

# A Proteomics Investigation of the HIV-1 Infection in T-cells

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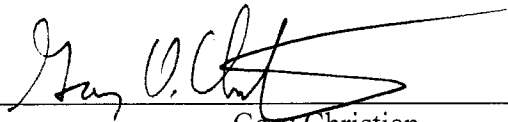
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**Abstract**

A Proteomics Investigation of the HIV-1 Infection in T-cells

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The defining feature of the acquired immune deficiency syndrome (AIDS) is the human immunodeficiency virus (HIV-1) infection of CD4<sup>+</sup> T lymphocytes, compromising the immune system, and rendering the body defenseless from common opportunistic infections. Infections with HIV-1 (virus type I) are associated with significant morbidity and mortality worldwide. Current therapies are partially effective and difficult to sustain due to toxicity and viral resistance. However, considerable progress has been made in the identification of cellular genes essential to pathogenesis and viral infection. This thesis is designed to take these studies to the level of proteomics by utilizing state-of-the-art technology to further investigate the HIV-1 infection on CD4<sup>+</sup> T-cells.

Classic proteomic methods including two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography (LC) coupled with mass spectrometry (MS) provide the backbone of the research. Innovative techniques including capillary electrophoresis (CE) in various separation modes and single-cell

analysis guides proteomics into the 21<sup>st</sup> century of bioanalytical science. Improvements to CE have also been discovered in a novel separation procedure involving fractionating LC in combination with 2D-CE to provide complete protein profiles of CD4+ T-cells.

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**Dedication**

For Roobers

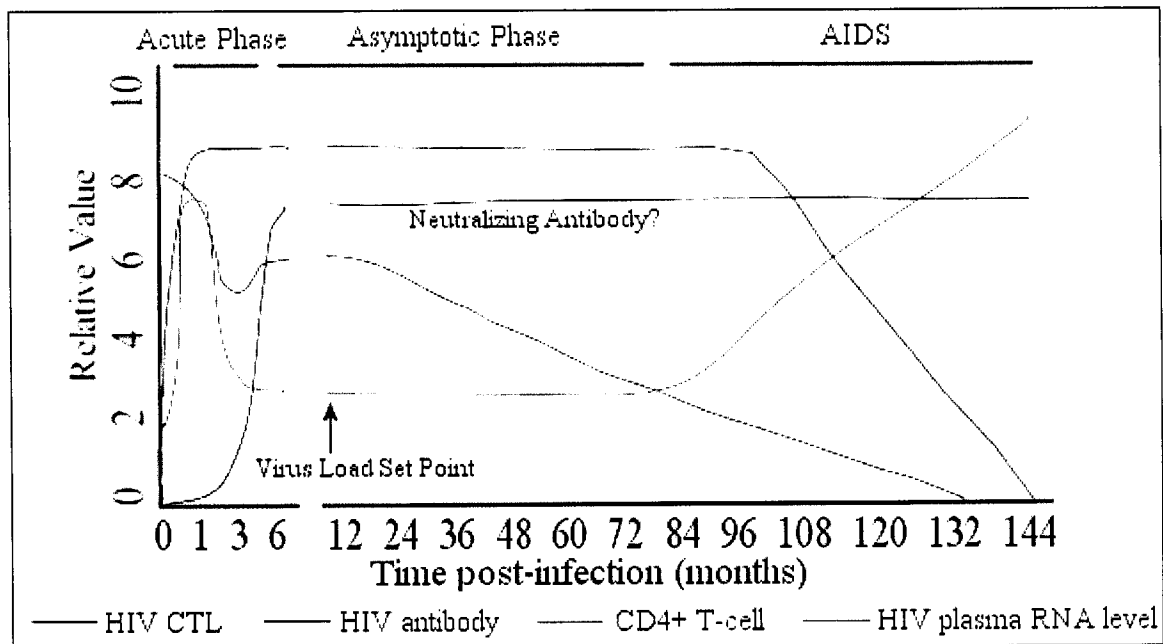
## **Chapter 1 – HIV-1 Infection Pathogenesis and Proteomics**

### **Section 1: The HIV Infection**

Since the first confirmed case of HIV was recognized in 1981<sup>1</sup>, HIV and AIDS have reached pandemic proportions primarily affecting children, teens, and young adults worldwide. According to the World Health Organization statistics, there are ~40 million people infected with HIV today, and since 1981 there have been over 25 million deaths. At this rate, it is possible that over the next ten years infections could reach 100 million.<sup>2</sup> This situation not only represents a human catastrophe, but also dramatically impacts on the economic and health fabric in countries with high HIV prevalence. Antiretroviral therapy has been a powerful tool in curtailing disease progression, but alone it is not the solution.

A variety of factors throughout the progression of the HIV infection including plasma virus load set point and increase of overall plasma virus load, rate of decline of CD4+ count, and HIV associated opportunistic infections, determine the outcome of the infection. Although there are numerous aspects influencing the course of disease progression, a balance between host and virus factors is the primary determinant of the outcome of the infection. Some infected people are termed long term non-progressors (LTNPs). These people have the ability to mount an immune response and to control virus multiplication with minimal loss of CD4+ T-cells resulting in an extremely low-to-undetectable plasma virus load. Others are unable to control virus multiplication and CD4+ T-cell count, and their health rapidly declines within two or three years from the

onset of infection. A timeline can be seen in Figure 1.1 with HIV associated factors. It is proposed that an innate immune response, adaptive immune response, and genetic factors, play some role in the mechanism responsible for CD4+ T-cell depletion. Despite these factors, a further understanding of CD4+ T-cells and their role in the HIV infection is required.



**Figure 1.1:** The rate decline in CD4+ count, plasma virus load set point, rate of increase in plasma virus load and immune response to HIV (CTL response and neutralizing antibody response) influence the progression of HIV disease. CTL=Cytotoxic T lymphocyte<sup>3</sup>

The CD4+ T lymphocytes (aka T helper cells) are the coordinators of the body's immune response. They provide help to B cells in the production of antibodies, as well as augmenting cellular immune response to antigens. One might assume that HIV causes AIDS by directly inducing the death of CD4+ T-cells or interfering with their normal function and by triggering other events that weaken a person's immune system. HIV-1

does directly induce the death of T-cells with a reported half-life of the infected population *in vivo* being 12-36 hours.<sup>4</sup> This is a fairly rapid death rate, but the fraction of infected CD4+ T-cells is relatively low. Recent evidence suggests that for both resting and activated CD4+ T-cells, the fraction of cells with stably integrated, replication-competent provirus is less than 0.1%.<sup>5</sup> Within the resting CD4+ T-cell population a higher but still small fraction of cells (1%) may harbor unintegrated HIV-1 deoxyribonucleic acid (DNA).<sup>5</sup> However, the case is not that simple. The infection itself is not the main culprit that causes the mass eradication of these cells. One mechanism other than direct HIV induced cell death is apoptosis, or programmed cell death. As the disease progresses, elevated levels of apoptosis correlate directly with the depletion of CD4+ T-cells. Various HIV proteins including gp120, Tat, Nef, and Vpu have been shown to induce cell death in uninfected cell populations.<sup>3</sup> Overall, apoptosis is a process that is normally used by individuals to eliminate redundant cell populations and defective cells, but HIV has morphed this mechanism to destroy both infected and uninfected cells.

In the late stages of infection, CXCR4 tropic HIV isolates preferentially infect T-cells. This infection induces membrane fusion between neighboring cells and forms a multinucleate cell termed a syncytium.<sup>6</sup> Mostly, these syncytia are short lived in the advanced stages of the infection, but both HIV infected and uninfected cells participate in syncytia formation, thus accelerating the progression. Membrane disruption can also be caused by the continuous budding of the viruses from infected cells.<sup>3</sup> This budding

comes into contact with not only CD4+ T-cells, but affects other cells of the immune system as well.

Despite our understanding of these mechanisms that determine the outcome of the infection, the question remains—what is unique about the less than 1% of CD4+ T-cells that become infected by HIV compared to the population?

The past few years have witnessed a significant surge of activity in methodology development in genomics and proteomics. Most new technologies provide substantial “proof of principle” and are easily adaptable to high-throughput screening methods. A method called surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has been developed for protein differential display, protein capture, protein identification, and a variety of other applications.<sup>7</sup> Modifications to Enzyme-Linked Immunosorbent Assay (ELISA)--the fundamental tool of clinical immunology--called rolling circle amplification (RCA) have provided much higher sensitivity.<sup>8</sup> Protein nanoarrays generated by a high-resolution patterning technique with very low non-specific binding of proteins have also been developed.<sup>9</sup> Two more techniques include random activation of gene expression (RAGE)<sup>10</sup> and discovery of localized proteins (DELphi)<sup>11</sup> have made significant advancements in generating normalized, genome-wide transcript and protein expression libraries, and alphavirus-based expression screening. Other genomic research areas including DNA arrays have revolutionized gene expression studies by providing a means to determine messenger ribonucleic acid (mRNA) levels for thousands of genes simultaneously. The

identification of genes influenced by viral infection has laid the groundwork for functional studies.<sup>12-15</sup>

The chapters of this thesis are designed to take these studies to the level of proteomics to further investigate CD4+ T-cells as well as the HIV-1 (virus type 1) infection on CD4+ T-cells.

## **Section 2: Proteomics**

First coined in 1995, the term “proteome” is derived from the words protein and genome.<sup>16</sup> As its name suggests, proteomics was designed to denote the protein complements of the genome in an attempt to describe the molecular basis of most physiological processes.

A proteome indicates the quantitative protein expression profile of an organism, tissue, or cell. The human genome contains approximately 30,000 genes. This information is static, and if every gene resulted in one protein then the proteome would directly correlate to 30,000 different proteins. However, one gene results in multiple protein products due to RNA splicing and post-translational modifications, which result in the production of roughly 100,000 differentially expressed proteins. These numbers represent the enormous variability in the composition of the proteome and the ability to form a vast number of phenotypes. The protein expression pattern for any given organism is dynamic due to a variety of factors including stress, drug effects, disease, or a

multitude of other environmental conditions. Proteomic analysis compares the protein expression profiles and links the observed pattern appearances to the causal effects. This comparison cannot be done with genomic data alone.<sup>17</sup>

### **Section 2.1: Protein Sample Preparation**

Although proteomics and genomics are complementary, genomics contains fundamental limitations because gene sequence is insufficient information to understand the function of the gene products. In contrast, proteins represent the most functional level of an organism's make-up as encoded in its genes. The information obtained at the protein level cannot simply be predicted from deciphering the organism's genome or by examining expression at the RNA level. A comprehensive protein-based approach provides an efficient strategy for a more complete understanding of coordinated and programmed gene expression encompassing the entire genome and of the contribution of post-translational modification to cell function.

A biological system contains a complex mixture of thousands of proteins greatly differing in their physico-chemical properties. Characterization of this mixture of proteins provides a challenge to the proteomics scientist. Proteins of extremely high abundance are intermixed with proteins of very low abundance and a dynamic range of  $10^5$ - $10^6$  in protein concentration has to be managed. Small proteins on the order of <10kD are present along with larger ones at upwards >200kD. There are soluble proteins beneath membrane bound ones and basic ones overlaying acidic ones. There are many

sources of contamination (i.e. even dust through the air) in the sample preparation as well as the prevention of unspecific protein absorption onto surfaces that must be considered. Proteins are soluble in a wide range of solutions ranging from secreted compounds able to be dissolved in their own media to membrane bound proteins that are nearly insoluble in the presence of the most robust chemical solvents. In spite of this, proteomics moves forward with a general strategy of sample preparation, protein separation by 2D-PAGE, protein digestion, and identification by LC tandem mass spectral analysis (LC/MS/MS). The Dovichi research group has an aim to drive proteomics into the next level of automation and sensitive detection by employing capillary electrophoresis with laser-induced-fluorescence (CE-LIF) to assist and possibly replace time consuming 2D-PAGE experiments.<sup>18</sup>

Prior to the launching of a proteomics project, many questions need to be addressed. For instance, what is the biological source used for the study? Tissue samples are useful because they display the *in vivo* situation, but they are often heterogeneous in nature because of various cell types or states inherent in the tissue. As a result, cultured cell lines are often preferred as the basis of a proteomics project. Once a cell line is determined, a few more questions arise. Is pre-fractionation necessary? Is the whole proteome of interest or simply a portion? Are the low-abundant proteins of interest or will the most abundant ones suffice? For this investigation, two cultured T-cell lines from malignant pleural effusion are employed in order to obtain information to further investigate the HIV-1 infection.

Once a biological source is determined, sample preparation is a crucial step that will ensure the reproducibility and overall effectiveness of the experiment. For the case of cultured cell lines, there are many ways to lyse a cell or population of cells. Lysis refers to cell disruption to break down the cell wall and solubilize the protein content from the cell. Depending on the cell make-up, both gentle as well as more vigorous lysis methods are available. Gentle lysis methods consist of osmotic lysis<sup>19</sup> where cells are suspended in a hypoosmotic solution, the freeze-thaw mechanism<sup>20</sup> where cells are rapidly frozen in liquid nitrogen and subsequently thawed till lysed, detergent lysis<sup>21</sup> where cells are suspended in a lysis solution containing an ionic detergent, and enzymatic lysis<sup>22</sup> where cells are treated with an enzyme in an isoosmotic solution. The gentle lysis methods are commonly employed when the sample of interest consists of easily lysed cells including cultured cells, blood cells, and some microorganisms. More vigorous lysing methods are used when solid tissue samples or cells with relatively tough cell walls are considered. These methods consist of sonication in a lysis solution<sup>23</sup>, a French pressure cell<sup>24</sup>, grinding<sup>25</sup> where cells are frozen and physically ground with a mortar and pestle, mechanical homogenization<sup>26</sup> for solid tissues, and glass bead homogenization<sup>27</sup> where cells suspended in lysis solution are vortexed in the presence of glass beads.

Once the sample is lysed, proteases are often activated that can severely degrade the proteins. To inhibit proteases, strong denaturants are often employed such as high concentrations of urea or sodium dodecyl sulfate (SDS). Proteases are less active at lower temperatures, so sample preparation at a chilled level is often preferred. Even if

these precautions are taken, proteases may still retain some activity. For this reason a number of commercial sources have developed extremely useful protease inhibitor “cocktail” tablets that are added directly to the lysis solution. For further protein purification, there are some precipitation methods available, but these risk losing portions of the protein profile. Precipitation and resuspension is often avoided when the aim of the experiment is the complete and accurate representation of all the proteins in the sample.<sup>28</sup>

### **Section 2.2: Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)**

First introduced by O’Farrell<sup>29</sup> and Klose<sup>30</sup> in 1975, 2D-PAGE is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological matter. This technique sorts proteins according to two orthogonal properties in two discrete steps. The first step is a separation based on isoelectric point (pI), termed isoelectric focusing (IEF). The second dimension step, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their respective molecular weight. Each spot that is visualized on the gel matrix ideally corresponds to a single protein expressed by the sample. Thousands of proteins can be visualized for certain samples. Along with the pI and molecular weight, amounts of protein can be determined quantitatively as well.

As previously stated, IEF separates proteins based on pI. Proteins are amphoteric molecules that can carry a positive, negative, or neutral charge depending on the environment. The sum of all the negative and positive charges of its amino acid side

chains and amino and carboxyl-termini equal a protein's net charge. When the net charge of a protein is equal to zero at a specific pH, the protein is said to have arrived at its pI. The presence of a pH gradient is crucial to the IEF method. In a pH gradient and with the application of an electric field, a protein will migrate to the position in the gradient where its net charge is zero. A protein with a positive charge will migrate towards the cathode becoming less and less positive moving through the pH gradient until it has reached its pI. The opposite is true for negatively charged proteins. If a protein should diffuse away from its pI, it immediately gains charge and migrates accordingly. This focusing phenomenon concentrates proteins at their specific pI's and allows them to be separated on the basis of very small charge differences. The overall resolution is determined by the slope of the pH gradient and electric field strength.

After isoelectric focusing, SDS-PAGE is used in the second dimension to further separate proteins based on molecular weight. For this dimension, the intrinsic electrical charge of the proteins is masked by the presence of SDS in the sample and the gel matrix. SDS is an anionic detergent that forms micelles composed of 70-80 molecules with the dodecyl hydrocarbon in the core and the sulfate head groups surrounding the make-up of the hydrophilic shell in the presence of water. Together with proteins, SDS forms a complex with a bead-on-a-string structure composed of micelles with a protein center connected by short polypeptide segments. Large amounts of SDS are incorporated in the complex in a ratio of approximately 1.4 grams of SDS per gram of protein.<sup>31</sup> The charge of the protein is effectively masked and the anionic complexes contain a slight net

negative charge per unit of mass due to the SDS. Along with SDS, reducing agents such as dithiothreitol (DTT) are also added to break the sulfide-sulfide bonds present in the proteins. Thus, separation within a polyacrylamide gel depends largely on the molecular weight of the protein. In fact, the logarithm of the molecular weight is proportional to the relative distance the SDS-polypeptide complex migrates down the gel. In order to visualize the spots after the two-dimensional process, gels are typically stained with Coomassie, silver, or a fluorescent dye.

### **Section 2.3: Protein Digestion and Mass Spectrometry**

After gel electrophoresis, the stained proteins need to be freed from the gel matrix and prepared for mass spectrometry identification.<sup>32</sup> The spots are either manually or robotically excised and then digested. There are many proteolytic reagents available for the digestion of proteins including pepsin, cyanogen bromide, and the most commonly used, trypsin. Pepsin is an enzyme that cleaves proteins into peptides on either side of leucines, phenylalanines, tyrosines, and tryptophans (with a few exceptions). Cyanogen bromide cleaves on the N-terminal side of methionines. Trypsin is an enzyme that hydrolyzes peptide bonds on the C-terminal side of arginine and lysine residues, except for the specific case when proline residues are on the C-terminal sides of the arginine or lysine. After digestion, the peptides are purified and filtered for mass analysis. In the most favorable cases, analysis of the tryptic fragments with LC/MS provides sufficient information for identification, but often MS/MS analysis is required to obtain the partial amino acid sequences of the fragments.<sup>33</sup>

Combining a reversed-phase (RP) chromatography with tandem mass spectrometry and database searching creates an impressive method for sequencing peptides. This analysis is fairly rapid, sensitive, and is able to sequence data on mixtures of peptides that have not been resolved by the first MS scan along with peptides with blocked N-termini. MS/MS has been successfully applied to sequence analysis and to the characterizations of posttranslational modifications such as glycosylation and phosphorylation.<sup>32</sup>

Once the amino acid sequences for a particular protein spot on a gel have been determined, they are then matched through a database search. There are many software tools available for proteome data exploration via the internet including DARWIN, MASCOT, PROWL, etc. SEQUEST, one of the most reliable databases, was developed at the University of Washington.<sup>18</sup> SEQUEST correlates uninterpreted tandem mass spectra of peptides with amino acid sequences from protein and nucleotide databases. Once the amino acid sequences are pieced together, they contain the information necessary for the identification of a specific protein to which they correlate. After identification, further gel analysis is required to determine which exact proteins appear/disappear or have higher/lower intensity when comparing two gel patterns.

There has been much important effort to eliminate manual electrophoresis for protein analysis. Isotope-coded affinity tag (ICAT) technology<sup>34</sup> relies on automated, multidimensional chromatography and electro-spray mass spectrometry to quantitate differences in protein expression with extremely high precision. In this process, the cysteine residues are isotopically tagged and isolated for separation by micro liquid

chromatography with tandem mass spectrometry. Another process has been developed termed direct analysis of large protein complexes (DALPC)<sup>35</sup> which has the capability of separation without a prior purification step. This technique uses a denatured protein complex that is separated by 2D chromatography. The first dimension separation uses strong cation exchange (SCX) chromatography and is followed by a second dimension separation on a reverse phase (RP) column. Each fraction is then eluted from the RP column into the mass spectrometer and subsequent SEQUEST algorithm search.

#### **Section 2.4: Capillary Electrophoresis (CE)**

In the early 1980's, a powerful separation technique was invented termed capillary electrophoresis (CE).<sup>36</sup> There are many different modes at which CE can operate, including capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary sieving electrophoresis (CSE), etc. MECC and CSE are the primary two modes that will be employed in this thesis.

The simplest form of CE is CZE. For this technique, a fused silica capillary is filled with a homogenous buffer solution, and constant field strength is applied throughout the capillary. Separation in CZE depends on the analyte's charge-to-size ratio due to the migration at different velocities. Separation of both anionic and cationic species is possible due to the inherent electroosmotic flow (EOF) present in the capillary when the potential is applied. Neutral species are unable to be separated and migrate with the EOF. The positively charged arginine and lysine residues can cause proteins to adhere to the negatively charged walls of the capillary. Many modifications are

necessary depending on the protein sample. Buffer composition, buffer concentration, buffer pH, and field strength are all parameters that must be adjusted to achieve maximum resolution in an electropherogram.<sup>37</sup>

MECC was developed in 1984 and does not stray far from traditional CZE.<sup>38</sup> In addition to the charge-to-size ratio, the separation mechanism also relies on hydrophobic interactions with micelles in the buffer. Micelles are typically formed in an aqueous solution with a hydrophilic exterior and a hydrophobic core surrounding an analyte. Above what is called the critical micelle concentration (CMC), micelles are formed. The most commonly used surfactant in MECC is SDS and its CMC is 8mM in water. With SDS micelles, anions migrate first due to electrostatic repulsions, and cations migrate last due to strong electrostatic attractions generated by the micelles. Neutral molecules can be separated based on hydrophobicity alone and migrate between the anions and cations. The same fine tuning applies for MECC as with CZE with an additional parameter being the surfactant concentration and composition.<sup>38</sup>

CSE is the capillary version of SDS-PAGE. Just as in SDS-PAGE, CSE separates proteins on the basis of size, but is able to separate much more rapidly and with much less sample. In order to separate by size, the fused silica capillary is filled with a low viscosity entangled polymer solution such as pullulan, dextran, agarose etc. to be used as the sieving medium. The capillary wall is also coated with a thin layer of polymer to eliminate EOF and reduce interactions with the capillary wall. When SDS is present in the sample, SDS-protein complexes form with similar charge-to-size ratios. These

charged complexes migrate through the porous sieving polymer where small molecules are able to pass quickly through the matrix and larger ones are retained by the medium and migrate later. Modifications for this technique include polymer type, concentration, and electric field strength in addition to SDS concentration.<sup>39</sup>

After a sample is lysed and the proteins are accessible, the proteins need to be fluorescently labeled in order to be detected by laser induced fluorescence (LIF), the detection method of choice for the Dovichi research laboratory. Fluorescein isothiocyanate is one of the most commonly used reagents for labeling by reacting with the unblocked N-terminus and the  $\epsilon$ -amine groups associated with the lysine residues. However, peak broadening and high background signal is often an issue as a result of incomplete labeling of the protein.<sup>40, 41</sup> There are  $2^X - 1$  possible fluorescent reaction products for a protein with X labeling sites. For the case of ovalbumin, which has 20 lysine residues, there are over 2 million possible fluorescent products with different electrophoretic mobilities. The resulting electropherogram is therefore composed of an extremely broad envelope and an essentially useless protein separation profile. With the addition of micromolar concentrations of SDS, FQ labeled proteins result in sharp, clean electropherograms. FQ reacts with the lysine residues forming a neutral product, and SDS ion-pairs with the unreacted and cationic lysine residues creating a neutral product as well. The labeled proteins thus merge together due to their chemical similarity providing a sharp, intense peak.

