<tr>
<td></td>
<td>add 1:90 to well 1</td>
<td>1:90 to well 5</td>
</tr>
<tr>
<td></td>
<td>1:180 to well 2</td>
<td>1:180 to well 6</td>
</tr>
<tr>
<td></td>
<td>1:360 to well 3</td>
<td>1:360 to well 7</td>
</tr>
<tr>
<td></td>
<td>(-) control well 4</td>
<td>(-) control well 8</td>
</tr>
<tr>
<td></td>
<td>6. wash PBS 5 min</td>
<td>6. wash PBS 5 min</td>
</tr>
<tr>
<td></td>
<td>7. Anti mouse 2° Ab (Link) 30 min.</td>
<td>7. Anti mouse 2° Ab Link 30 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. Other domains of fibronectin are also involved with this adhesion process and may involve interaction with collagen, heparin and other cell surface glycosaminoglycans. The conformation and orientation of adsorbed fibronectin is also important and has an effect on cell spreading and strength of adhesion of endothelial cells. Fibronectin addition to serum-free medium promotes cell adhesion. More significant effects are observed with BHK, CHO and other cell lines by coating of cultureware with fibronectin at 1-5 ug/cm². 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.

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 & PHYSICAL FORM:
1 milligram per vial, lyophilized.

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

RECONSTITUTION & USE:
Equilibrate vial to room temperature. Resuspend in one milliliter sterile distilled water. Allow 30 minutes for material to go into solution. DO NOT AGITATE OR SWIRL. If entire amount of material is not to be used immediately, transfer into appropriate aliquots and store at -20°C. It is recommended that solubilized product is used within two weeks. DO NOT STORE IN FROST-FREE FREEZER. AVOID MULTIPLE FREEZE THAWS.

Human Fibronectin is generally used in the concentration range of 1-5 micrograms per cm² 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.
QUALITY CONTROL: 

- 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: 


Coating Procedure

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

1) Dilute fibronectin to desired concentration using serum-free culture Ca++, Mg++ free medium or buffer at pH 7.9. The final solution should be sufficiently dilute so that the volume added will cover the surface evenly.
   
   Example: If the final coating concentration will be 5 µg/cm², dilute the material to 50 µg/ml and add 1 ml/35 mm dish, 3 ml/60 mm dish, etc.
   
   NOTE: Because of the CAPS component in the HFN preparation, buffers of media containing Ca ++ and/or Mg ++ added to the HFN may result in the formation of insoluble metal hydroxides. This will not occur if the buffering capacity of the diluent brings the pH to 8.0 or lower.

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

3) Incubate at room temperature for 1 hour.

4) Aspirate remaining material.

5) Rinse plates carefully with dH₂O - 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, begin growing

[Diagram]

HuAoSMC

K9 Fibroblasts

Vector Red

Lev

V.R. - makes 3 mLs

- figure out optimal ratios

Levamisole
Title: H&E staining of paraffin embedded tissue slides

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

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

III. Procedures:

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

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

3. Protocol Guidelines
   a. Deparaffinize and hydrate tissue to deionized H₂O (VRL.SOP.)
   b. Stain in Richard-Allen II hematoxylin for 1 minute at room temperature (RT). (Harris hematoxylin substituted for 12 minutes at RT is an acceptable substitution)
   c. Rinse in tap water until glass is clear.
   d. Differentiate by dipping 5-10 times in 1% acid alcohol.
   e. Place in ammonia water to blue. (<10 sec)
   f. Rinse in warm running water for 30 sec.
   g. Place in 80% Alcohol for 1 min.
   h. Place in 1% working eosin for 30 sec.
   i. Serially place in 3 changes of 95% Alcohol for 30 sec each change.
   j. Place in absolute alcohol for 1 min.
k. Clear in 3 changes of Xylene for 2 min each change.
l. Coverslip with Permoun and coverslip in fume hood.