A superior alternative to fluorescent dyes is the use of fluorogenic reagents which only become fluorescent upon reacting with a protein. Typical fluorogenic reagents consist of O-phthaldialdehyde (OPA)<sup>42</sup>, naphthalene-2,3-dicarboxaldehyde (BQCA)<sup>43</sup>, and 3-(2-furoyl)quinoline-2-carbaldehyde (FQ)<sup>44</sup> with the latter being employed in the Dovichi laboratory due to the absorption maximum at 480nm coinciding with the 488nm line of argon ion lasers. The derivatization reaction is fairly rapid.

For labeled proteins, LIF detection is the most sensitive method available. The first reported on column LIF detection system for CE was developed in 1985.<sup>45</sup> The Dovichi laboratory employs post column LIF detection with a sheath flow cuvette which is slightly more sensitive than on-column detection.<sup>46</sup> Sheath flow cuvettes use a flowing surrounding buffer to hydrodynamically focus the sample stream as it exits the capillary where a laser beam, positioned approximately 30 microns below the capillary tip, is focused to excite the sample. The signal is collected, passed through a band pass filter, directed by an avalanche photodiode detector (APD), and it is then sent to the computer for data visualization.<sup>47</sup>

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## **Chapter 2 – Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) and Mass Spectrometry (MS) of SupT1 and CEM Cell Lines**

### **Section 1: Introduction to 2D-PAGE and MS Identification**

Currently, 2D-PAGE is the core technology of proteomics, surpassing all other techniques with its capability of resolving thousands of proteins in one separation procedure. The technique has been perfected since its introduction in 1975 with the replacement of classical first-dimension carrier ampholyte pH gradients with well-defined immobilized pH gradients.<sup>1</sup> This change has resulted in higher resolution, improved inter-laboratory reproducibility, increased protein loading capacity, and an extended basic pH limit where resolution was previously lost due to cathodic drift. For an extensive description of the 2D-PAGE process, refer to section 2.2 of chapter 1.<sup>2-4</sup>

After separation with 2D-PAGE and spot digestion, MS is used for the identification of peptides and proteins. Newly translated proteins consist of 20 amino acids covalently linked by amide bonds forming linear heteropolymers. The polypeptide backbone has a repeating mass of 56 atomic mass units (amu) with amino acid side chains contributing masses ranging from 1 to 130amu. Protein structures are extremely diverse due to the variety of molecular entities constituting the side-chain groups. To completely understand the functional and mechanistic properties of proteins, the linear sequence of amino acids must be deciphered along with an accurate molecular weight determination. Accompanying amino acids, proteins also frequently undergo post-

translational modifications including phosphorylation, glycosylation, amidation, acetylation, etc. Currently, MS is the method of choice for protein sequence analysis and the elucidation of post-translational modifications. Refer to section 2.3 of chapter 1 for supplemental information on protein digestion and MS identification.<sup>5-9</sup> For this investigation, two distinct T-cell lines (SupT1 and CEM) are used for 2D-PAGE/MS analysis with the intention of identifying novel proteins specific to T-cells providing insight into the HIV-1 infection.

## **Section 2: Materials and Experimental Design**

All reagents are purchased from Sigma unless otherwise stated. Water is generated from an in-lab filtration system (Barnstead). Each buffer is filtered through a 0.22 $\mu$ m vacuum filter (Millipore). All organic solvents are purchased from Fisher unless otherwise stated.

CEM and SupT1 are the CD4<sup>+</sup> T-cell lines used (American Type Culture Collection, ATCC). CEM is a T lymphoblastoid cell line derived by Foley<sup>10</sup> and obtained in November, 1964 from a peripheral blood buffy coat of a 4-year old Caucasian female with acute lymphoblastic leukemia. SupT1 is a T lymphoblastoid cell line derived from malignant cells collected from the malignant pleural effusion of an 8-year old Caucasian male with acute lymphoblastic leukemia. All cellular supplements including RPMI media, fetal-bovine serum (FBS), l-glutamine, penicillin, and streptomycin are supplied by the microbiology department at the University of Washington. All HIV-1

infected cells are infected at a BSL-3 facility with the LAI strain at the microbiology department by Angelique Van't Wout at a multiplicity of infection (MOI) of 2 to ensure that 100% of all cells are infected. To prepare the cells for 2D-PAGE experiments approximately 10 million cells are used from each of the respective cell lines. The cells are spun at 1200rpm for 5 minutes to rid the cells of excess media, washed with phosphate buffered saline (PBS) (Gibco), and spun down again to form a washed cell pellet. The pellet is then placed in lysis buffer at a concentration of approximately 0.1mL per million cells. The lysis buffer consists of 7M urea, 2M Thiourea, 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS), and 2% (v/v) Pharmalyte 3-10 (Amersham). Prior to lysis, an EDTA-free protease inhibitor cocktail tablet (Roche) is added to the lysis buffer in a concentration of 1 tablet per 9mL of buffer to prevent proteolysis of the sample. The lysate sample is mixed thoroughly by pipet and immediately sonicated in a Branson Sonifier 250 high powered sonicator (VWR Scientific) for one minute in a 2°C environment. The lysed sample is then placed in 3000Da molecular weight cut-off ultracentrifuge tubes and spun at 13,000g's for 1 hr. The remaining lysate is then aliquotted, frozen in -80°C conditions, and ready for 2D-PAGE application.

A 300µL aliquot of lysate is used per gel run. The lysate is lyophilized in a speed vac (Savant) for one hour or until the volume of the sample is reduced by half. Rehydration buffer composed of 8M urea, 2%(w/v)Triton X-100, a few grains of bromophenol blue, DTT, and 0.5% 3-10 IPG buffer (Amersham) is then introduced to the

lysate at a final volume of 350 $\mu$ L. The resulting sample is allowed to rest for several minutes and is then applied to pre-made, immobilized 18cm IEF strips (Amersham), pH 3-10, for first dimension isoelectric focusing analysis. To prevent evaporation of the sample, a layer of mineral oil (Pharmacia Biotech) is applied over the strips and rehydration occurs for approximately 16 hrs. The strips are then placed on an IPGphor Platform (Pharmacia Biotech) and electrophoresed according to the following protocol at 20°C and 50 $\mu$ A per strip; 1) 500V for 1hr 2) 1000V for 1hr 3) 8000V for 4hrs 30min. for adequate focusing. After the strips have been focused they are ready for second dimension analysis. The strips are equilibrated with SDS equilibrium buffer composed of 50mM Tris-HCl pH 8.8 (Biorad), 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a few grains of bromophenol blue, and 100mg of DTT per 10mL of buffer for 15 minutes. The strips are then rinsed with Tris-Glycine-SDS (TGS) buffer (Biorad) for 5 min. and placed securely atop a polymerized SDS-PAGE gel composed of acrylamide/bis 30%T, 2.67%C stock (Biorad), 1.5M Tris-HCl pH 8.8, 10% (w/v) SDS, 10% (w/v) ammonium persulfate (APS), 1% N,N,N',N'-tetramethyl-1,2-diaminoethane (TEMED) (Invitrogen), and water. To ensure that the strip is flush against the gel matrix, it is sealed with a warmed agarose solution that is allowed to harden prior to the introduction of an electric field. The gels are then placed in a Protean II xi Cell (Biorad), and the upper and lower buffer reservoirs are filled with TGS buffer. The gels are run at a constant current of 16 $\mu$ A/gel for 10 minutes followed by 24 $\mu$ A/gel for approximately 5hrs or until the bromophenol blue line has migrated 1mm shy of the end of the gel matrix. The gels are then released from their

glass holder plates, rinsed in water, and silver stained according to the Biorad silver staining protocol. Stained gels are visualized by scanning the gel (Epson) to a computer.

To prepare the proteins for MS analysis, a rigorous in-gel digestion protocol is employed. The spots are excised by hand with any available sharp tool and placed in a clean water bath. The spots are then destained in a solution of 30mM  $K_3Fe(CN)_6$  and 100mM  $Na_2S_2O_3$  and subsequently washed in water to the point where only a clear gel piece remains with the affixed protein(s) of interest. The gel piece is then cut into smaller pieces in order to increase the surface area for the remaining steps. The gel pieces are placed in acetonitrile (ACN) and dried. Sequence-grade modified porcine trypsin (Promega) is added at a final concentration of 12.5-20ng/ $\mu$ L in 50mM  $NH_4HCO_3$ , 5mM  $CaCl_2$  and incubated on ice for one hour. After incubation to allow the proteins to become trypsinized, the excess liquid is discarded and a fresh, non-trypsin solution consisting of 50mM  $NH_4HCO_3$ , 5mM  $CaCl_2$  is added. The gel pieces are then allowed to incubate at 37°C for 12-15 hrs. After the second incubation, the supernatant is removed and saved for it contains the peptides of interest. To ensure all peptides are freed from the gel pieces, 20mM  $NH_4HCO_3$  is added, and the solution is sonicated for 15 minutes in a sonicating water bath (Cole-Parmer). This supernatant is combined with the prior supernatant. Three subsequent additions of 50% ACN/5% formic acid are sonicated with the gel pieces and combined with the previous supernatants as well. This solution is then frozen and lyophilized to a few microliters. The remaining solution is reconstituted with 0.3% trifluoroacetic acid (TFA). To desalt, concentrate, and purify the sample, a pipette

tip with a bed of chromatography media (C18) fixed at its end (ZipTip, Millipore) is used according to the ZipTip protocol.

Prior to LC/MS/MS injection, 3% TFA is added to the sample and spun at 1500 rpms for one minute. The sample is then pressure loaded ( $\sim 2\mu\text{L}$ ) onto a C12 chromatography column and connected to the LC (Agilent) MS/MS (LCQ, ThermoFinnigan) system. Gradient elution for the LC portion is employed at the following percentages of ACN 1) 5-20%, 1min. 2) 20-60%, 20min. 3) 60-90%, 5min. 4) 90%, 10min. 5) 90-5%, 5min., and lastly 6) 5%, 10min. to reequilibrate the column. MS/MS data is taken over the first 60 minutes, and the spectrum is analyzed in a Bioworks browser. After visualization, the amino acid sequences are matched to a SEQUEST database and the identification is confirmed. All identifications are performed on the SupT1-line gel.

### **Section 3: Experimental Results and Discussion**

Approximately 240 gels have been generated on both SupT1 and CEM cell lines to optimize the protein expression pattern display. The optimized gel profiles are shown in Figures 2.1-2.4 for SupT1, SupT1-LAI (specific HIV-1 strain), CEM, and CEM-LAI, respectively. Molecular weight information along the vertical of the gel profile begins at 200kDa and progresses down to 10kDa. Typically, proteins  $<10\text{kDa}$  are not visible on the gel due to the composition of the gel matrix. Along the horizontal, the pH range for these gels is 3-10 to display the most information possible of each proteome.

Currently, there are no publications on proteomic studies for the SupT1 CD4+ T-cell line, but recently, a biotechnology research group from Athens, Greece published a protein profile for the CEM CD4+ T-cell line.<sup>11</sup> Figure 2.3 has a similar protein expression profile for the CEM line when compared to the aforementioned publication and a higher intensity and spot capacity for the entire set when compared to other lymphocyte publications.<sup>12-15</sup> Using the spot detection feature on Melanie IV software (Genebio) for gel analysis, upwards of 1000 spots are detectable on both cell lines (visibly ~300 spots). Many differences as well as similarities are noticeable between the two different cell lines themselves as well as their infected counterparts. To further understand these phenomena, identifications have been performed on Figure 2.1, resulting in Figure 2.5. Across the entire gel, 153 protein identifications have been confirmed on 136 excised spots as shown in Table 2.1.



Figure 2.1: SupT1 Protein Expression Gel

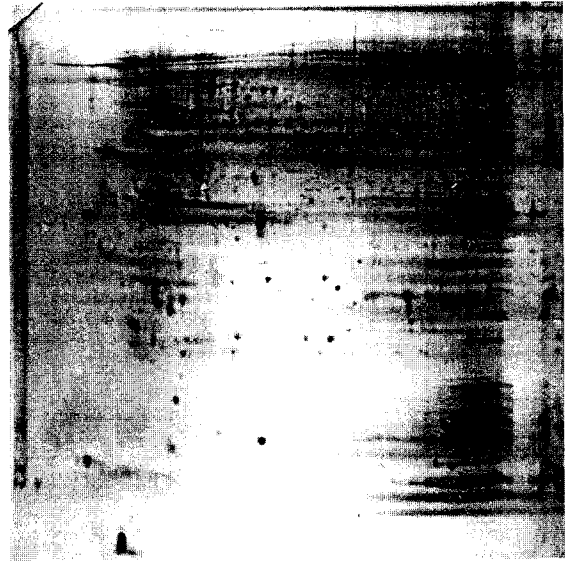


Figure 2.2: SupT1-LAI Protein Expression Gel



Figure 2.3: CEM Protein Expression Gel

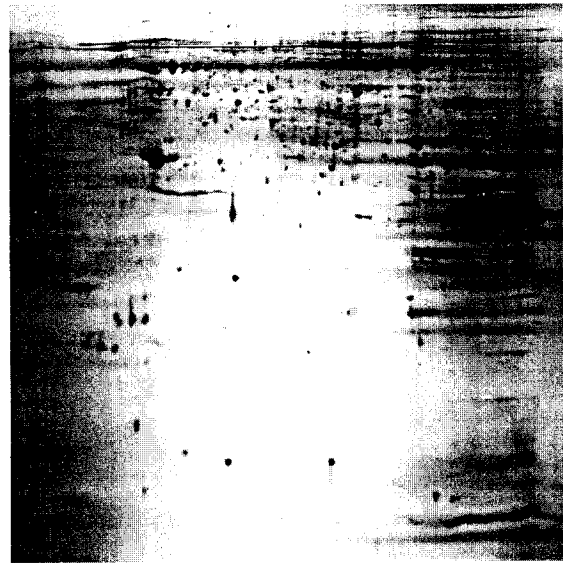


Figure 2.4: CEM-LAI Protein Expression Gel

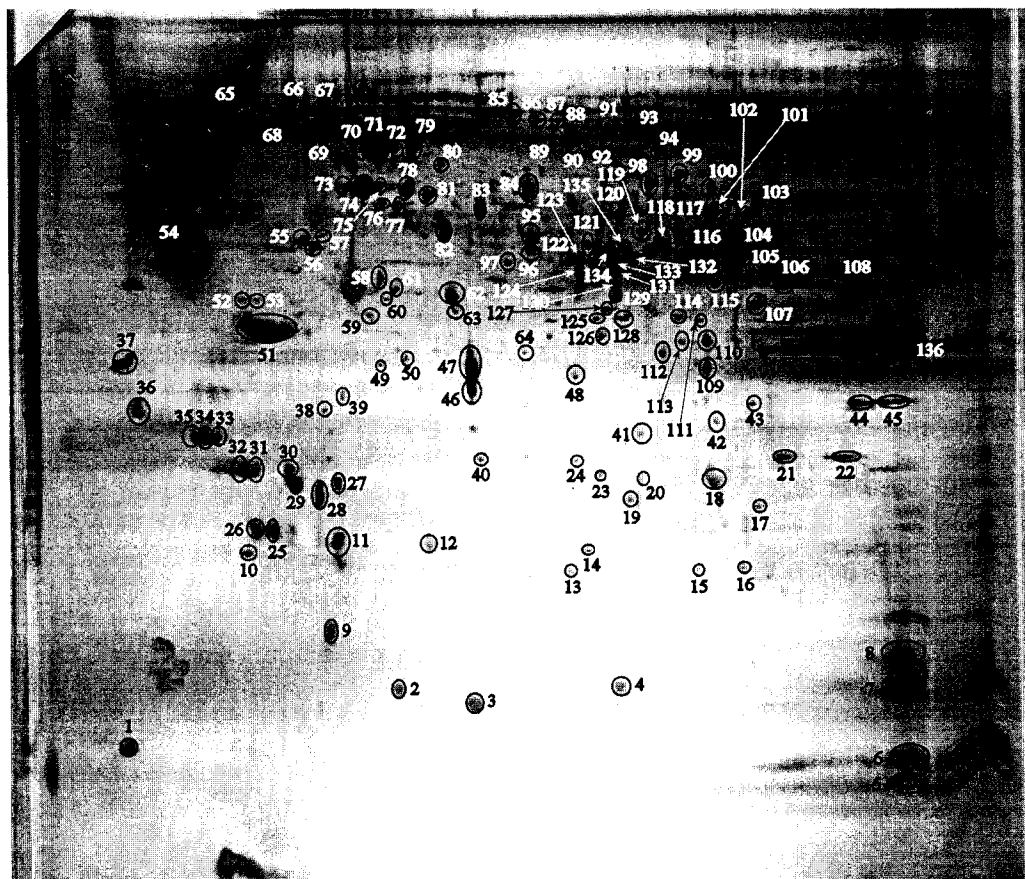


Figure 2.5: Annotated SupT1 Protein Expression Gel

Table 2.1: Identifications from Figure 2.5

Spot #	Identification
1	ribosomal protein P2, <b>and/or</b> hypothetical protein XP_049091
2	stathmin 1
3	ribosomal protein P2, <b>and/or</b> hypothetical protein XP_049091
4	cofilin 1
5	H2A histone family member Z, <b>and/or</b> H2A histone family member C
6	H2B histone family member F, <b>and/or</b> histone H4, <b>and/or</b> H2A histone family member Z
7	hypothetical protein XP_043245
8	histone H4
9	eukaryotic translation initiation factor 4C
10	multicatalytic endopeptidase complex
11	chromobox homolog 5, heterochromatin protein 1 homolog alpha (HP1 alpha)
12	glutathione transferase <b>and/or</b> RAB11A, member RAS oncogene family <b>and/or</b> peroxiredoxin 2

Table 2.1 (continued)

13	guanylate kinase 1 <b>and/or</b> RAB2, member RAS oncogene family
14	RAB2, member RAS oncogene family
15	proteasome subunit, beta type, 2
16	KIAA0186 gene product <b>and/or</b> dihydrofolate reductase
17	hydroxyacyl-Coenzyme A dehydrogenase, type II, <b>and/or</b> PDGF associated protein
18	triosephosphate isomerase 1
19	hypoxanthine phosphoribosyltransferase 1 <b>and/or</b> anti-oxidant protein 2
20	phosphoglycerate mutase 1 (brain)
21	proteasome subunit, alpha type, 4
22	small nuclear ribonucleoprotein polypeptide A'
23	endoplasmic reticulum luminal protein ERp28 precursor
24	glutathione-S-transferase like
25	chromobox homolog 1, heterochromatin protein 1 homolog beta (HP1 beta)
26	chromobox homolog 1, heterochromatin protein 1 homolog beta (HP1 beta)
27	RAN binding protein 1 (RANBP1)
28	Rho GDP dissociation inhibitor (GDI beta)
29	Rho GDP dissociation inhibitor (GDI beta)
30	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
31	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta, <b>and/or</b> eta, <b>and/or</b> theta polypeptide, <b>and/or</b> 14-3-3 protein gamma-subtype
32	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta, <b>and/or</b> eta, <b>and/or</b> theta, <b>and/or</b> zeta polypeptide, <b>and/or</b> 14-3-3 protein gamma-subtype
33	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide
34	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide
35	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide
36	Human pre-mRNA splicing factor SF2p32, complete sequence, gene product <b>and/or</b> protein tyrosine phosphatase, receptor type, C-associated protein
37	nascent-polypeptide-associated complex alpha polypeptide
38	chloride intracellular channel 1 <b>and/or</b> survival of motor neuron protein interacting protein 1 <b>and/or</b> ribonuclease HI, large subunit
39	N-ethylmaleimide-sensitive factor attachment protein, alpha <b>and/or</b> hypothetical protein XP_001526
40	proteasome activator subunit 1
41	proteasome subunit, alpha type, 1
42	phosphatidylinositol transfer protein, beta
43	ribosomal protein S3a
44	LDHA <b>and/or</b> voltage-dependent anion channel 1

Table 2.1 (continued)

45	LDHA and/or voltage-dependent anion channel 1 and/or ATP synthase gamma chain, mitochondrial precursor and/or ribosomal protein S3 and/or L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain
46	lactate dehydrogenase B
47	lactate dehydrogenase B <b>and/or</b> ribosomal protein P0
48	U2 small nuclear RNA auxillary factor 1
49	protein phosphatase 2, catalytic subunit, alpha isoform
50	pyruvate dehydrogenase (lipoamide) beta chain precursor
51	SET translocation (myeloid leukemia-associated)
52	laminin receptor 1 <b>and/or</b> nucleophosmin 1
53	laminin receptor 1
54	No identification confirmed due to low protein expression
55	ATP synthase beta chain, mitochondrial precursor <b>and/or</b> tubulin, beta <b>and/or</b> tubulin, alpha 3
56	suppression of tumorigenicity 13 (colon carcinoma)
57	dynactin 2 <b>and/or</b> ATP synthase beta chain, mitochondrial precursor <b>and/or</b> tubulin, beta <b>and/or</b> tubulin, alpha 3
58	alpha 2 actin
59	N-ethylmaleimide-sensitive factor attachment protein, gamma
60	beta actin
61	chromosome 14 open reading frame 3
62	adenosine deaminase
63	envelope glycoprotein <b>and/or</b> differentiated embryo chondrocyte expressed gene 1
64	transaldolase 1
65	tumor rejection antigen (gp96) 1
66	heat shock protein HSP 90-alpha (HSP86) <b>and/or</b> heat shock 90kD protein 1, beta
67	heat shock protein HSP 90-alpha (HSP86) <b>and/or</b> heat shock 90kD protein 1, beta
68	dna-type molecular chaperone HSPA5 precursor
69	lamin B1 <b>and/or</b> L-plastin
70	heat shock 70kD protein 8
71	heat shock 70kD protein 8
72	heat shock 70kD protein 8 <b>and/or</b> lamin B2
73	heterogeneous nuclear ribonucleoprotein K <b>and/or</b> 60kD heat shock protein, mitochondrial precursor (HSP60)
74	heterogeneous nuclear ribonucleoprotein K <b>and/or</b> 60kD heat shock protein, mitochondrial precursor (HSP60)
75	heterogeneous nuclear ribonucleoprotein K <b>and/or</b> 60kD heat shock protein, mitochondrial precursor (HSP60)
76	FK506-binding protein 4 (T-cell)
77	FK506-binding protein 4 (T-cell)
78	chaperonin containing TCP1, subunit 5 (epsilon)
79	heat shock 70kD protein 9B (mortalin-2)
80	No identification confirmed due to low protein expression
81	T-complex protein 1, theta subunit

Table 2.1 (continued)

82	glutathione synthetase and/or eukaryotic translation initiation factor 2, beta
83	protein disulfide-isomerase ER60 precursor
84	t-complex 1, alpha subunit
85	heat shock protein HSP 90-alpha (HSP86) and/or heat shock 90kD protein 1, beta
86	CCCTC-binding factor (zinc finger protein) and/or B-lymphocyte antigen precursor
87	heat shock protein HSP 90-alpha (HSP86)
88	villin 2
89	glycyl-tRNA synthetase
90	glycyl-tRNA synthetase
91	villin 2
92	moesin
93	moesin
94	tumor necrosis factor type 1 receptor associated protein <b>and/or</b> thyroid autoantigen 70kD
95	heterogeneous nuclear ribonucleoprotein H1
96	heterogeneous nuclear ribonucleoprotein H1
97	proteasome 26S subunit, ATPase, 2
98	chaperonin containing TCP1, subunit 6A (zeta 1)
99	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
100	tyrosyl-tRNA synthetase
101	dihydrolipoamide dehydrogenase precursor
102	pre-B-cell colony enhancing factor
103	adenylyl cyclase-associated protein
104	enolase alpha, lung specific <b>and/or</b> enolase 1 <b>and/or</b> enolase 3, beta muscle <b>and/or</b> phosphogluconate dehydrogenase
105	proteasome 26S subunit, ATPase, 5
106	citrate synthase precursor
107	isovaleryl Coenzyme A dehydrogenase <b>and/or</b> casein kinase 2, alpha 1 polypeptide
108	phosphoglycerate kinase 1 <b>and/or</b> acetyl-coenzyme A acetyltransferase 2
109	aldo-keto reductase family 1, member B1 <b>and/or</b> malate dehydrogenase 1, NAD (soluble)
110	BUB3 homolog
111	BUB3 homolog <b>and/or</b> cytosolic acyl coenzyme A thioester hydrolase <b>and/or</b> poly(rc)-binding protein 2 (alpha-cp2)
112	annexin 1
113	protein kinase, AMP-activated, gamma 1 non-catalytic subunit
114	BUB3 homolog <b>and/or</b> aldo-keto reductase family 1, member A1
115	Tu translation elongation factor, mitochondrial
116	suppression of tumorigenicity 13 (colon carcinoma)
117	hypothetical protein XP_003585 <b>and/or</b> 3-phosphoglycerate dehydrogenase <b>and/or</b> SEPTIN 6
118	La 4.1 protein
119	TATA binding protein interacting protein 49kDa

**Table 2.1 (continued)**

120	tubulin, alpha 3 and/or chaperonin containing TCP1, subunit 2 (beta) and/or 3-oxoacid CoA transferase precursor
121	human rab GDI
122	tumor susceptibility gene 101
123	proliferation-associated 2G4
124	proliferation-associated 2G4 and/or adenosylhomocysteinase
125	lysozyme precursor
126	transaldolase (EC 2.2.1.2)
127	purine-rich element binding protein A
128	mitochondrial ribosomal protein S22
129	proliferation-associated 2G4 <b>and/or</b> neural precursor cell expressed, developmentally down-regulated 5
130	proliferation-associated 2G4 <b>and/or</b> mitogen-activated protein kinase kinase 1
131	proliferation-associated 2G4
132	eukaryotic translation elongation factor 1 gamma <b>and/or</b> eukaryotic initiation factor 4A-like NUK-34
133	proteasome 26S subunit, non-ATPase, 11
134	enolase 1
135	eukaryotic translation elongation factor 1 gamma
136	glyceraldehyde-3-phosphate dehydrogenase <b>and/or</b> glyceraldehyde-3-phosphate dehydrogenase, muscle

For all identifications, the following criteria are employed with the SEQUEST search database. X correlation factors are 1.8, 2.5, and 3.5 for +1, +2, and +3 charge states, respectively. At least two peptides are necessary for all confirmations. All peptide coverages are over 70% for b and y series ions, and protein coverage is 5% at a minimum and in most cases up to 50% for all correlating peptides.