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

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

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

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

8. Trouble shooting
   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
IV. References

V. Attachments
NA.

VRL-SOP.307 2000 / RLD
Vector Red for sorting project
06-20-01

Checking for toxicity & flow

Fibroblasts in Vector Red

A

Make 3 mLs of Vector Red

Well 1 → 10 mLs Vector Red

1:10
2 → 200 uLs VR + 1.8 mL media

1:100
3 → 20 mLs VR + 1.99 mL media

1:500
4 → 4 mLs VR + 1.994 mL media

1:1000
5 → 2 mLs VR + 1.998 mL media

B

Levamisol 1 drop = 1 mL

1:1
1 → 2 drops Lev + 2 mLs media

1
2 → 1 drop Lev + 2 mLs

1:100
3 → 20 mLs Lev + 3 mLs

1:1000
4 → 10 mLs Lev + 3 mLs

1:500
5 → 5 mLs Lev + 2 mLs

1:4
6 → 4 drops Lev + 2 mLs

How many uLs is one drop? 38 uLs
Jun 21, 01  Vector Red + Levamisole on fibroblasts.

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

Under microscope:

(A) Vector Red (Gibre)

- About 38% dead (floating)
  in clusters, some exploded
  (2mLs VR)

- 10% dead (floating)
  (20mLs VR)

- 21% dead
  control

(B) Levamisole

- 4 mLs VR

- 2.5% dead
  (20mLs VR)

- 2% dead
  (2mLs VR)

- < 5% dead
  (50mLs Lev)

- < 1% dead
  (50mLs Lev)

- 90% dead
  (160mLs Lev)

Not confident although areas are.

- Dead probably
  more b/c wells
  are confluent.

- Had most Lev
  in it.  (about 160mLs)
June 21, 01

Separately 4 at a time

Trypsinize each well (1 mL trypsin)

Spin down

Resuspend in 1 mL of media

---

Title: Cell Counting with a Hemocytometer

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

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

III. Procedures:

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

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

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

4. Trouble shooting
   Same as VRL.SOP.601
<table>
<thead>
<tr>
<th>SOP Number</th>
<th>University of Tennessee Medical Center Vascular Research Lab</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VRL-SOP-607</td>
<td>Standard Operating Procedures</td>
<td>2 of 2</td>
</tr>
</tbody>
</table>

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
Making Vector Red 7-22-01 need 285 ml

100 ml Tris - HCl

Pour every 2.5 ml and 1 drop A, B, & C solutions

10 ml 1 M + 90 PBS acrosol
Syringe filtered sterilized (using .02 micron filter)

Making Levamisol

2 drops of Levamisol per 2.5 ml of 160 mM Tris - HCl

80 ml
Smooth Muscle Cells

7-23-01

16 hrs. Tagging Markers

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

- 200 µL VR
- 1.8 mL 5% FBS
- 1.75 mL red
- 1.99 mL red

- 50 µL VR
- 1.945 mL 5% FBS
- 1.99 mL 5% FBS

- 2 µL
- 1.945 mL 5% FBS
- 1.99 mL 5% FBS

Control

7/4 VE 11/5 VE 12/6 VE

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

Leumisol

- 50 µL LEV
- 1.95 mL red
- 1.99 mL 5% FBS

- 10 µL LEV
- 1.985 mL 5% FBS

- 5 µL LEV
- 1.985 mL 5% FBS

- 2 µL LEV
- 1.985 mL 5% FBS

Control

4/1-2

5/5

FACS

Trypan blue

PET

FACS

Trypan blue

Mixed accident
Tris buffered saline

Trypsinized
Re-suspend in medium
Aspirate
Re-suspend in paraformaldehyde

Trypsinized
Dilute in media → 5mM2
Take sample for trypan blue - 25 uL sample
Spin, fix
1500 rpm"para formalddehyde"

Aspirate media, wash w/ CMF strip w/ 1 mL trypsin dilute to 5mM2
- take sample for trypan blue - 25 uL → Results
- Spin remaining in centrifuge 1500 rpm for 5 min.
- Aspirate
- Bring up pellet in 1 mL formaldehyde
- Take to flow tubes
- Take to flow

FACS
Fluorescent Activated Cell Sorter/Scanner
### Cell Cytometer Results (Trypan Blue)

<table>
<thead>
<tr>
<th>Cell #</th>
<th>Concentration</th>
<th>Viable</th>
<th>Non-viable</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR 1</td>
<td>200 μL VR</td>
<td>0</td>
<td>3</td>
<td>$\times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td></td>
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<td>1</td>
<td>2</td>
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<td>control</td>
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![Diagram](image)
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 aqeuously 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 PIPE REAGENTS DIRECTLY FROM BOTTLES. Drop volumes of each component may be different due to solvent characteristics. Proper concentrations of substrate components in the working solution are assured only by using the drop dispensers.