Closer inspection of the gel profiles and identifications leads to many differences as well as similarities in protein expression. The protein cofilin 1 (spot #4, Figure 2.5) appears to be up-regulated in the uninfected SupT1 gel and down-regulated in the infected line. Other notable proteins include tyrosine 3-monooxygenase (spot #33-35), villin 2 (spot #88, 91), and glycyl-tRNA synthetase (spot #89, 90), all of which appear to

be up-regulated in the uninfected SupT1 line. For the HIV-1 infected cell lines, up-regulation appears to occur in the lower molecular weight ranges (10-30,000Da) and at higher pI's (8-10). Identifications are not performed on the HIV infected gels, so no HIV associated proteins have been identified. Expression between the SupT1 and CEM gels are similar, but contain minute differences due to the different tumor origination of each cell line. These differences pertain to minor up-regulation in the SupT1 line at lower molecular weight ranges (10-30,000Da) and at mid-range pI's (5-8). Approximately 10-15 different spots are located in this region on the SupT1 line when compared to CEM. Between two cultured cell lines, the general similarity is as predicted. Comparison between the two HIV-1 infected cell lines shows that their 2D-gels are almost identical with minor differences at the high pI range where some up-regulation is noted for the CEM-LAI gel (Figure 2.4). Overall, similarities between all gels are noted in the largest and most intense spot formation, but the minor differences are new areas of exploration. These minor differences are difficult to detect and quantify on a 2D-gel format. More sophisticated experimentation employing CE with higher sensitivity to exploit these differences is necessary. However, this gel and identification study has laid the groundwork for further functional studies on these CD4+ T-cell lines as will be evident in following chapters.

## Notes to Chapter 2

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## **Chapter 3 – One- and Two-Dimensional Capillary Electrophoresis (CE) of SupT1**

### **Section 1: Introduction to Capillary Electrophoresis (CE)**

Since its introduction in early 1980's<sup>1</sup>, capillary electrophoresis (CE) has led to a myriad of invaluable including genetic analysis, analysis of pharmaceuticals (containing nitrogenous bases), pharmaceuticals with chiral centers, counter-ion analysis in drug discovery, carbohydrate analysis for the determination of post translational modifications, and of most concern to this thesis, protein separation and characterization. For proteome studies, CE with laser induced fluorescence detection (LIF) may become an effective replacement for manual slab gel electrophoresis due to its automation, quantitation, speed, peak capacity, low sample volume (femtoliters), low detection limits (zeptomole range), and high efficiency at various different modes. The primary modes of CE employed in this investigation are CSE and MECC. For a detailed review of these modes refer to chapter 1, section 2.4.<sup>2-4</sup>

Combining two orthogonal modes of CE (i.e. CSE and MECC), results in an even more powerful separation. By first separating a sample in CSE mode, molecular weight information is obtained, and then further separation by MECC mode allows one to determine hydrophobicity and analyte charge information in conjunction. One- and two-dimensional CE has been well established in the Dovichi laboratory<sup>5-10</sup>, and this thesis reports the first CD4+ T-cell CE protein separations. This chapter is designed to complement the two-dimensional gel electrophoresis data from the previous chapter with

one- and two-dimensional CE data in order to exploit the analytical capabilities of CE while simultaneously providing further insight toward the CD4+ T-cell proteome. Supplemental experiments also include separation/fractionation by liquid chromatography (LC) couple with two-dimensional CE to provide enhanced protein separations.

## **Section 2: One-Dimensional CE**

### **Section 2.1: Methods and Experimental Design**

Refer to chapter 2, section 2 for general information on water, buffers, reagents, solvents, and cell culture. For all CE experiments, cells are lysed in a different manner than for gel electrophoresis. Both CEM and SupT1 CD4+ T-cell lines are pelleted as previously described, placed in a 0.1-1% SDS (w/v) solution, and high-power sonicated for one minute to ensure complete lysis. Urea is unable to be used due to interference with the labeling of proteins. The labeling reagent, 3-(2-furoyl)quinoline-2-carbaldehyde (FQ) (Molecular Probes), is prepared by dissolving in methanol and drying for convenient preparation of aliquots to obtain a concentration of 100nM/vial. The labeling reaction is simple and is shown in Figure 3.1.

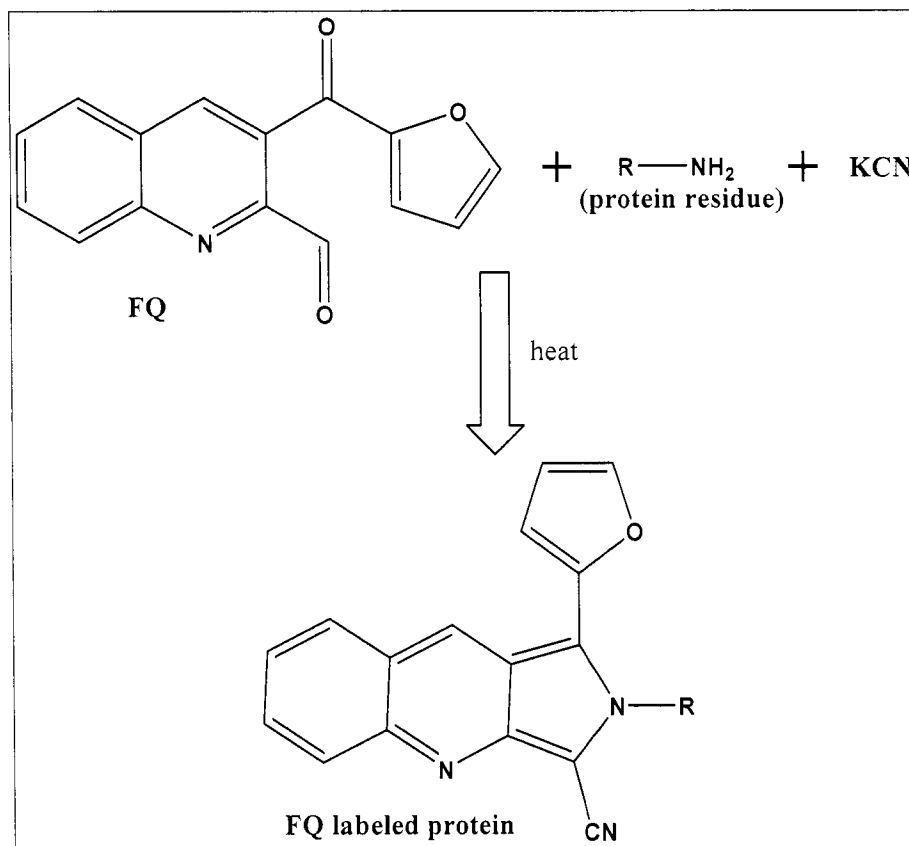


Figure 3.1: FQ labeling reaction

The lysed cell extract is denatured by heating to a temperature of 95°C for 4 minutes. In a dried FQ vial, 5µL of 5mM KCN is added to solubilize and activate the FQ and is mixed with the denatured cell extract. This solution is then heated to 65°C for 5 minutes. The remaining lysate is quenched in running buffer and should appear slightly orange in color.

All CE experiments are performed on a home-built CE instrument as diagrammed in Figure 3.2. A 5mW argon-ion laser (Uniphase) emits a 488nm wavelength beam directed at the sheath flow cuvette by the use of optical mirrors (Melles Griot). The laser

line is visualized by a microscope (Melles Griot) with a 10X objective lens (Melles Griot). An injection block built in-house (University of Washington Machine Shop) is used to contain the buffer reservoir and samples necessary for each run. A 30 $\mu\text{m}$  inner diameter, 150 $\mu\text{m}$  outer diameter, 37mm in length, fused silica capillary (Polymicro) is placed in the injection block and the sheath flow cuvette (Figure 3.3).

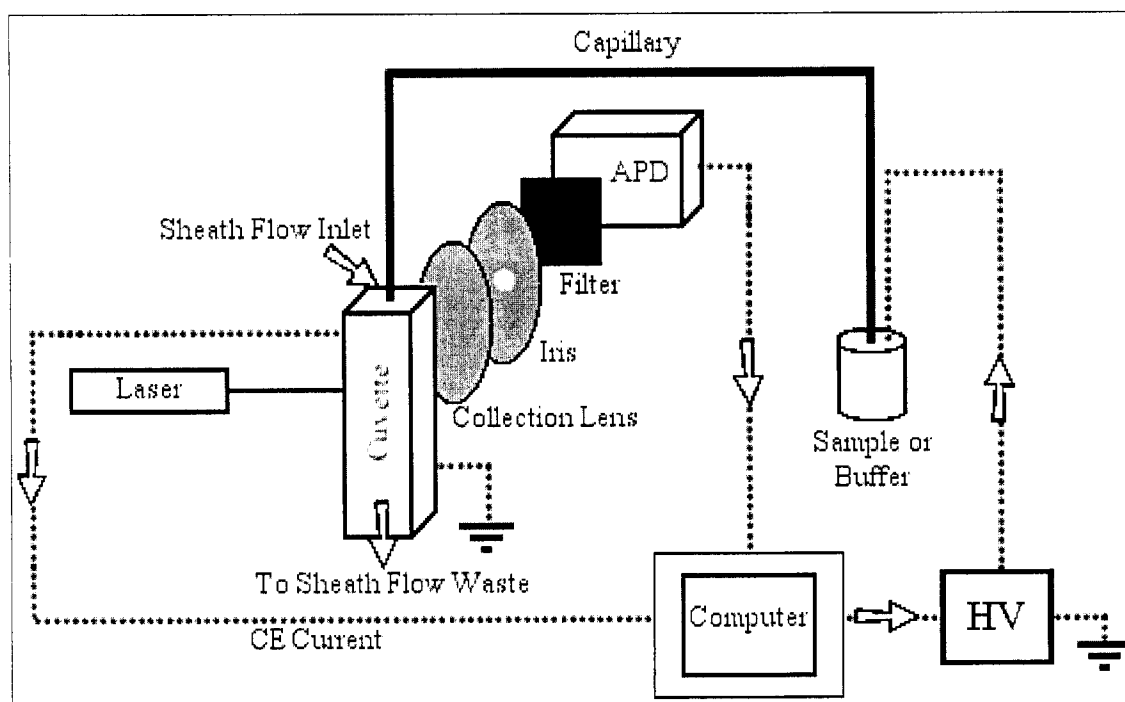


Figure 3.2: One-Dimensional CE Instrument

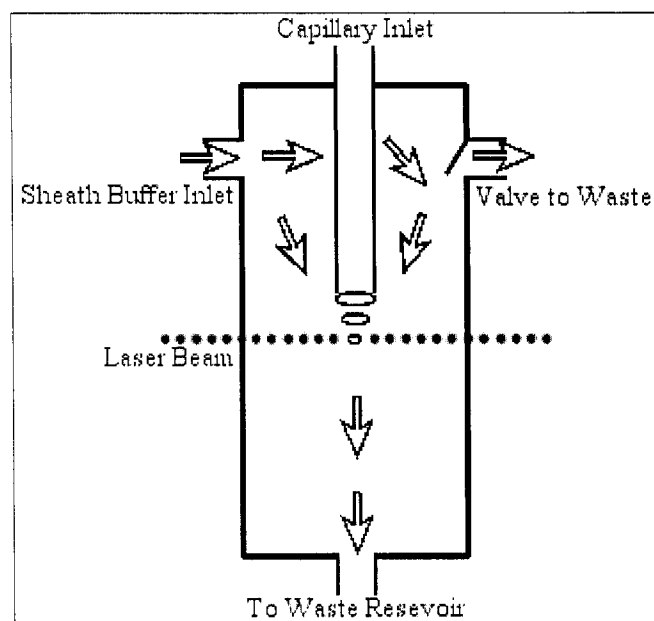


Figure 3.3: Sheath Flow Cuvette

The capillary is prepared by rinsing with 1M NaOH for 1 minute, water for 1 minute, and finally running buffer for 1 minute. MECC separations require no capillary modifications, whereas CSE separations employ a dynamic capillary coating, UltraTrol (Target Discovery), which is introduced to the system prior to the running buffer. For MECC separations, the optimal running buffer is composed of 50mM CHES, 50mM Tris, 15mM SDS, 5%(v/v) methanol, adjusted to a pH of 8.7. CSE running buffers are 100mM CHES, 100mM Tris, 3.5mM SDS, 5%(w/v) 513kDa dextran, adjusted to pH 8.3. Sample injections and runs are performed electrokinetically with a high voltage power supply (Spellman). Typically, injections are on the order of 2-5s at 5kV, and runs are completed anywhere from 6-20 minutes at 17kV for MECC and -16kV for CSE. As the sample migrates through the buffer-filled capillary, it is separated and hydrodynamically focused

after exiting the cuvette. The laser excites the labeled protein, and the fluorescence, after passing through a bandpass filter (Thorlabs) and adjustable iris (Thorlabs), is collected by a 50 $\mu$ m inner diameter fiber optic cable (Thorlabs) and directed to an avalanche photodiode detector (APD) (Perkin Elmer). The fluorescent signal and current are monitored by a computer at a data acquisition rate of 10Hz. All data manipulation and visualization is performed by IGOR Pro (Wavemetrics) software.

## **Section 2.2: Experimental Results and Discussion**

Approximately 400 electropherograms have been generated for both the SupT1 and CEM CD4+ T-cell lines in both MECC and CSE modes. The primary focus has been on the SupT1 cell line due to its lack of exposure in the literature. In the interest of brevity, all data reported is on the SupT1 lysates. CEM data is similar.

Buffer compositions, concentrations, polymers, coatings and coating procedures, field strength, capillary length and size, injection volume, pH, sample preparation, as well as the addition of organic solvents to the buffer, have all played a role in optimization. An example electropherogram for the SupT1 cell line is noted in Figures 3.4 and 3.5 for MECC and CSE separations, respectively.

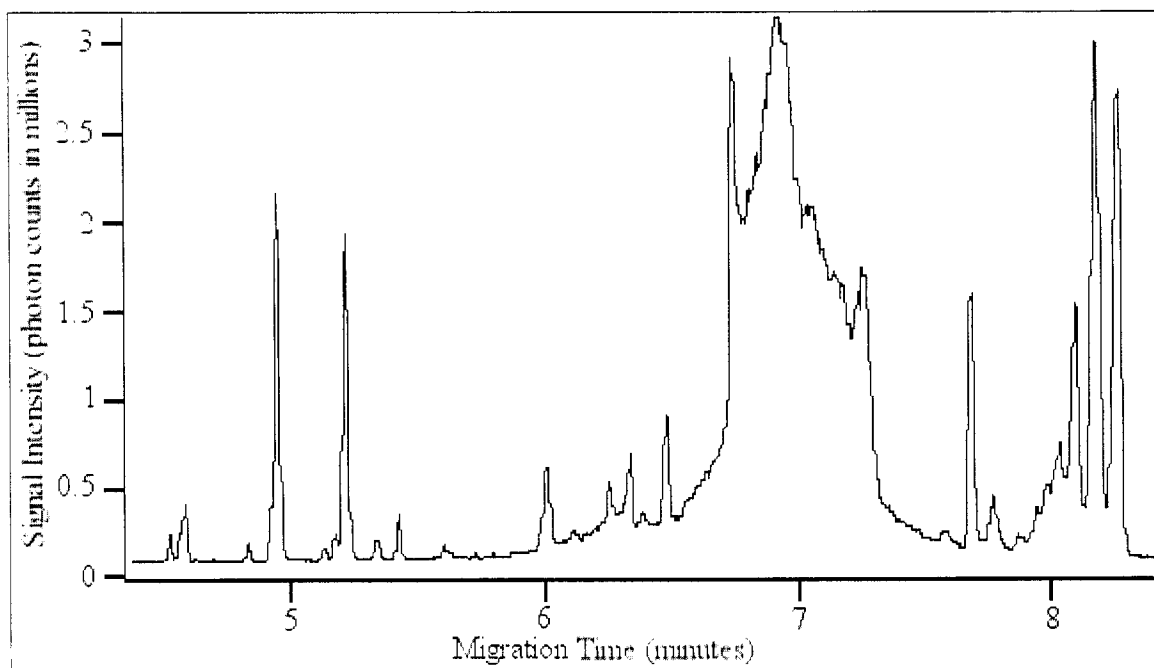


Figure 3.4: MECC Separation of SupT1 Homogenate, 5kV injection for 3s, 17.2kV separation

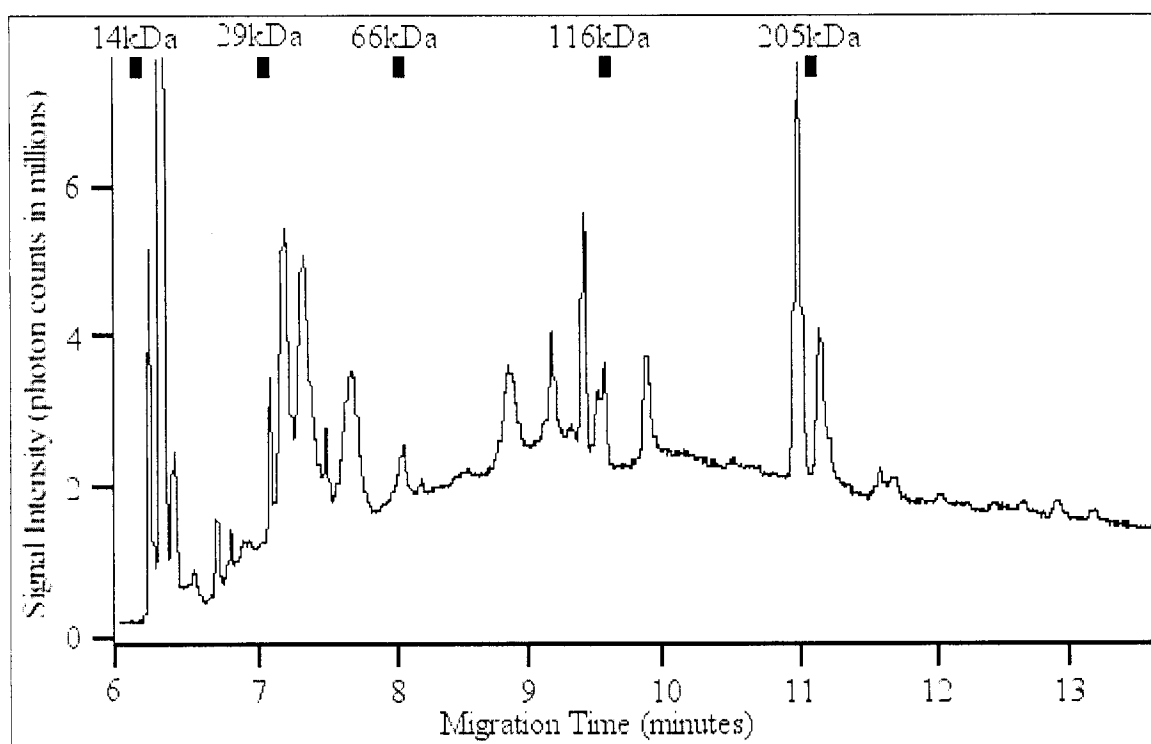


Figure 3.5: CSE Separation of SupT1 Homogenate, -5kV injection for 2s, -16kV separation

Upon close inspection, 35 peaks are visible within each electropherogram, which is a 7-fold improvement in resolving capability from initial experiments. Efficiencies for most peaks are approximately 200,000 plates with a maximum value of 700,000 plates. MECC separations are twice as fast due to the absence of a sieving polymer matrix introduced to the buffer of CSE separations as well as the increased separation potential. The molecular weight ladder atop Figure 3.5 is from a separation of standard proteins of known molecular weight. Applying linear regression analysis, a standard molecular weight ladder can be obtained, providing useful protein molecular weight information. Peaks are mostly baseline resolved with the exception of the large envelope at 7 minutes in the MECC separation. This region of unresolved components can be attributed to a cluster of hydrophobic proteins which have similar charge states. In order to improve the resolution, peak capacity, and overall separation within each electropherogram, a two-dimensional strategy is employed.

### **Section 3: Two-Dimensional CE**

#### **Section 3.1: Methods and Experimental Design**

All two-dimensional experiments require identical sample preparation to that of one-dimensional runs. However, slight modifications to running conditions and experimental setup are necessary. A diagram of the two-dimensional instrument can be seen in Figure 3.6, with a close-up view of the home-built capillary interface in Figure 3.7. Both interfaced capillaries are of identical dimensions to that of a one-dimensional experiment. The interfaced capillaries are aligned via two outer sleeves and positioned at

an approximate  $30\mu\text{m}$  distance apart. All components are held together by UV glue (Kemxert Corp.) cured upon exposure to long wave-length ultraviolet light (320-380nm). A second power supply is introduced to propel the second dimension buffer and separation. All optics and data collection remain, and data visualization is performed in both IGOR (Wavemetrics) as well as MATLAB (MathWorks) software.

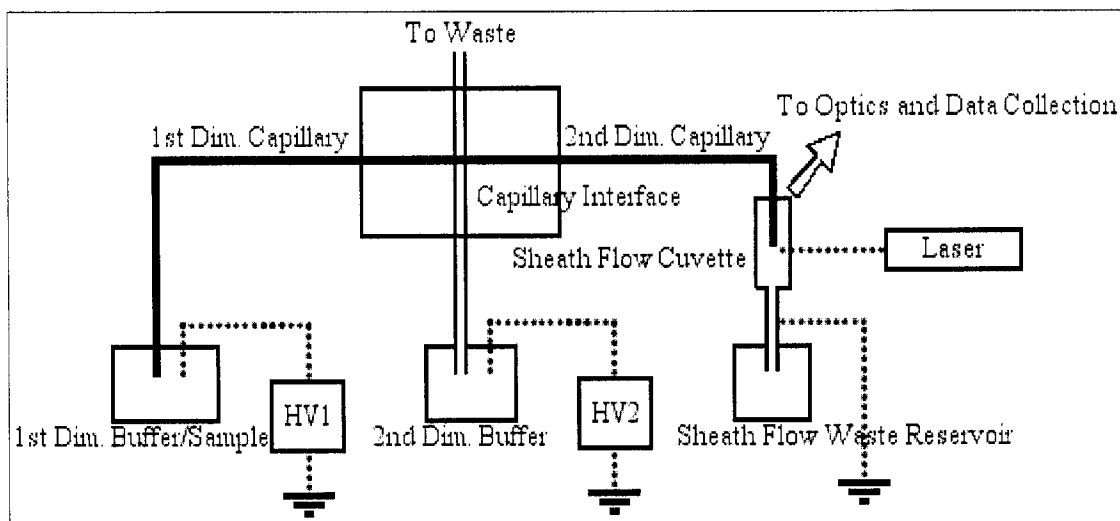


Figure 3.6: Two-dimensional CE Instrument

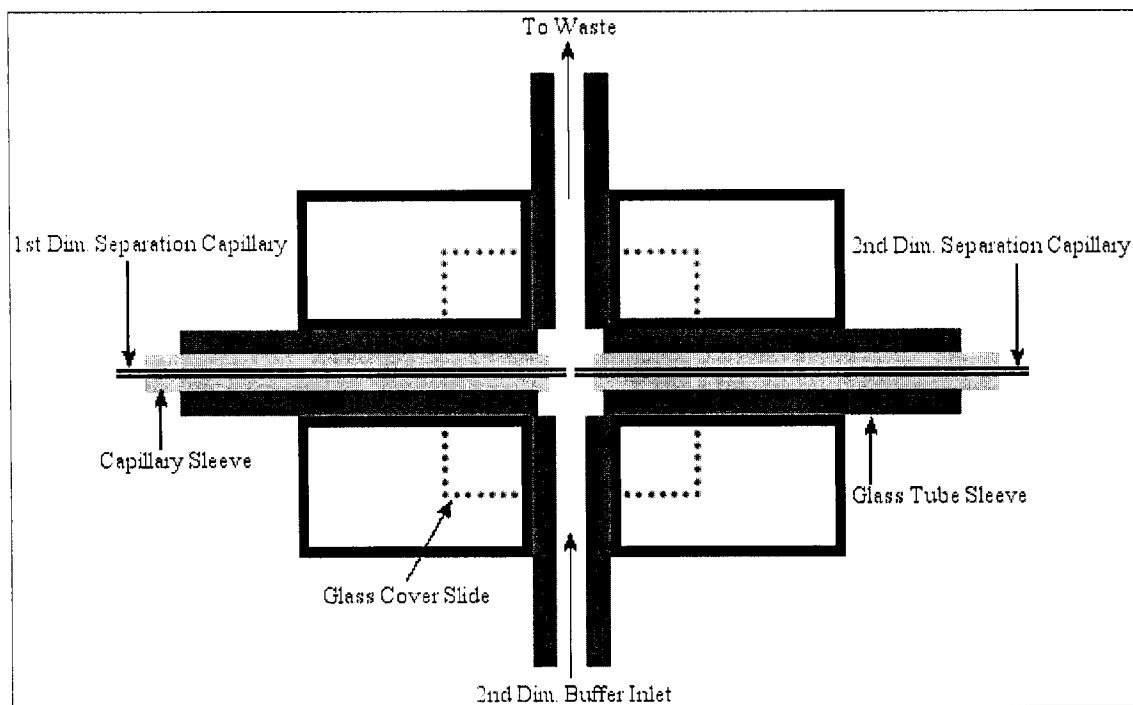
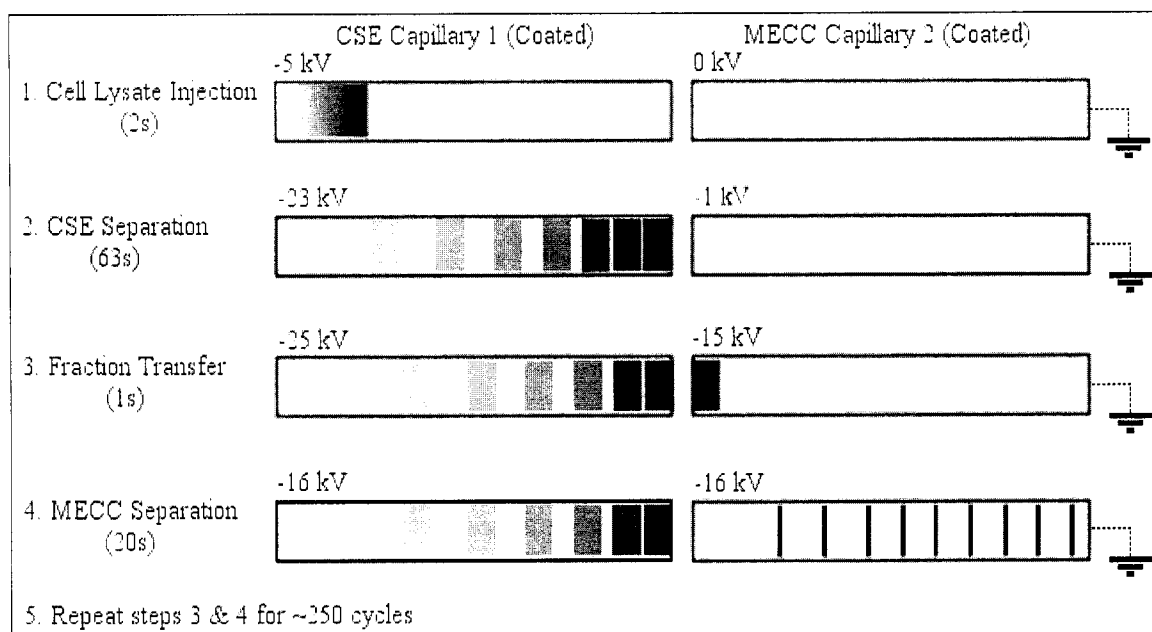


Figure 3.7: Capillary Interface

A diagram of a mock two-dimensional run can be seen in Figure 3.8. In the first dimension (CSE), the buffer is composed of 100mM CHES, 100mM Tris, 3.5mM SDS, 5%(w/v) 513kDa dextran, adjusted to pH 8.3. The second dimension (MECC) buffer is composed of 100mM CHES, 100mM Tris, 15mM SDS, adjusted to pH 8.7. Both capillaries are dynamically coated as previously stated and the MECC dimension is in reverse mode. Preparation of both capillaries is complex. Through the second dimension buffer inlet, both capillaries are cleaned with 1M NaOH for 2 minutes, water for 2 minutes, UltraTrol for 2 minutes, and finally second dimension buffer for 3 minutes. The first dimension capillary is filled with its respective buffer via the injection block while purging the interface to prevent buffer cross contamination. The sample is the

injected electrokinetically as previously stated, and a fast CSE separation is performed in the first dimension (<63s, at -22kV). At this point, the first component from the first dimension is at the end of the first capillary. A net -10kV potential is applied for 1s to transfer a small plug from the first capillary to the second. A -16kV MECC separation is then performed on that small plug in approximately 20s. This iterative process continues until all plugs from the first capillary have been transferred and separated in the second dimension. This process is completely automated, and separations are performed in <80 minutes.



**Figure 3.8:** Two-Dimensional Experimentation Specifics

### Section 3.2: Experimental Results and Discussion

Approximately 140 two-dimensional electropherograms have been generated on the SupT1 and CEM CD4+ T-cell lines. Figure 3.9 displays the optimal raw electropherogram for the SupT1 T-cell line. The total separation window is about one hour with very good resolution. When analyzing the raw data, it appears that there are approximately seven visible peaks. An expanded view of the final peak from Figure 3.9 displays that three peaks are interwoven to appear as one (Figure 3.10). The peaks indicated by the circles represent the most intense peak extracted from that cluster, with two other peaks that are baseline resolved denoted by the squares and x's.

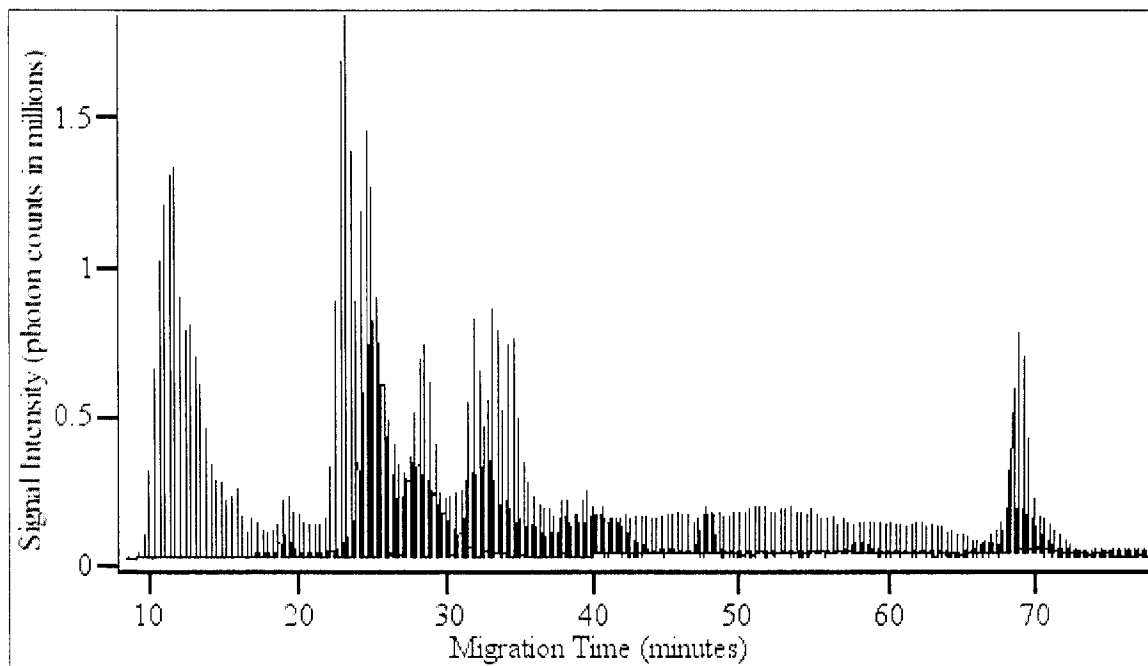


Figure 3.9: Two-Dimensional Raw Data

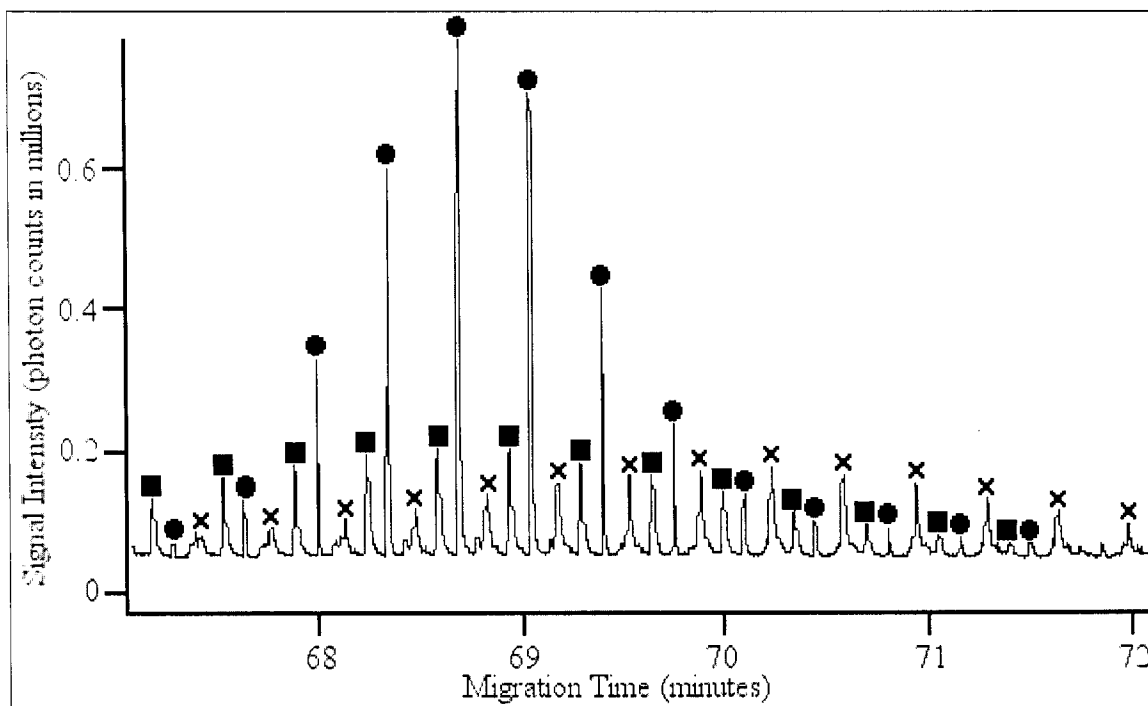


Figure 3.10: Two-Dimensional Raw Data Expansion

In order to visualize the data, a gel image is generated in MATLAB to display the individual peaks on a two-dimensional platform (Figure 3.11).<sup>10</sup> It appears from the gel image that only ~30 peaks are visible. The three peaks outlined in Figure 3.10 are more pronounced, appearing as the final three spots of high molecular weight.

The gel image can also be portrayed as a landscape image to better display peak intensity (Figures 3.12 and 3.13). In the landscape image it is easier to recognize peak shape and intensity with ~50 peaks visible. One major concern remains. If 30 peaks are visible in a one-dimensional CE format for both CSE and MECC separations, one should see 900 peaks in a 2D-CE experiment employing identical separation modes in each

dimension. Most of the proteins lie on an intense ridge in the low molecular weight region and between 6 and 16 seconds in the MECC separation dimension. The entire separation window is not being fully utilized. This hydrophobic region is exhibiting numerous protein-protein interactions rendering them unable to be resolved even within two dimensions. By dividing the homogenate into smaller fractions and running a series of two-dimensional fractions, it is hypothesized that some of these protein-protein interactions will desist and a more complete separation will be obtained. This data is presented in section 4.

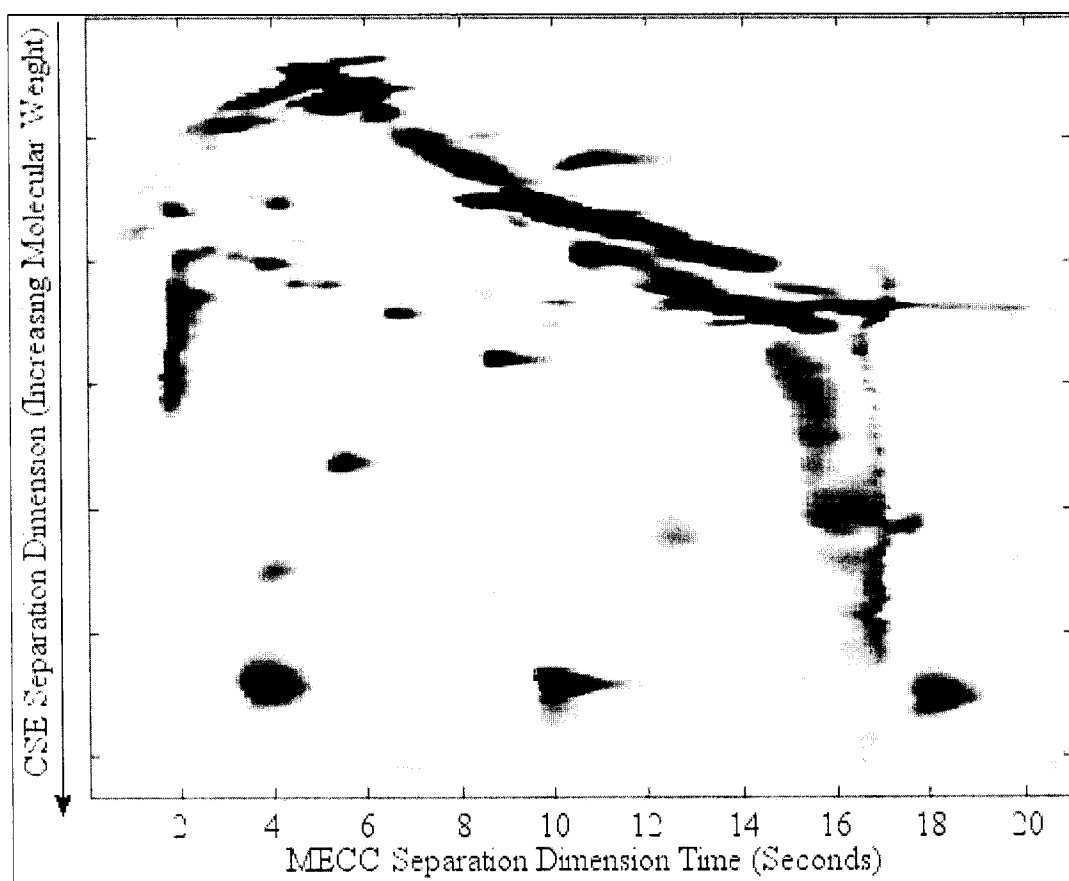


Figure 3.11: Two-Dimensional Gel Image

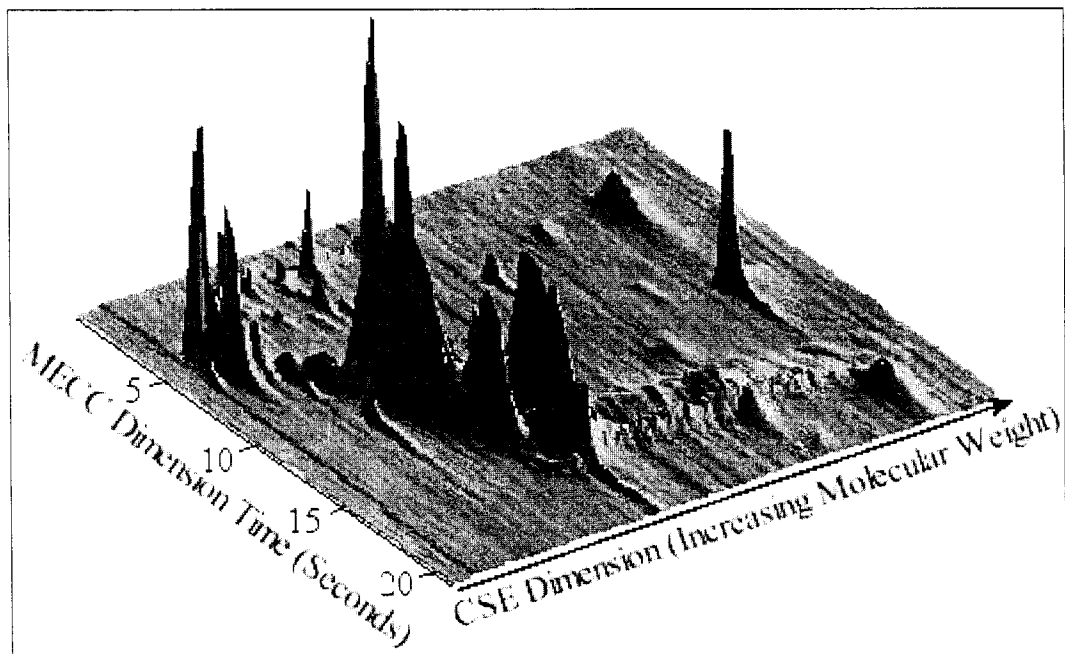


Figure 3.12: Two-Dimensional Landscape Image

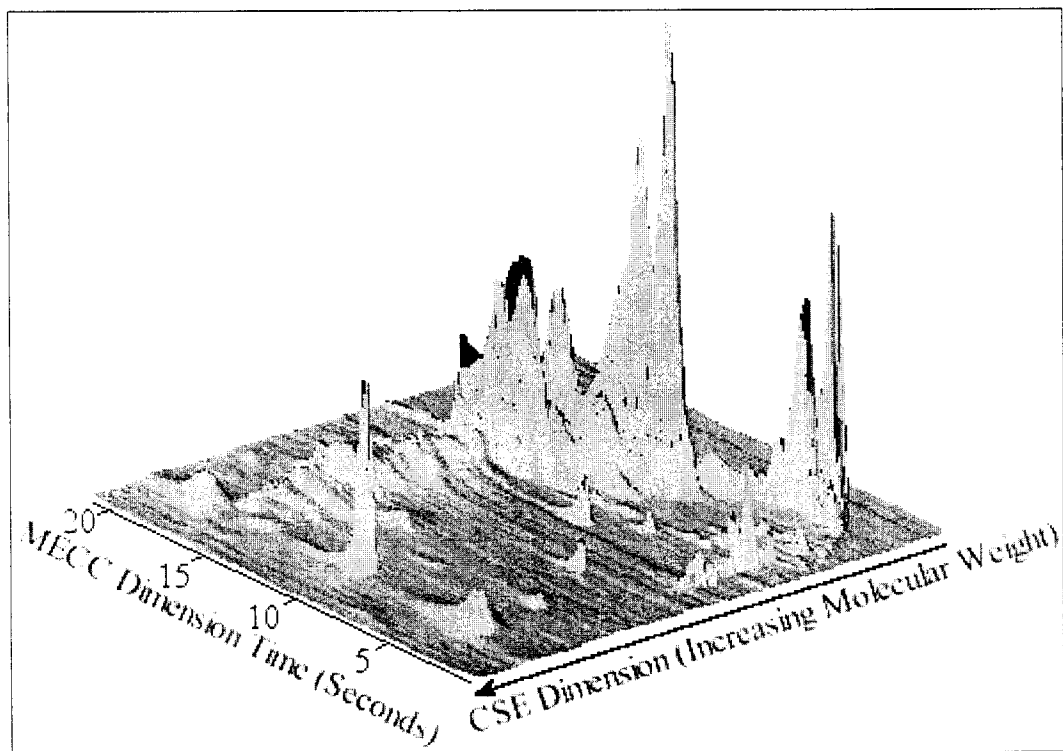


Figure 3.13: Two-Dimensional Landscape Image (Rotated 180°)

## **Section 4: Liquid Chromatography (LC)/Two-Dimensional CE**

### **Section 4.1: Methods and Experimental Design**

All LC experiments were performed courtesy of the Fred Hutchinson Cancer Research Center facility (Seattle, WA). The HPLC is from Vision Workstation complete with automated fractionation. All experiments are run in reverse phase employing a Zorbax 300sb-C8, 4.6x250mm, with 5 $\mu$ m packing (Agilent). Buffer A is composed of 0.1% formic acid in water, while buffer B contains 0.1% formic acid in acetonitrile. SupT1 sample homogenates are lysed by sonication for one minute in water only to prevent clogging the LC column. Total injection volume is 400 $\mu$ L. Gradient elution is employed along the following gradient: 98-70% buffer A, 2-30% buffer B for 40 minutes, 50% buffer A, 50% buffer B for 5 minutes, 10% buffer A, 90% buffer B for 3 minutes with a flow rate of 200 $\mu$ L/minute. The sample is separated along the column, passes through a UV detector, and 200 $\mu$ L fractions are collected. Usable fractions vary between each run, but on average 10 protein fractions are collected corresponding to the absorbance intensity on the UV detector. Those fractions which do not absorb in the UV spectrum are saved and run as blanks. All collected fractions are lyophilized to 2-3 $\mu$ L, in order to discard the volatile components of the LC buffers, and quenched in CE running buffer to a total volume of 30 $\mu$ L. Samples are then labeled and introduced to the two-dimensional CE system as previously described.