INSTRUCTIONS FOR USE:
Prepare the Vector® Red substrate working solution immediately before use.

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

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

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

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

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

For aqueous mounting in VECTASHIELD® Mounting Medium: Tap excess buffer from sections and count. 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 with Texas Red® or rhodamine excitation and emission filter systems. Vector® Red fluoresces visibly 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, precipitate may form in some reagents upon prolonged storage. This will have no effect on the quality or intensity of the staining. Do not filter the reagents or working solution.
4. Do not put sodium azide in the buffer used for the Vector® Red working solution; it will prevent staining.
5. For tissue, using 0.1% Tween® 20 in the Vector® Red working solution may increase the sensitivity and crispness of staining especially when non-enzymatic antigen retrieval methods are used in the immunostaining protocol. Tween® 20 should not be added to the Vector® Red substrate solution for membrane applications.
6. When using neural tissue, Vector® Red is not recommended for visualizing processes, fibers, or terminals (inadequate staining may occur).
7. Endogenous alkaline phosphatase activity (other than the intestinal isoenzyme) can be inhibited by the addition of levamisole (Cat. No. SP-5000) to the buffer prior to the preparation of the working solution. Intestinal alkaline phosphatase activity can be inhibited, before immunostaining, with several tissue pretreatments (Bulman AS and Heydeman 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.
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.
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 excitation 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." 
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
Fluorescent wavelengths

488 NM
ARGON LASER

SSC = RALS = ORTHOGONAL LS

FL-1

FL-2

FL-3

FSC = FALS

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

525 NM
575 NM
HSP Project → Flow System

2000 mL Erlenmeyer flask

gas permeable tubing

2000 mL media

pump → pulsitik pump

pulsed dampener

stepper cart

connectors

pressure transducer

computer program

cut-off 25 mL pipets used to keep vessel stable

→ no curvature desired

tie-down to tie vessels to connectors

must incubate flask in water to keep temp. of system @ 37°
Want from ATLS K9

SMC from Aorta  

fibroblasts from Carotid  

endothelial Iliac

---

may be mixed or sorted.

Try FACS/Flow test using Vector Red

---

Want to eventually try to sort fibro + SMC

on arteries b/c so thin nearly impossible to separate at this time.
1. 7.15-7.75 T75
2. 1 ml 7.15-7.75
3. 3 ml
4. Spin
5. T25 new
6. Change media
7. T75
8. T75
9. T75
10. Count
11. Wash in T:25
12. Slide
13. SMC
14. SMC
15. SMC
16. SMC
17. SMC
18. SMC
19. SMC
20. SMC
21. SMC
22. SMC
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90. SMC
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92. SMC
93. SMC
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95. SMC
96. SMC
97. SMC
98. SMC
99. SMC
100. SMC

For slides 5-7, possibly commentary.
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<th>Cells</th>
<th>Media</th>
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<tr>
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<td>200 µL VR</td>
<td>rabbit fibro</td>
<td>DMEM</td>
</tr>
<tr>
<td>3</td>
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<td>K9 fibro</td>
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<tr>
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<td>200 µL VR</td>
<td>K9 fibro</td>
<td>DMEM</td>
</tr>
<tr>
<td>5</td>
<td>control</td>
<td>SMC² T/G ATCC</td>
<td>SMGM2</td>
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<td>SMC² T/G ATCC</td>
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<td>IMDM</td>
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<td>8</td>
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</table>

Note: Media entries include DMEM, SMGM2, and IMDM. Other entries are sample conditions and cell types.
To plate cells from specimen/tissues:

1. Get about a 1 cm² block.
2. 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, forceps) in other side of petri dish.
   d. Use sterile gloves.
3. Put pieces of minced tissue in 6-well plates.
4. Place 1 drop of FBS on each piece of tissue. Typically, use IMDM for media.
5. Turn over (invert) well-plate.
6. Place in incubator upside-down for 1 hr.
7. Take out, re-invert.
8. Place only enough media to barely cover bottom of well plate.
9. Add media in so that it does not touch the specimen but comes very close as it surrounds it.
10. Let sit overnight in incubator.
11. Add enough media to just cover specimen.
12. Let sit for 3 days or until specimen unattaches from bottom of well.
13. Watch under microscope for cells to grow.
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

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<th>% Total</th>
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Histogram Statistics

File: FIB/SMC 8/3/01.002  
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Patient ID: TAG=TUBE  
Acquisition Date: 3-Aug-1  
Gate: G1  
Gated Events: 4548  
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Histogram Statistics

File: FIB/SMC 8/3/01.002  
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Patient ID: TAG=TUBE  
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**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

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**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

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**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

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

Histogram Statistics

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

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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

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Histogram Statistics

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

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Histogram Statistics

File: FIB/SMC 8/3/01.007
Patient ID: TAG= TUBE
Gate: G1
Total Events: 5000

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Histogram Statistics

File: FIB/SMC 8/3/01.007
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Gate: G1
Total Events: 5000

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Histogram Statistics

File: FIB/SMC 8/3/01.007
Patient ID: TAG= TUBE
Gate: G1
Total Events: 5000

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**File:** FIB/SMC 8/3/01.008  
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### Histogram Statistics

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**Acquisition Date:** 3-Aug-1  
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**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

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SSC-H

**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

0 200 400 600 800 1000

FSC-H

**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

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Counts

**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

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Counts

**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

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Counts

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**HUSMC's 7/24/01.007**

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Counts

**VECTOR RED 6/21/01.002**

**HUSMC's 7/24/01.007**

0 10 20 30 40 50 60 70 80

Counts

File: HUSMC'S 7

Patient ID: TAG=

Gate: G1

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File: HUSMC'S 7

Patient ID: TAG=

Gate: G1

Total Events: 50

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Patient ID: TAG=TUBE
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Histogram Statistics

File: HUSMC'S 7/24/01.009
Patient ID: TAG=TUBE
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Histogram Statistics

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

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Histogram Statistics

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Patient ID: TAG=TUBE  
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Gate: G1  
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**Sample ID:** HUMAN AORTIC SMCS  
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**File:** HUSMCS'S 7/24/01.011  
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**Sample ID:** HUMAN AORTIC SMCS  
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**Acquisition Date:** 24-Jul-1  
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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)

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File: VECTOR RED 6/21/01.001  
Sample ID: VECTOR RED  
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Gate: G1  
Gated Events: 6389  
Total Events: 10000  
X Parameter: FL2-H (Log)

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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)

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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

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**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

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**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  
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File: HUSMC'S 7/24/01.001
Patient ID: TAG=TUBE
Gate: G1
Gated Events: 2133
Total Events: 4425

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File: HUSMC'S 7/24/01.002
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Gate: G1
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**Histogram Statistics**

File: HUSMC'S 7/24/01.003
Patient ID: TAG=TUBE
Gate: G1
Gated Events: 2905
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File: HUSMC'S 7/24/01.010
Sample ID: HUMAN AORTIC SMCS
Patient ID: TAG= TUBE
Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5000

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Histogram Statistics

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

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Acquisition Date: 24-Jul-1
Gate: No Gate
Gated Events: 5000

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File: HUSMC'S 7/24/01.004  
Patient ID: TAG=TUBE  
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Histogram Statistics

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

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Histogram Statistics

File: HUSMC'S 7/24/01.004  
Patient ID: TAG=TUBE  
Gate: G1  
Gated Events: 3294  
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**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

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**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

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**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

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**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

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**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

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**Histogram Statistics**

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

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3 Fluorescent wavelengths

488 NM ARGON LASER

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