**Section 4.2: Experimental Results and Discussion**

According to the HPLC UV detector, 10 fractions out of 40 generated signal. The remaining 30 fractions were collected and run as blanks on the two-dimensional CE instrument which generated nominal or no signal. The 10 protein fractions created intense two-dimensional patterns comparable to the homogenate runs from section 3. These fractions can be seen as both a gel and landscape image in Figures 3.14-3.33.

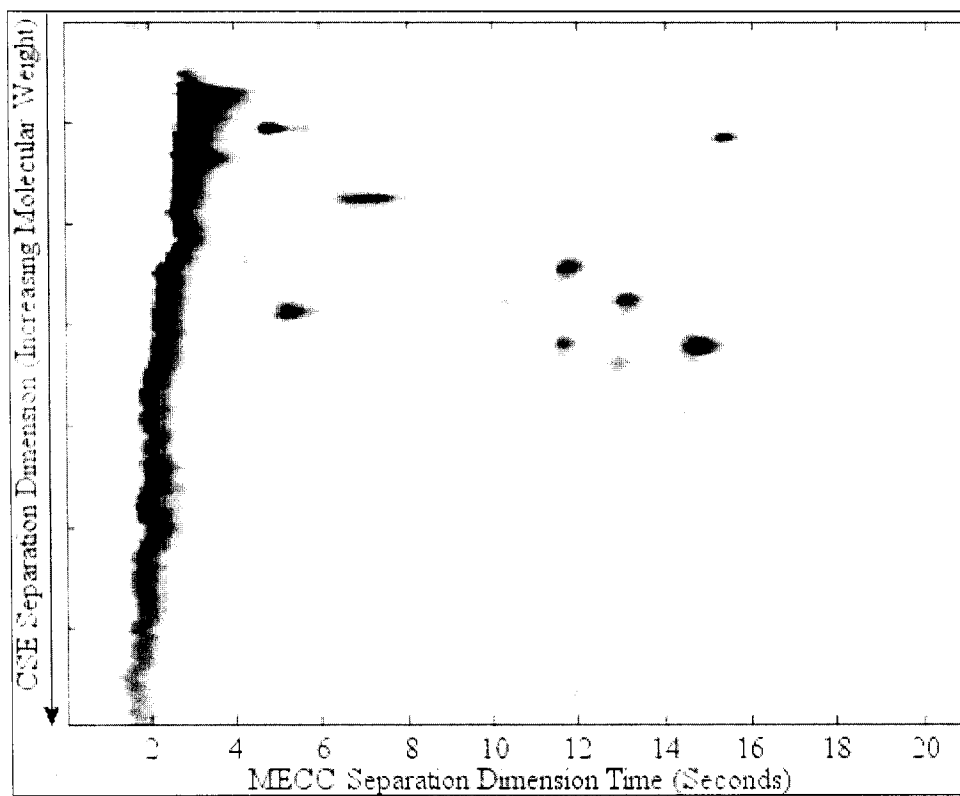


Figure 3.14: Fraction #1-gel image

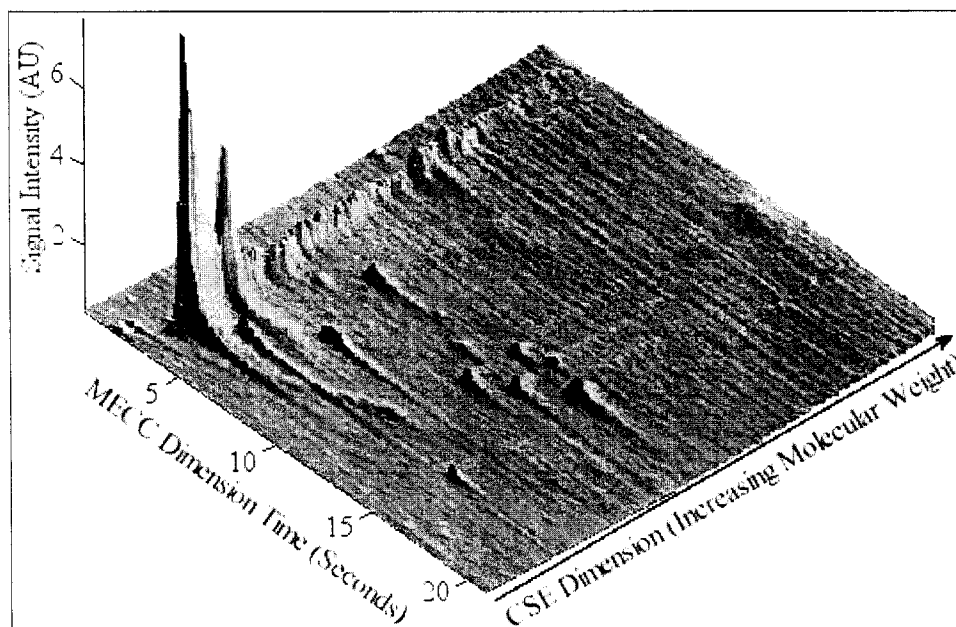


Figure 3.15: Fraction #1-landscape image

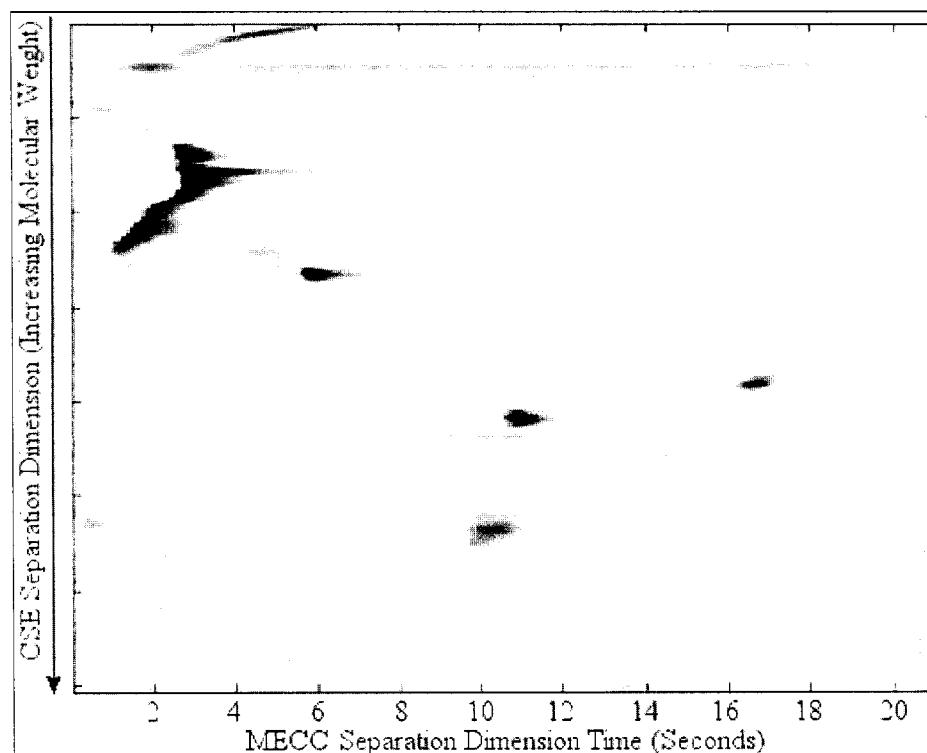


Figure 3.16: Fraction #2-gel image

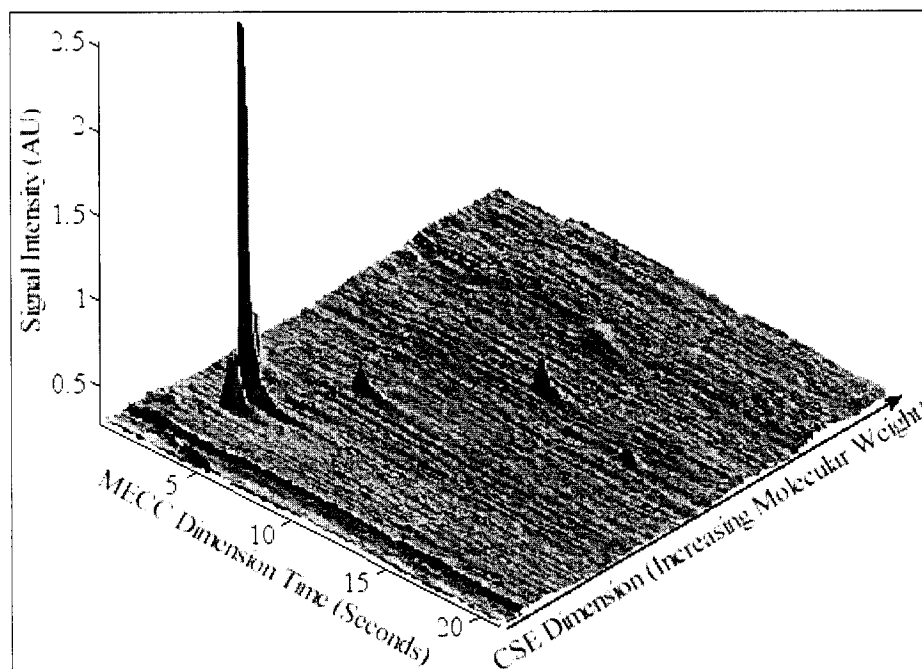


Figure 3.17: Fraction #2-landscape image

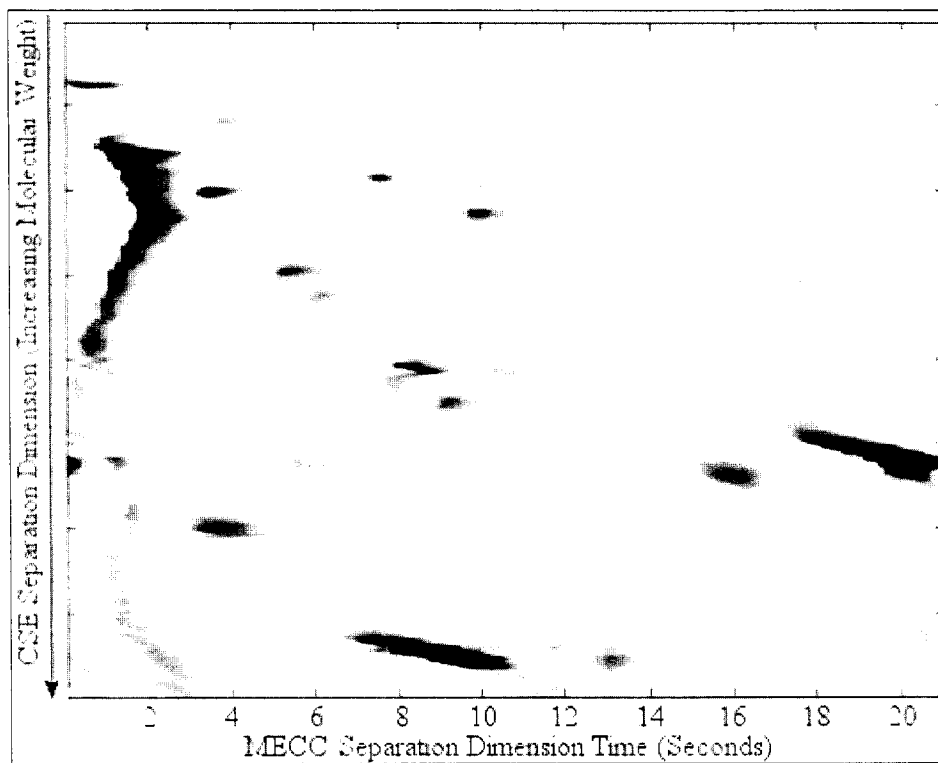


Figure 3.18: Fraction #3-gel image

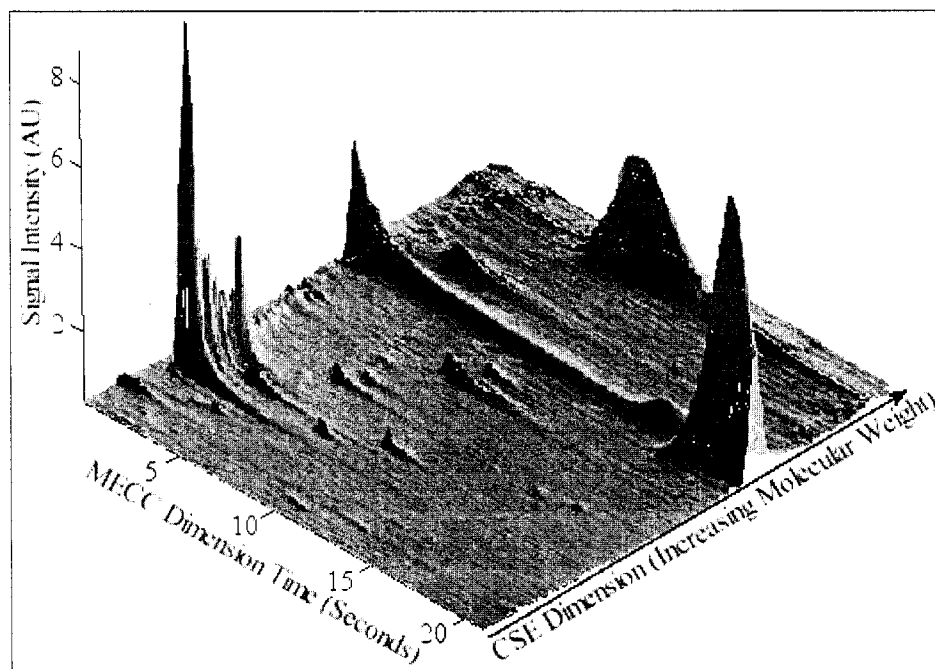


Figure 3.19: Fraction #3-landscape image

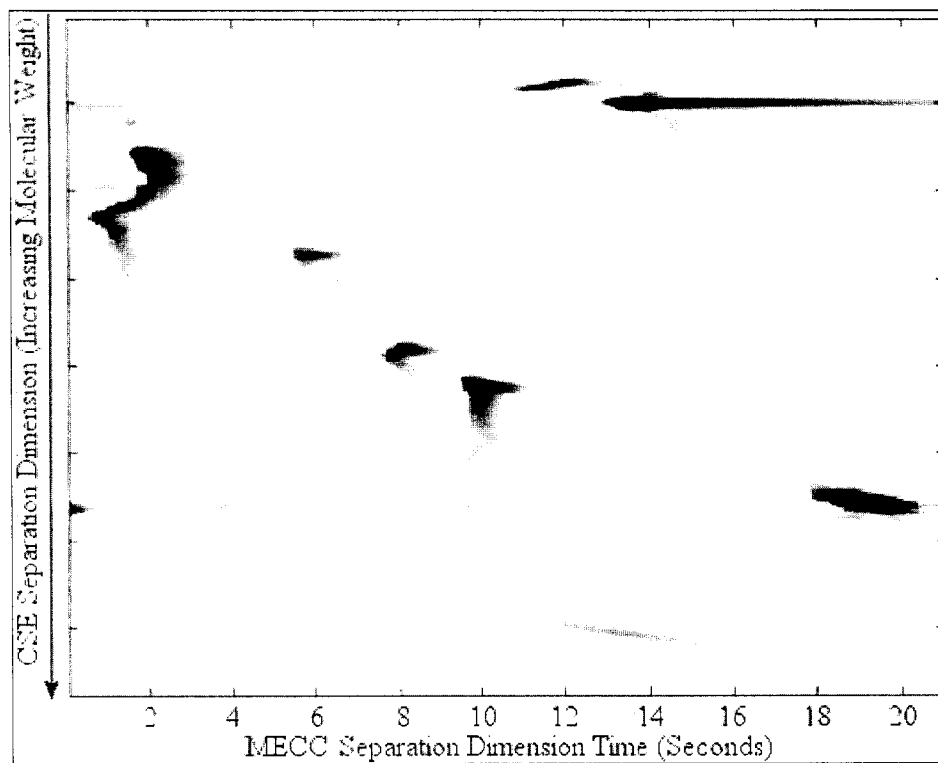


Figure 3.20: Fraction #4-gel image

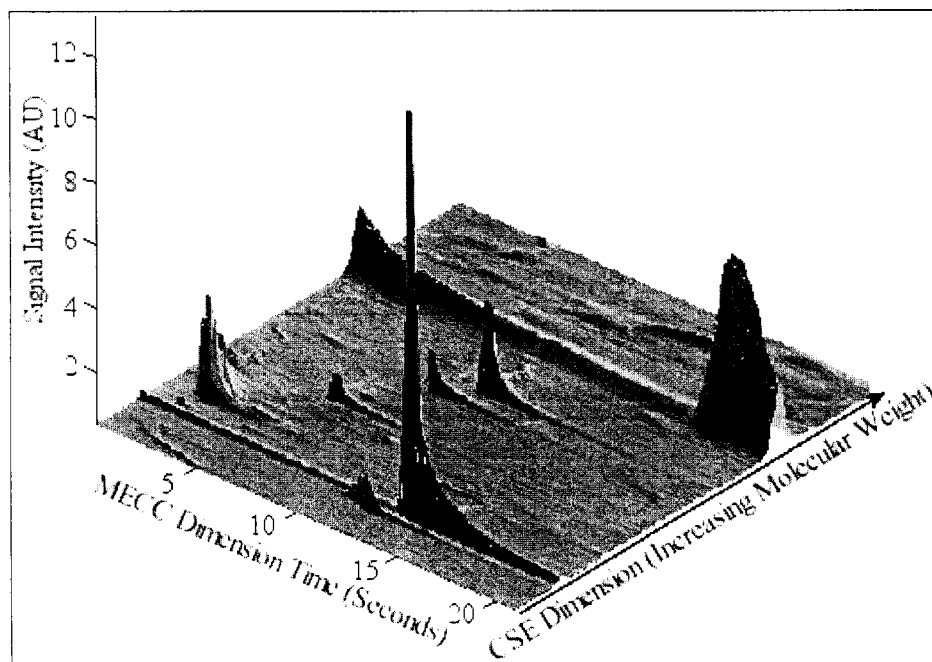


Figure 3.21: Fraction #4-landscape image

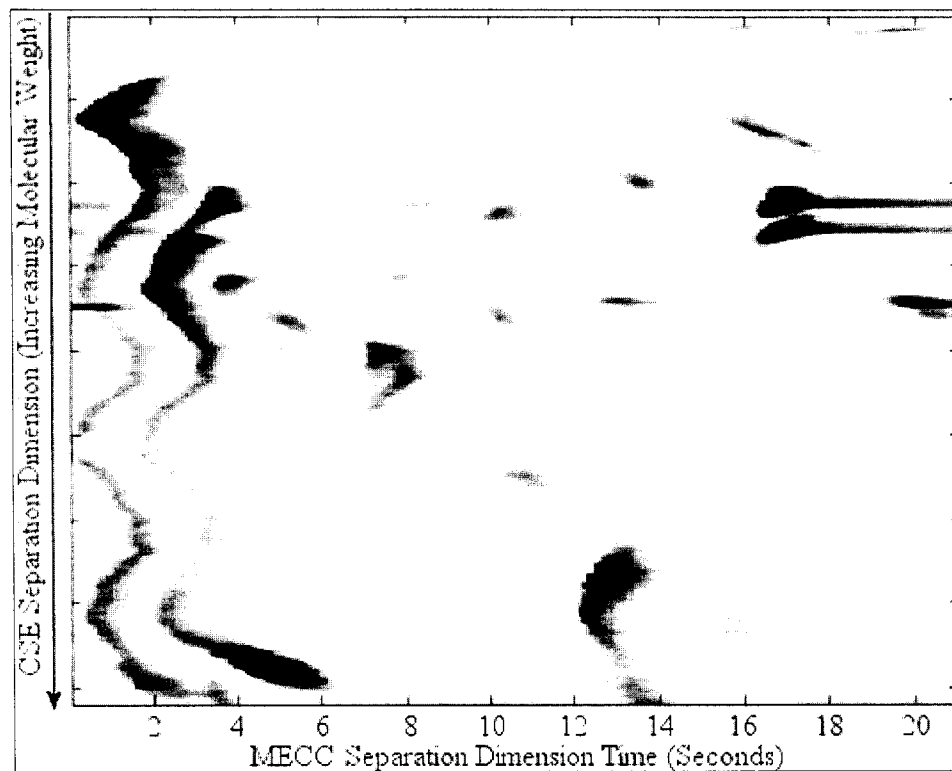


Figure 3.22: Fraction #5-gel image

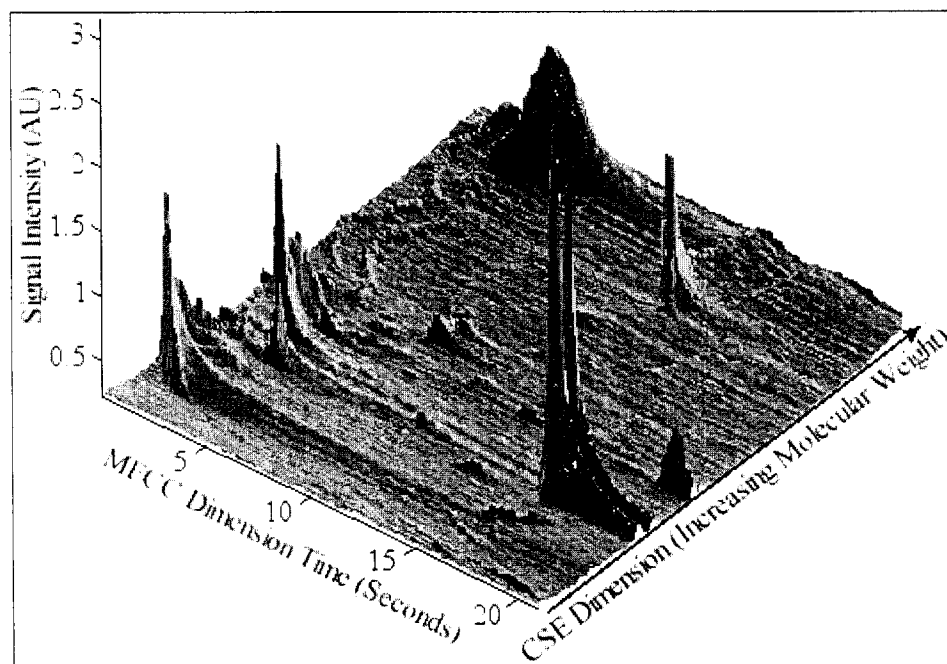


Figure 3.23: Fraction #5-landscape image

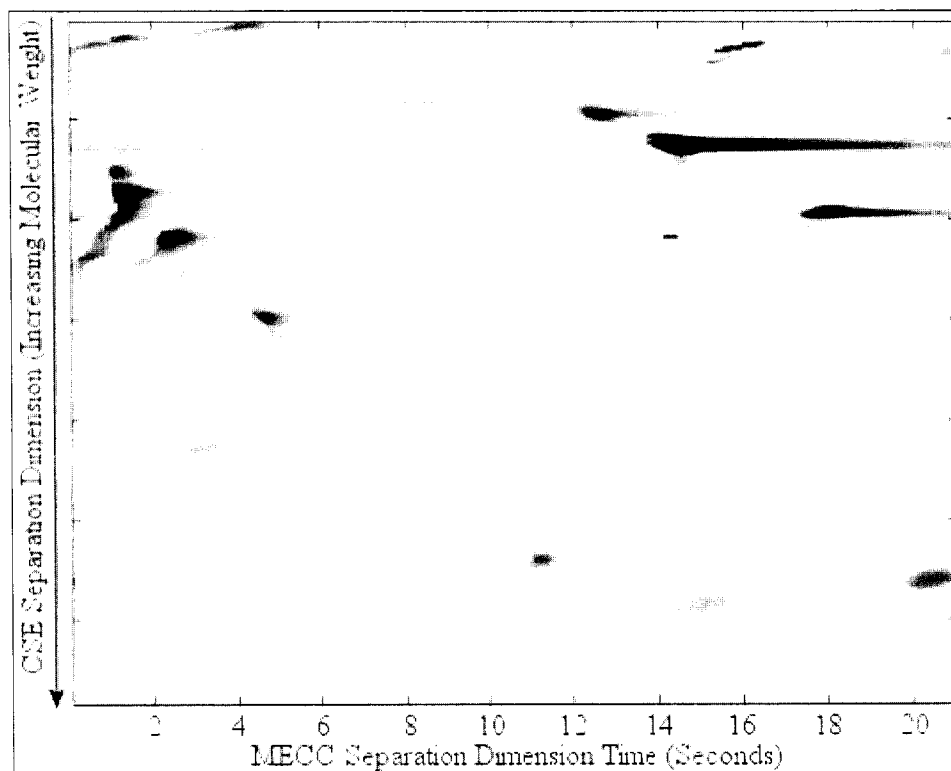


Figure 3.24: Fraction #6-gel image

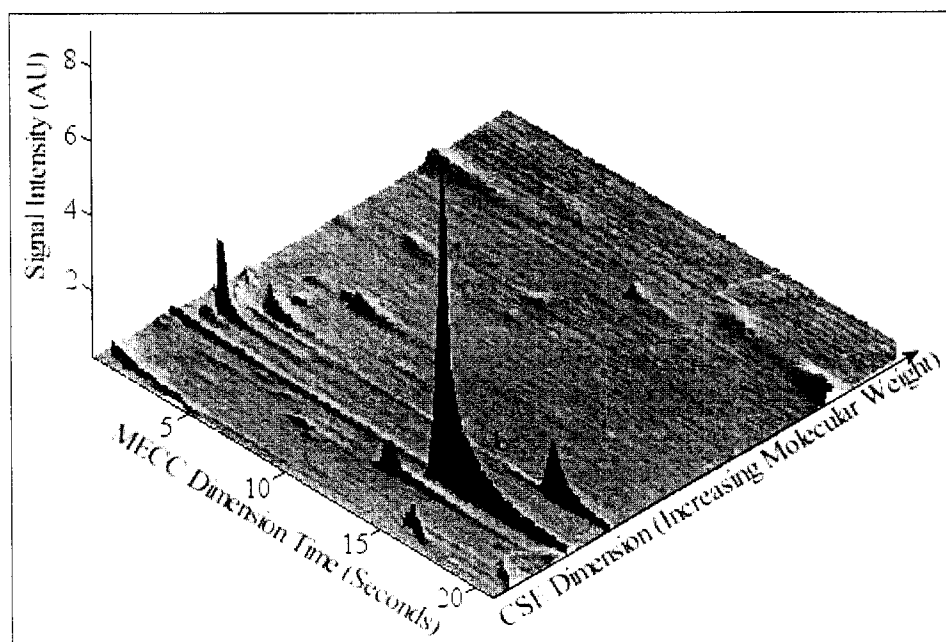


Figure 3.25: Fraction #6-landscape image

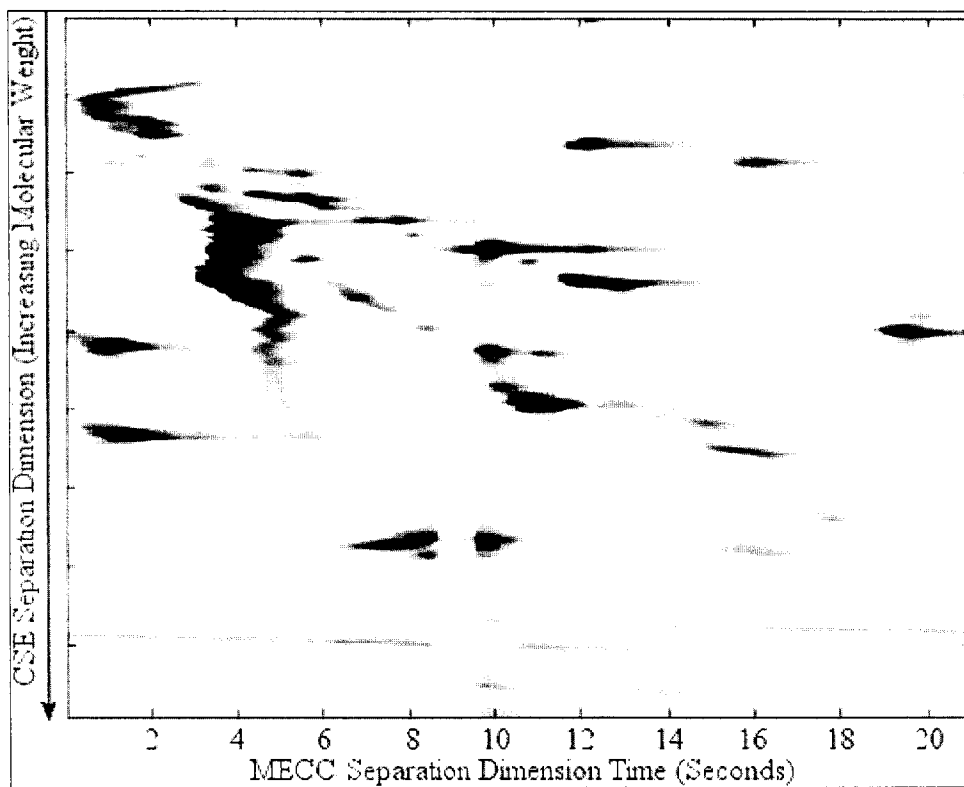


Figure 3.26: Fraction #7-gel image

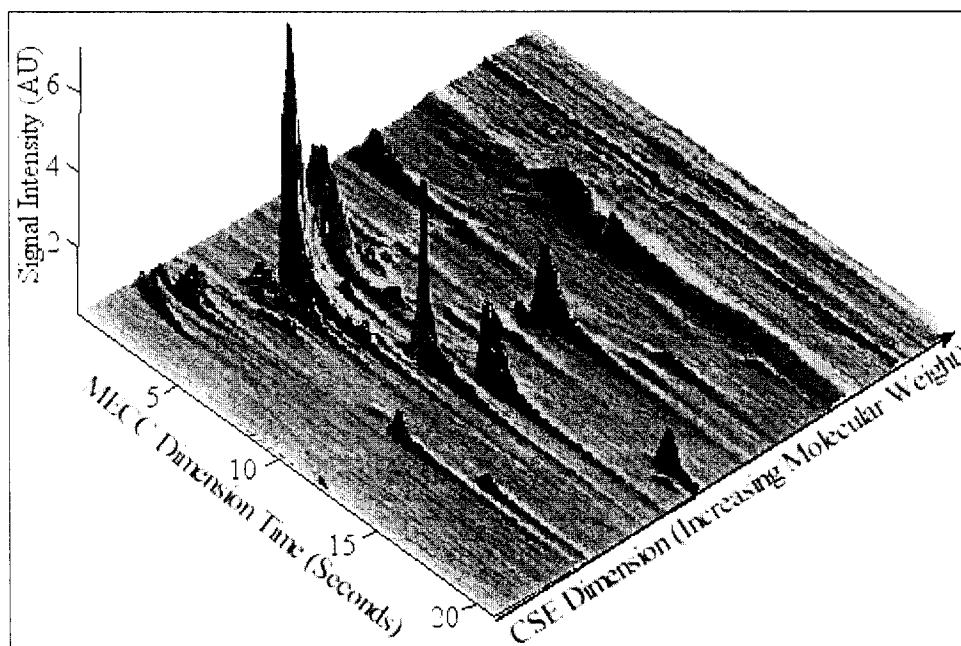


Figure 3.27: Fraction #7-landscape image

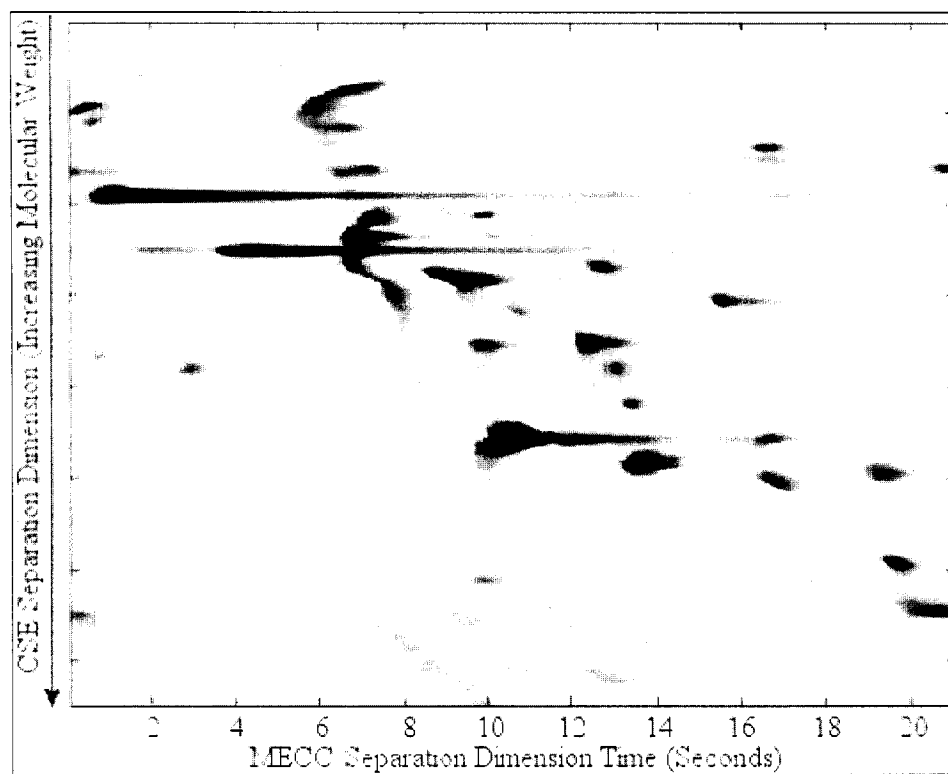


Figure 3.28: Fraction #8-gel image

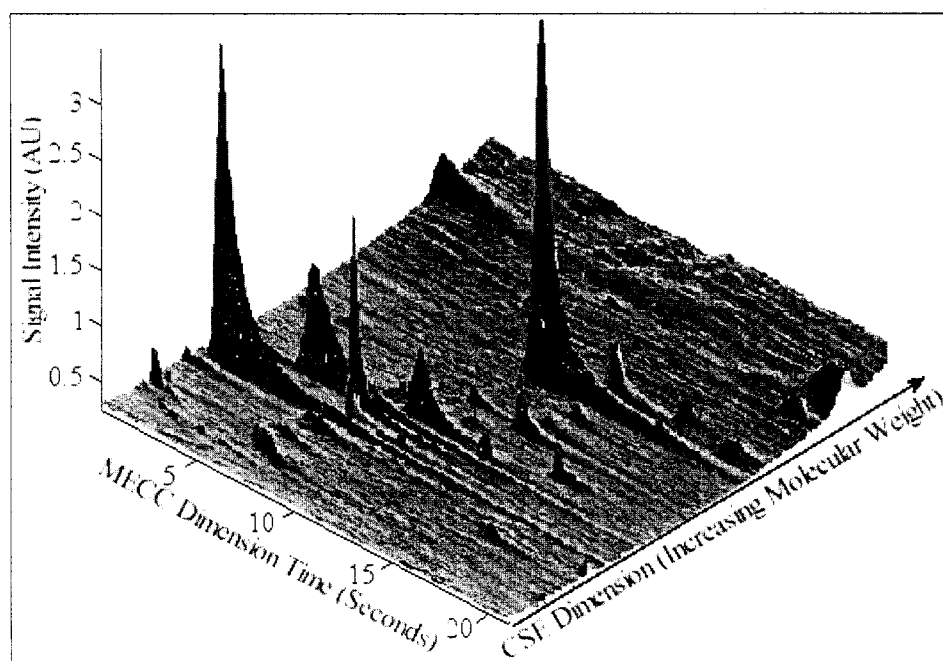


Figure 3.29: Fraction #8-landscape image

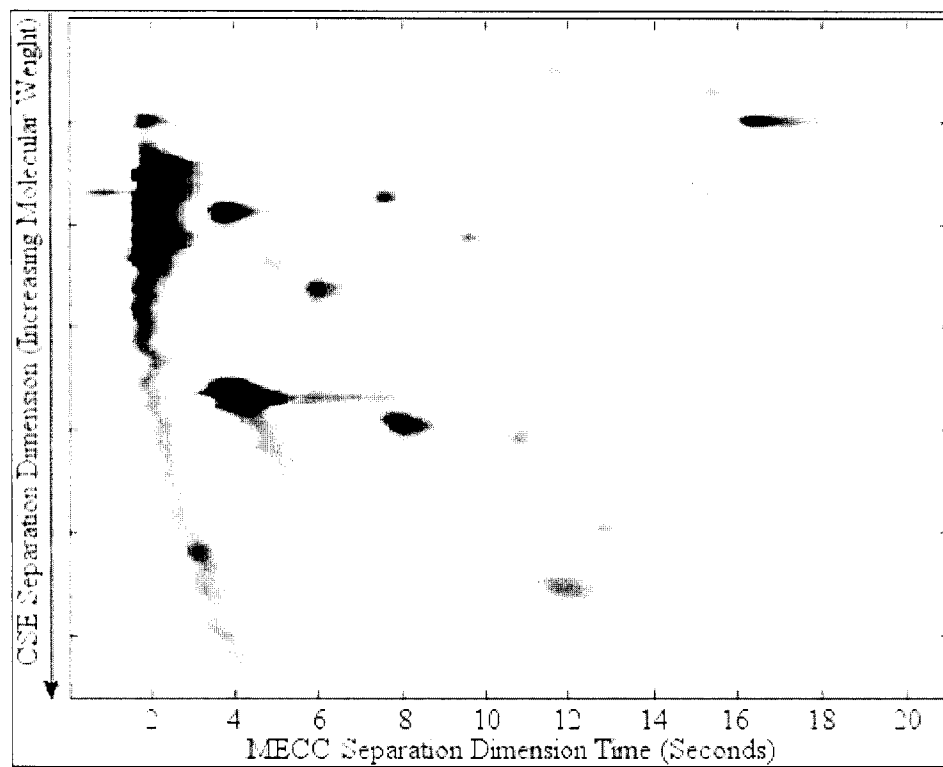


Figure 3.30: Fraction #9-gel image

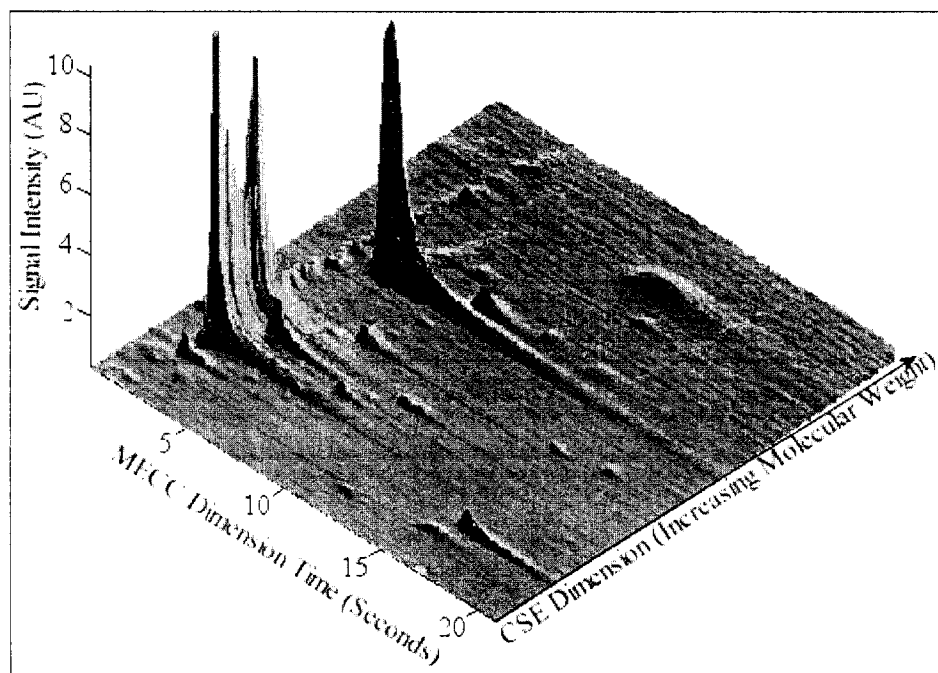


Figure 3.31: Fraction #9-landscape image

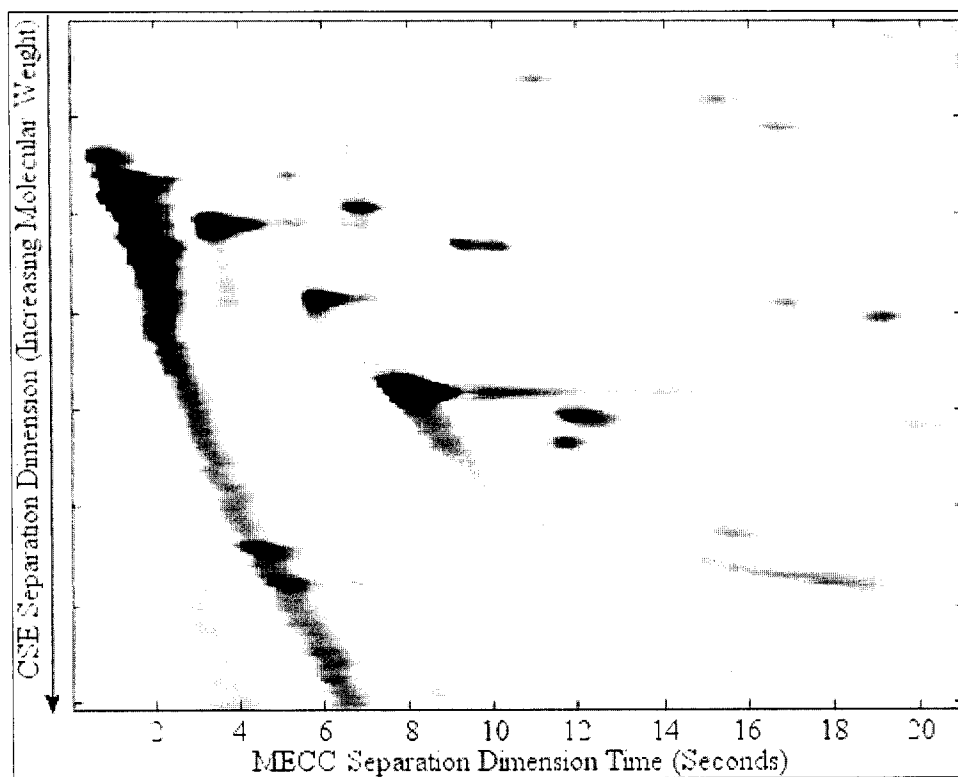


Figure 3.32: Fraction #10-gel image

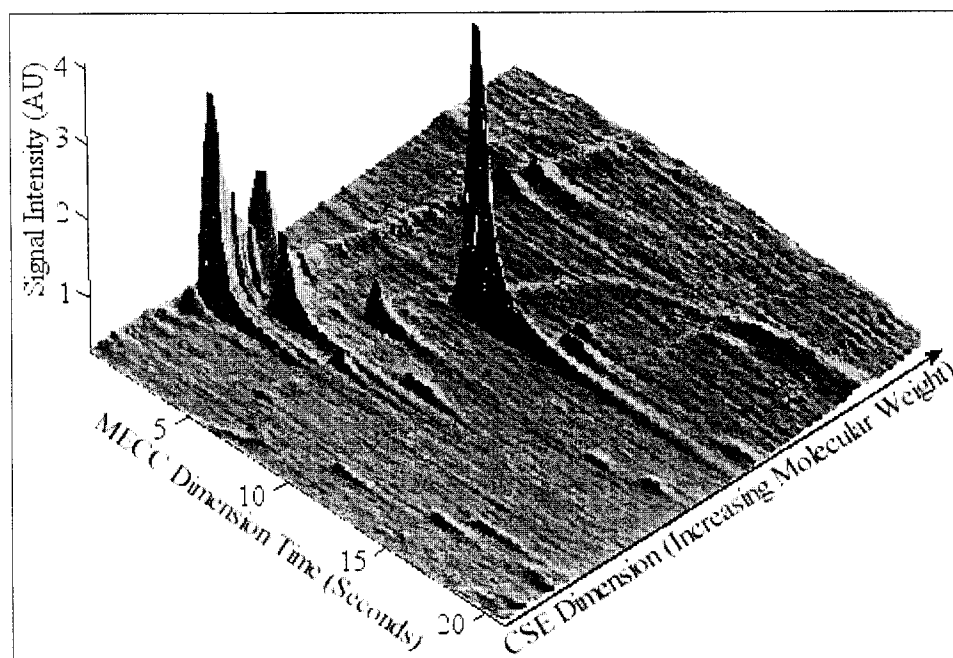


Figure 3.33: Fraction #10-landscape image

The profile generated by each fraction is unique. When combining all fractions to view the homogenate two-dimensional profile, almost the entire 2D map is utilized with minor overlap of a few peaks in the low molecular weight region. In comparison to the optimal two-dimensional run from section 3, this is approximately a 4-fold improvement in the total number of peaks resolved. Due to the decreased amount of protein in each fraction, there are less protein-protein interactions, allowing each two-dimensional run to be more efficiently separated. The protein content also aids in more complete labeling of each fraction. With less protein interactions, each protein can unfold more easily in the denaturing process, prior to FQ introduction allowing more amine groups to be exposed.

While this process has provided a more complete profile of the proteins within the SupT1 CD4+ T-cell line, there remain some limitations. The focus of CE in this thesis is to construct experiments that are quick and automated. While both portions of this section are automated (LC and CE), they are not coupled together and require further sample preparation between steps. This results in increased experimental time. Also, currently there are no identifications correlating the peaks to specific protein(s). The original aim of these experiments was to obtain fractions and divided each fraction into two parts. One part would be used for two-dimensional CE and the other for LC/MS/MS to identify the proteins in each fraction. The exquisite sensitivity of the CE-LIF system still provided intense peaks in the two-dimensional electropherogram. However, due to the small amount of protein present in each fraction, running a portion of the sample through another LC system for identification was not possible. In addition, each fraction

does not result in one or two proteins, but multiple proteins, as evident by each figure in this section. Improvements in the LC gradient are necessary in order to further separate the homogenate.

### Notes to Chapter 3

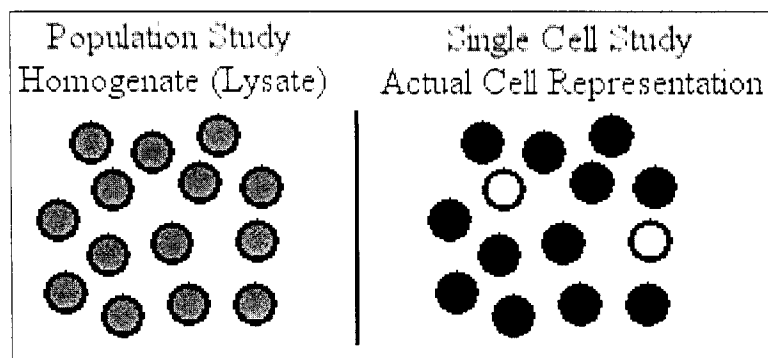
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## Chapter 4 –Single Cell Analysis

### Section 1: Why Single Cells?

Qualitative and quantitative chemical information at the single cell level is of vital interest in the biological and medical sciences. Individual cells contain a variety of components ranging from small molecules to proteins. Knowledge at the single cell level enhances the understanding of diverse cellular processes including intracellular and intercellular communication (i.e. transport processes, exocytosis, endocytosis, secretion, and receptor mediated signal transduction), cell differentiation, physiological effects of external stimuli (i.e. drug application, environment, and toxins), disease states (i.e. cancer, HIV) and gene expression.<sup>1</sup> Analysis of single cells becomes especially imperative in answering the aforementioned question—what is unique about the less than 1% of CD4+ T-cells that become infected by HIV compared to the population?

Experiments involving cell homogenates provide information regarding average protein expression. By looking solely at the average state of the tissue or cell, potentially important information about low-level expression can be overlooked by extrapolating average expression to a single cell. This situation is suggested in Figure 4.1. These two situations could possibly have identical electropherograms, but the composition may be strikingly different. In the homogenate representation it appears that each cell contains identical quantities and internal composition. However, the representation could just as easily be a few under expressed cells within an overly expressed population.



**Figure 4.1:** Lysate vs. Single Cell Within a Population

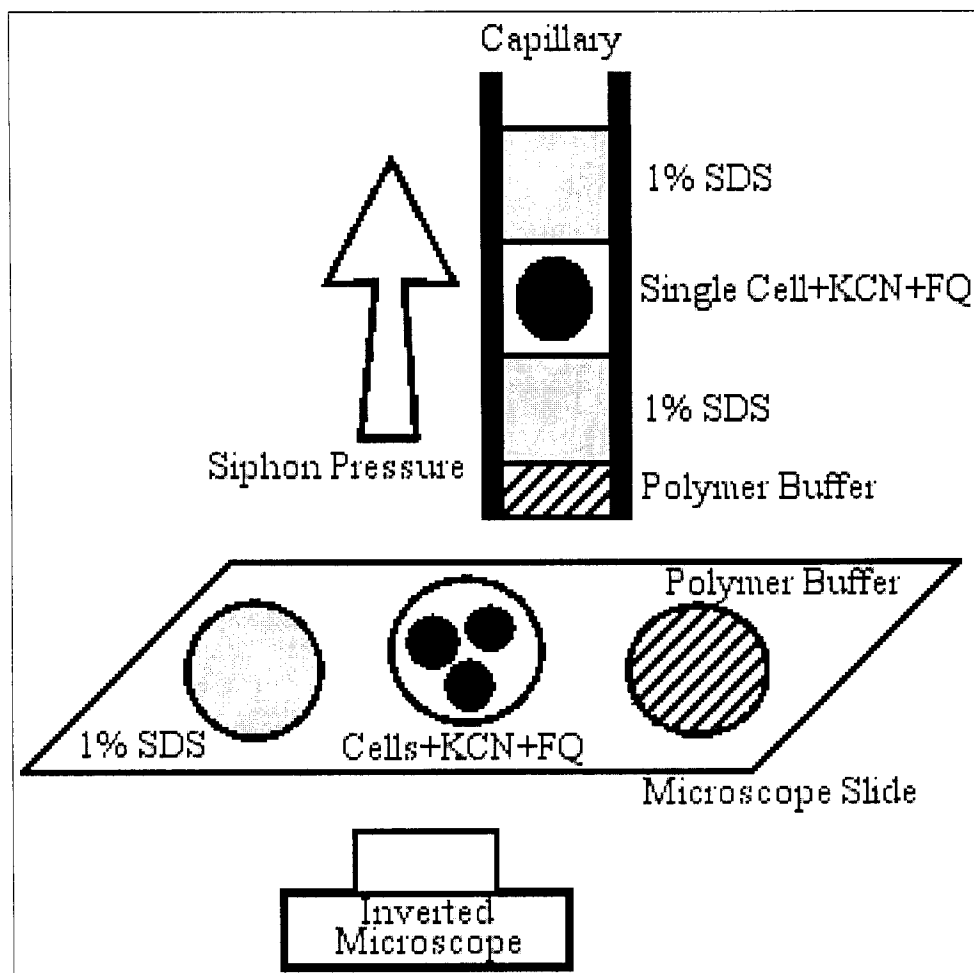
The primary reason that single cells studies have not become commonplace is due to the limited amount of material contained within each cell. Single cell analysis requires the most sensitive of analytical techniques capable of quantitatively monitoring minute chemical compositions associated with dynamic cellular events (i.e. the proteome). The majority of molecules found within a cell are typically present in the femtomole to zeptomole ( $10^{-15}$ – $10^{-21}$  mol) range.<sup>2</sup> For instance, a typical, single mammalian cell is  $10\mu\text{m}$  in diameter with a volume of  $500\text{fL}$  equating to a mass of  $500\text{pg}$ .<sup>3</sup> Assuming each cell contains about 10% protein by weight, and an average protein has a molecular weight of  $25\text{kDa}$ , the cell will contain  $\sim 2\text{fmols}$  of protein. If 10,000 proteins are expressed in a single cell at any moment, the average amount of protein is  $\sim 200\text{zmol}$ . Quantitative detection at this microscopic level is a challenge for conventional bio-analytical techniques due to lack of sensitivity. Electrochemical and laser induced fluorescence detection have been used to obtain sufficiently low mass detection limits for quantitative determination of compounds in single cells with some developments noted in the mass spectrometry and radiochemical schemes.<sup>4,5</sup> The

Dovich laboratory is well established in single cell analysis employing laser-induced fluorescence detection since the late 1990's<sup>6-14</sup>, and it is with this method that CD4+ T-cell analysis is performed in a one-dimensional format.

## **Section 2: Methods and Experimental Design**

All single cell experiments have been performed on the SupT1 CD4+ cultured T-cell line as mentioned in the previous chapters. All CE instrumentation is in the one-dimensional format as described in chapter 3, with the addition of an inverted fluorescence microscope (Olympus) positioned directly above the injection block and a micromanipulator (SOMA Scientific) attached to the injection block to accurately position the capillary over a single cell.

Single T-cells are injected hydrodynamically via gravity instead of electrokinetically according to the following protocol.<sup>13</sup> SupT1 cells are harvested and washed three times in PBS at a concentration of cells that are visible under a microscope objective. On a microscope slide, three wells are divided containing 1%SDS in well 1, cell suspension in PBS, 5mM KCN, and 100nM FQ in well 2, and CSE separation polymer (composition from chapter 3) in well 3. Injections are pulsed for one second according to Figure 4.2.



**Figure 4.2:** Single Cell Injection Method. A single cell with labeling reagent, FQ, is sandwiched between the detergent, SDS, to ensure complete lysis. The polymer is injected to ensure the cell is contained within the capillary while heated.

After all components are injected onto the capillary, a buffer solution identical to the polymer separation sieving matrix (minus the polymer) is heated to 95°C, and the capillary is placed in the solution for five minutes. This process denatures, lyses, and labels the proteins within the injected single cell. After five minutes, the sample is placed

back into a polymer well, and a one-dimensional separation is performed at -16kV. All data manipulation is then viewed in IGOR (Wavemetrics) software.

### Section 3: Experimental Results and Discussion

A total of 40 single cell experiments have been run on the SupT1 CD4+ cultured T-cell line, of which 39 produced an intense electropherogram. For the one failed run, a cell is either not properly lysed or unable to be injected due to adherence to the microscope slide. A typical single cell electropherogram is noted in Figure 4.3.

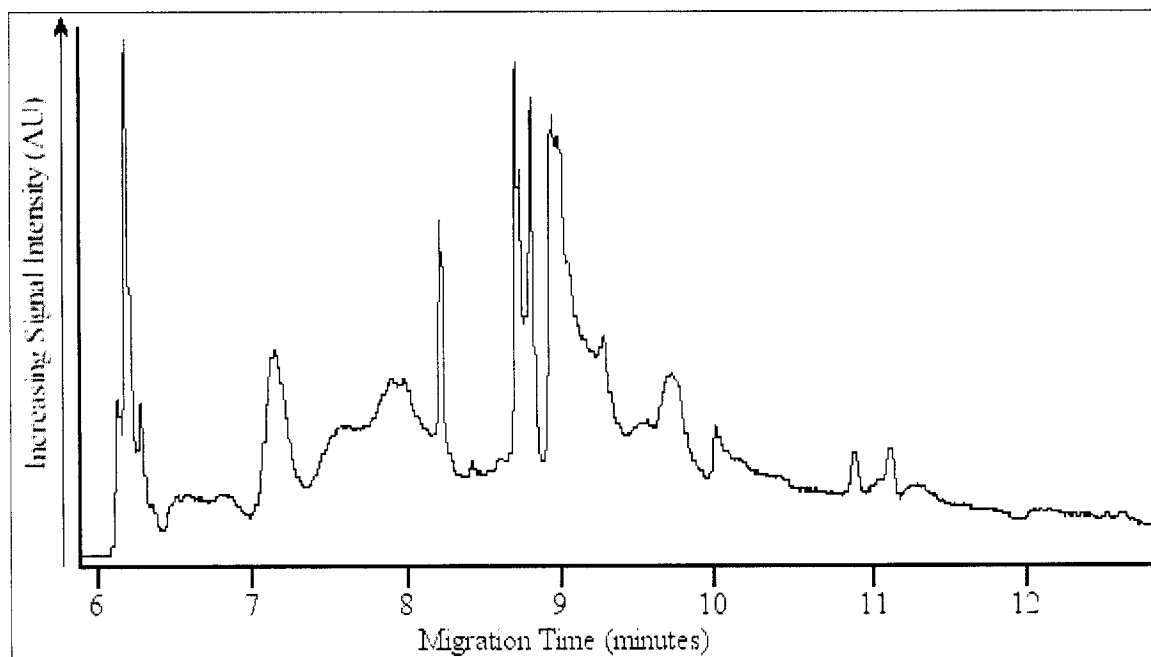


Figure 4.3: Typical SupT1 single cell electropherogram

Efficiencies for most peaks are similar to homogenate one-dimensional CSE runs shown in chapter 3. Not much information can be generated from one single cell electropherogram. When condensing all 39 runs into one electropherogram, many unique properties are present as shown in Figure 4.4.

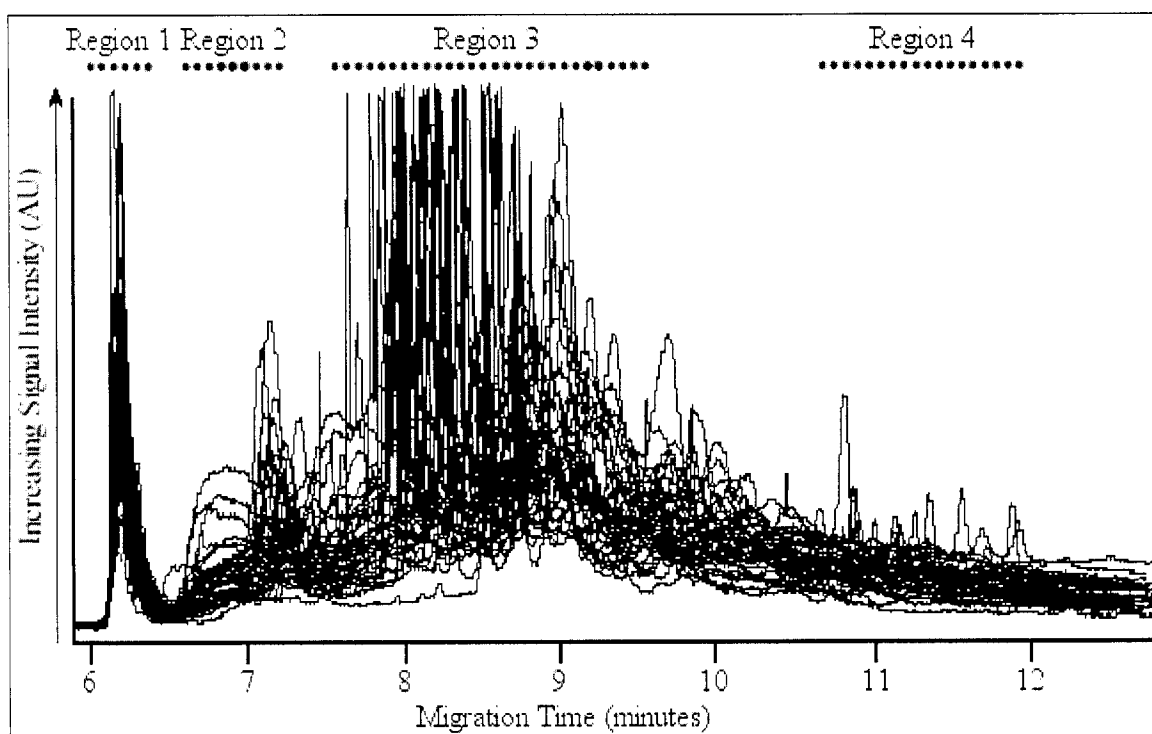


Figure 4.4: Compiled single cell electropherograms

At first glance, Figure 4.4 appears to be a garbled array of peaks migrating within a 6 minute time frame. However, upon closer inspection, the compiled electropherogram can be divided into 4 distinct regions/clusters of peaks each with their own similarity and variability. An enhancement of each region is shown in Figures 4.5-4.8.

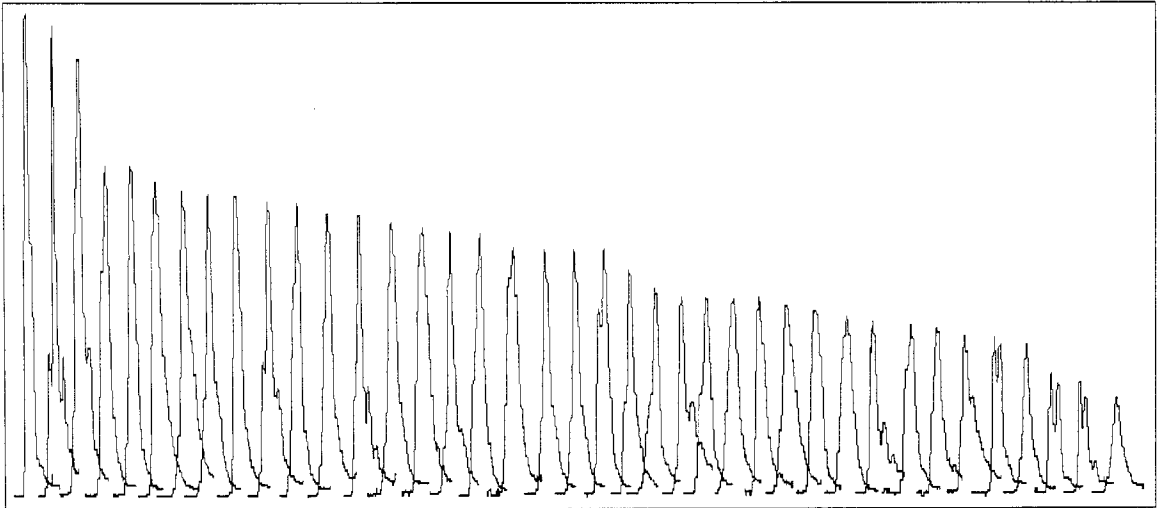


Figure 4.5: Compiled region 1 from Figure 4.4

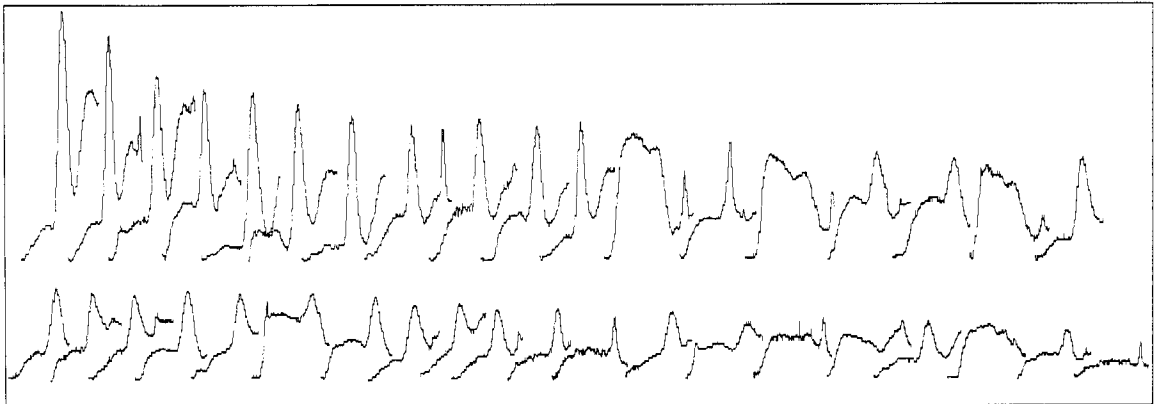
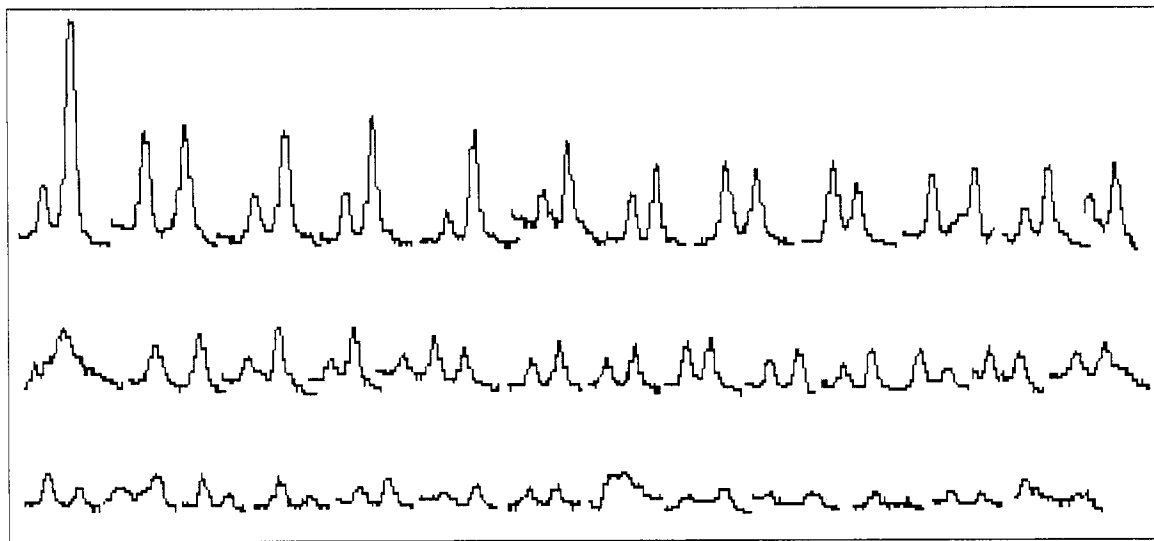


Figure 4.6: Compiled region 2 from Figure 4.4



Figure 4.7: Compiled region 3 from Figure 4.4



**Figure 4.8:** Compiled region 4 from Figure 4.4

Figure 4.5 represents the initial migrating peak from each electropherogram. The migration time for this first peak is reproducible within 99% confidence, but the variability in peak shape and intensity is evident. The intensity of this peak can differ by up to a factor of 4. Usually (75% of the time) this first peak is simply one peak. However, at times (25%) it appears as either a triplet or a doublet of varying intensity. Referring back to Figure 3.5, the homogenate run contains a triplet as the first peak. Employing single cell experiments shows that approximately 75% of the time certain cells are not expressing three discernable proteins (or groups of proteins) within that region, but merely one.

A similar story can be told for the second region of peaks outlined in Figure 4.6. In the homogenate run from Figure 3.5, this second region appears to be a series of well-

resolved intense peaks. However, for each single cell experiment this region is either one or two peaks or even no peaks at all.

Further demonstrating cell-to-cell variability within a cultured T-cell population are regions 3 and 4 in Figures 4.7 and 4.8. Figure 4.7 represents the highest variable region containing anywhere from two to seven distinct, well-resolved peaks of varying intensity. Figure 4.8 represents the final two peaks of every single cell run, which again vary in intensity. Overall, as expected, there are some similarities in migration time of certain peaks and distinct regions of peaks between each single cell experiment. Intensity and resolution characteristics vary greatly, representing the enormous variability even within a cultured T-cell line. Most of these differences within a cultured cell line are due to the varying DNA content inherent to the cell line. Because DNA content often depends on the stage of the cell cycle, this may account for differences in protein expression intensity. For example, as the cell cycle proceeds, proteins involved specifically with cell division in the G2/M phase are often up regulated compared to the G1 phase, which occurs directly after cell division. Since cells cannot be sorted based on cell cycle by physical inspection under a microscope, these experiments did not discriminate based on the cell's current state. Cells are able to be sorted via flow cytometry. However, this is not performed due to the amount of time required to sort the cells leading to protein degradation. To further explore these phenomena, primary T-cells will be analyzed from multiple blood donors in the following chapter.

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## Chapter 5 –Primary Single Cell Analysis

### Section 1: Introduction to Primary T-cells and Extraction from Blood

Cultured cell lines are advantageous in that they can provide reproducibility between experiments within a population. Cultured cells can also provide a virtually endless supply of samples with which to optimize experimental conditions without increasing variability. However, cultured cell lines are only able to display the *in vitro* situation, which is the major disadvantage. By obtaining primary cells from multiple blood donors and extracting the CD4+ T-cells from the blood, *in vivo* conditions can be monitored. Variability between T-cell donors can be explored as well as variability within the individual donor cell population. This chapter exhibits the T-cell variability between four separate blood donors implementing the advantages of single-cell analysis as previously mentioned in chapter 4.

Isolation of T-cells (lymphocytes) from whole human blood is often required for clinical investigations such as histocompatibility testing, assay of cell-mediated immune responses, and many other areas of immunological research. In order to separate out T-cells from whole blood, the introduction of a sterile, ready to use density gradient medium is necessary to filter the blood of undesired products. The most widely used commercially available product is Ficoll-Paque PLUS™ which uses a simple and rapid centrifugation procedure based on the method developed by Boyum in 1968.<sup>1-3</sup> Specifically, Ficoll-Paque PLUS is an aqueous solution of density 1.077g/mL containing 5.7g Ficoll 400 and 9g sodium diatrizoate with 0.0231g calcium disodium

ethylenediaminetetraacetic acid in every 100mL. Ficoll 400 is a synthetic high molecular weight polymer (400kDa) of sucrose and epichlorohydrin which is readily soluble in water. Unfortunately, specific molecular information is not available to the public. However, it is known that the molecules of Ficoll 400 are highly branched, approximately spherical, and compactly coiled with a Stokes' radius of 10nm. Compared with other linear polysaccharides of the same molecular weight (i.e. dextran), Ficoll 400 has a low intrinsic viscosity and low osmotic pressures. Sodium diatrizoate, the sodium salt of 3,5-diacetamido-2,4,6-triiodobenzoic acid, is a convenient compound to use with Ficoll 400 considering it forms solutions of low viscosity and high density. This compound is outlined in Figure 5.1. The introduction of sodium diatrizoate to Ficoll-Paque PLUS is primarily for obtaining optimal density and osmolarity necessary for the efficient removal of other cells from lymphocytes.

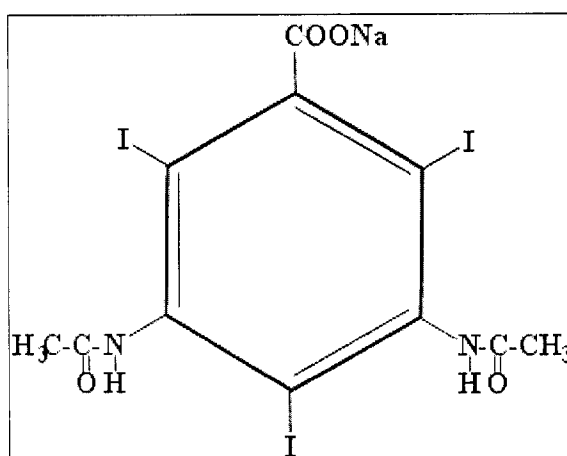


Figure 5.1: Sodium Diatrizoate

In addition to Ficoll-Paque PLUS, a relatively new technology (1991) called RosetteSep™ has been developed to enrich and isolate specific cell types including all T-cells, CD4+ only, CD8+ only, and B-cells.<sup>4</sup> RosetteSep links unwanted nucleated cells to red blood cells using a cocktail of bi-specific tetrameric antibody complexes such that red blood cell rosettes form around targeted nucleated cells. During a standard density centrifugation, these rosettes pellet along with free red blood cells, while the desired cells are highly enriched at the interface between the plasma and the density separation medium. It is the combination of RosetteSep and Ficoll-Paque PLUS that makes possible the isolation of pure CD4+ T-cells from whole blood.

## **Section 2: Methods and Experimental Design**

Whole blood is supplied from the Puget Sound Blood Center (Seattle, WA) from four separate donors in 500mL quantities. Each 500mL quantity is transferred from the hospital bag to a sterile bottle at room temperature. The RosetteSep Human CD4+ T-cell Enrichment Cocktail (Stem Cell Technologies) is added to the whole blood at 50µL/mL. At room temperature, this cocktail and blood sample is allowed to incubate for 20 minutes. The 12.5mL enriched blood sample is then diluted with an equal volume of phosphate buffered saline (PBS) (BioWhittaker) for a total volume of 25mL. The diluted sample is then layered over 12.5mL of Ficoll-Paque PLUS (Amersham) taking care not to mix the layers. The solution is spun at 2000rpm for 20 minutes at which time the solution should separate into 4 distinct layers. The bottom densest layer contains red

blood cells and rosetted undesired cells. On top of that layer is the remaining Ficoll-Paque. A small layer of enriched CD4+ T-cells should be barely visible above the Ficoll-Paque layer. The least dense layer is the remaining plasma. A pictorial representation is seen in Figure 5.2. Approximately 2mL of enriched CD4+ T-cells are extracted from the Ficoll-Paque/plasma interface and transferred to a sterile centrifuge vial. These remaining cells are extensively washed with PBS at a total volume of 50mL and spun at 1700rpm for 10 minutes. The supernatant is discarded and the remaining pellet is resuspended in 2mL of ACK lysing buffer (BioWhittaker) to lyse any leftover erythrocytes that were not filtered. This solution is incubated for no more than 2 minutes at room temperature and the volume is adjusted to 50mL using PBS. The solution is spun at 1200rpm for 10 minutes. The pellet is resuspended in 0.5mL of PBS and is ready for single cell analysis. Single cell CE runs are performed in an identical fashion to that in chapter 4. Twenty single cells are electrophoresed and analyzed from each blood donor.

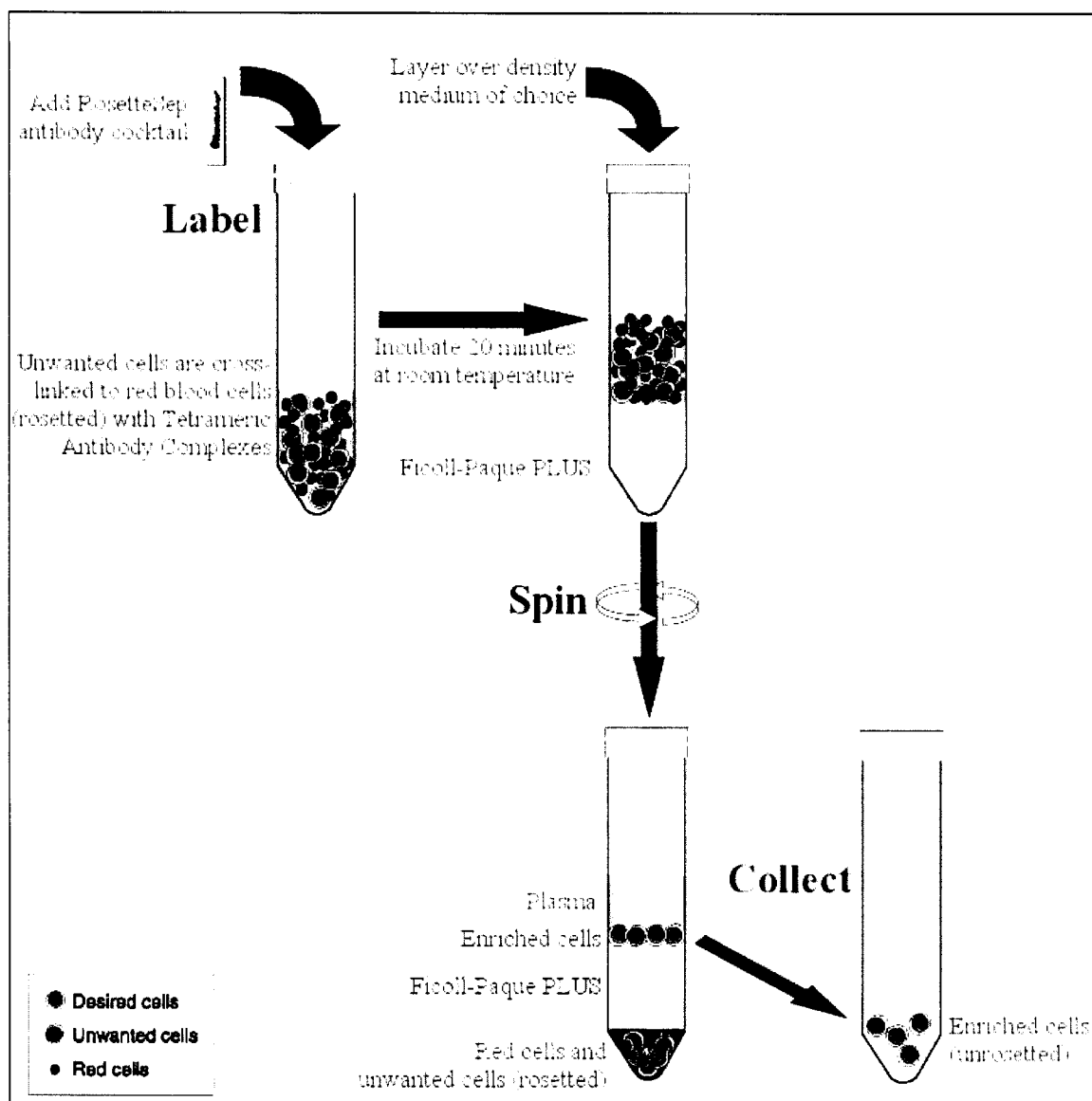


Figure 5.2: CD4+ T-cell Isolation (Courtesy of StemCell Technologies, Catalog #15022)

### Section 3: Experimental Results and Discussion

As previously stated, 20 single cell CE runs have been performed on each of 4 blood donor CD4+ T-cell isolation sets. Experimental success rate is 100% in that each single cell injected produced a clean and intense electropherogram with a CSE separation profile. Compiled electropherograms are displayed for each donor in Figures 5.3-5.6. In addition, a “blank” run is conducted injecting the supernatant without an isolated CD4+ T-cell. This is shown in Figure 5.7, proving that each peak generated from a single cell is due to the proteins within that cell and not from the supernatant.

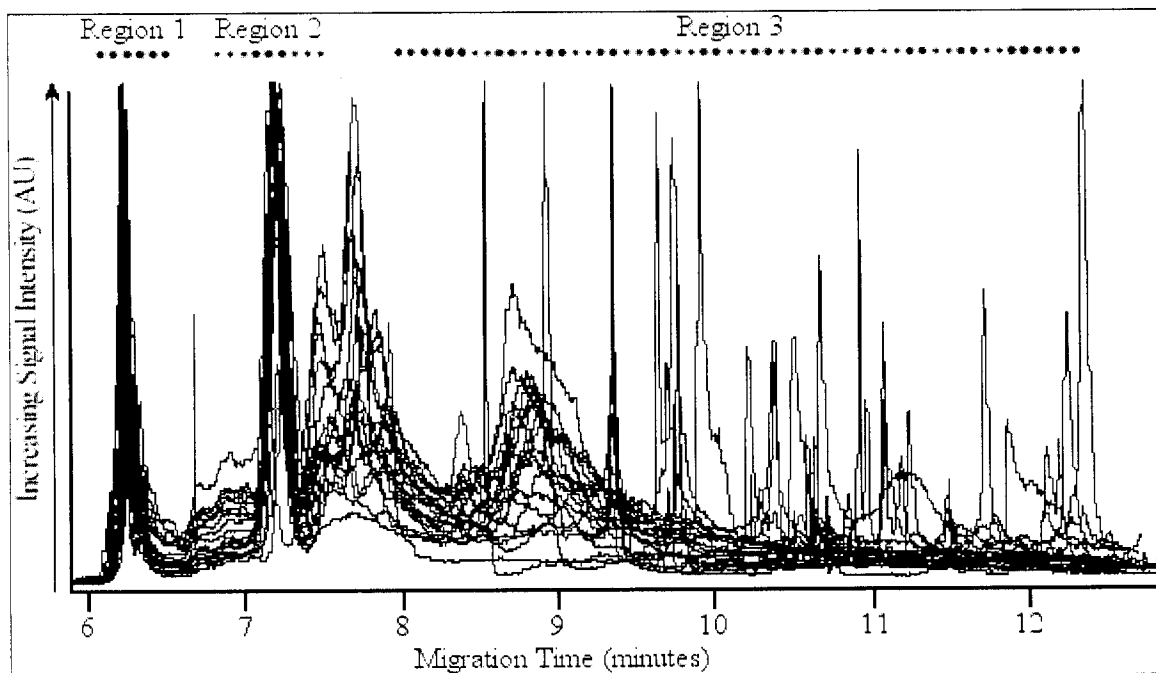


Figure 5.3: Compiled Electropherograms from Blood Donor #1

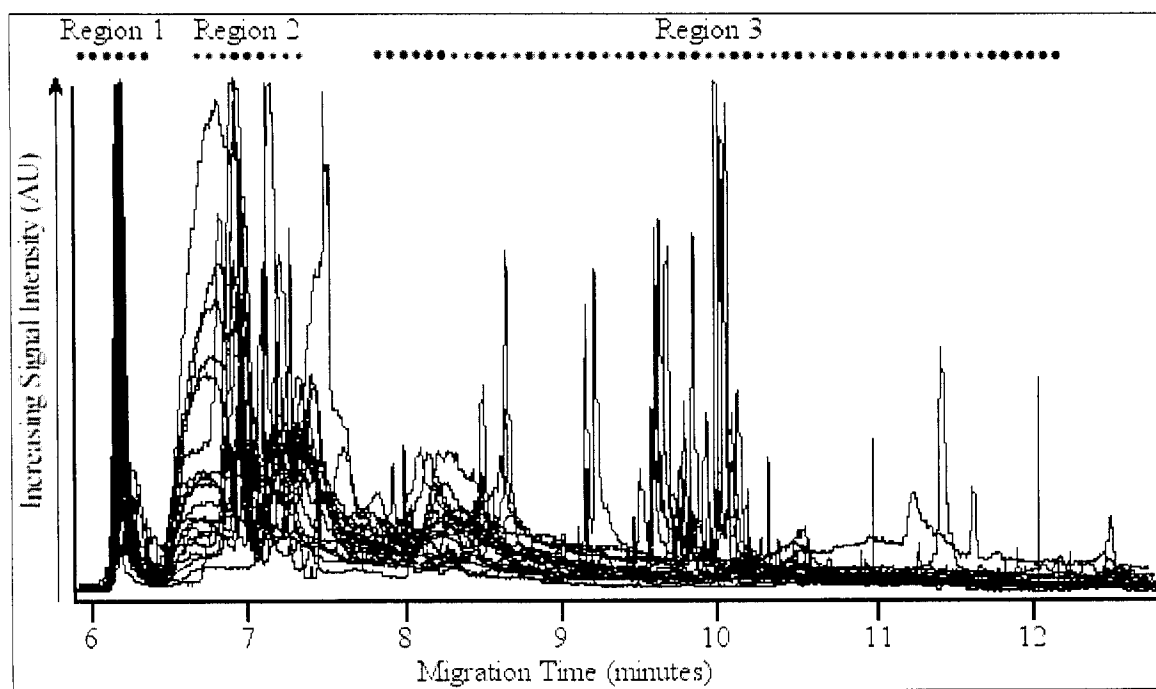


Figure 5.4: Compiled Electropherograms from Blood Donor #2

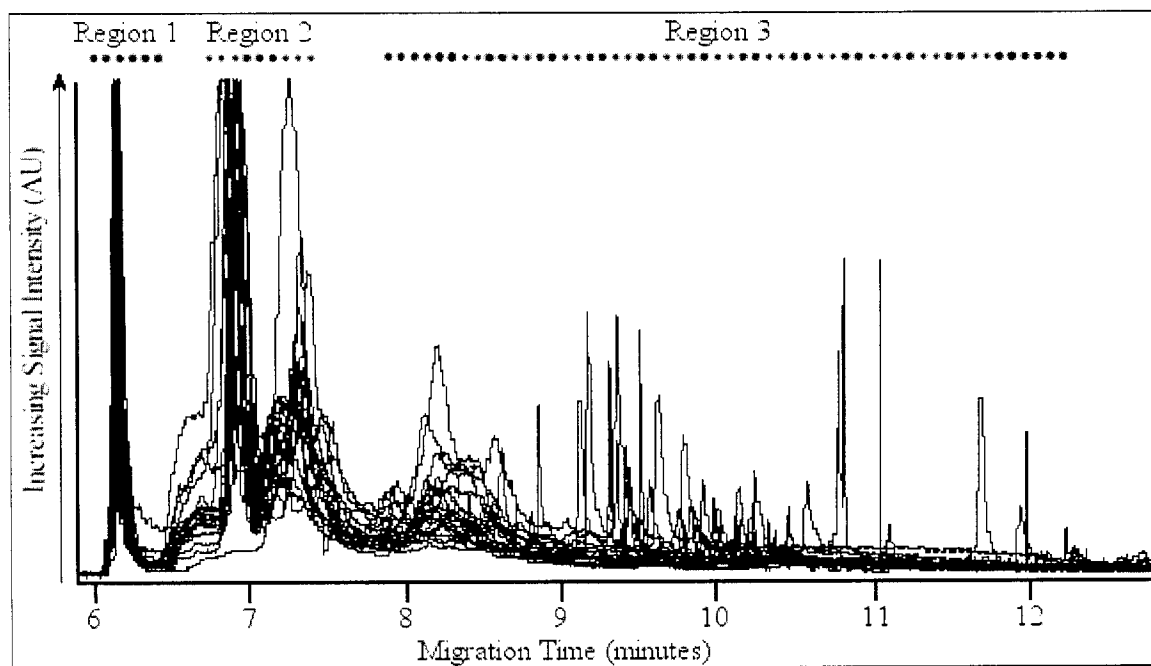


Figure 5.5: Compiled Electropherograms from Blood Donor #3

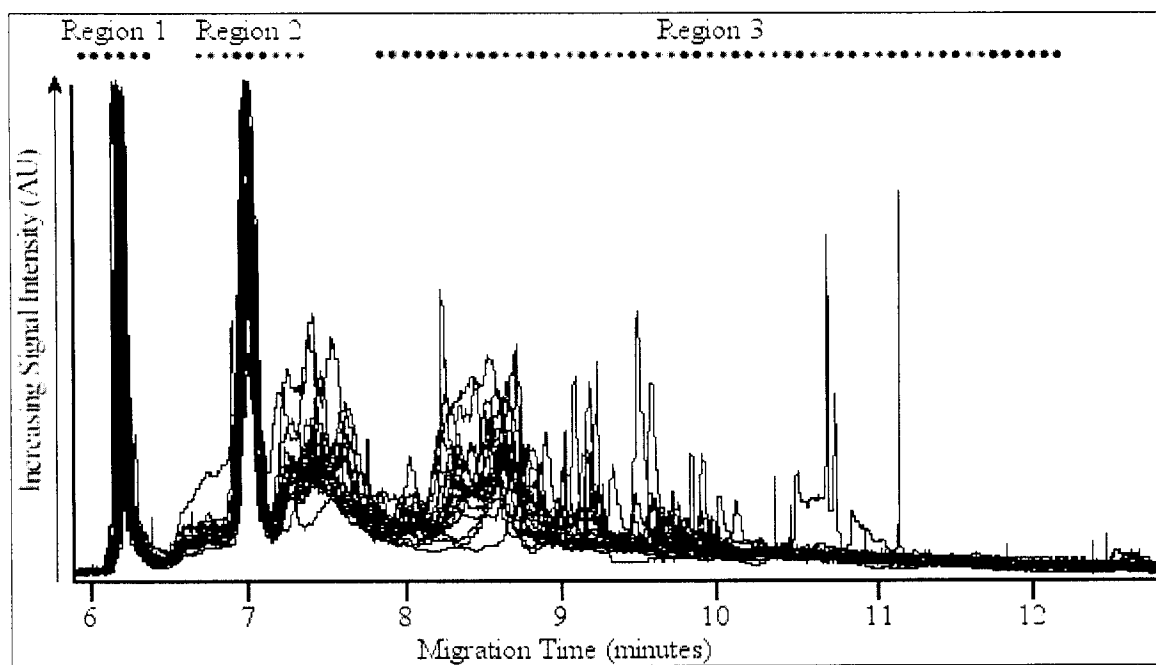


Figure 5.6: Compiled Electropherograms from Blood Donor #4

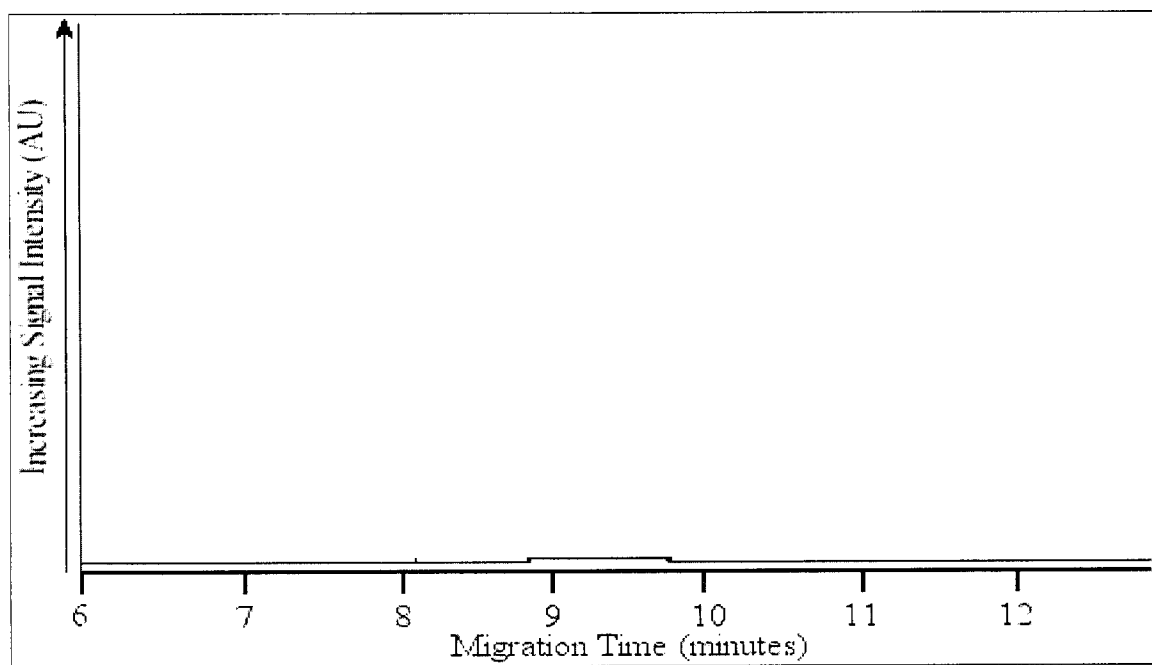
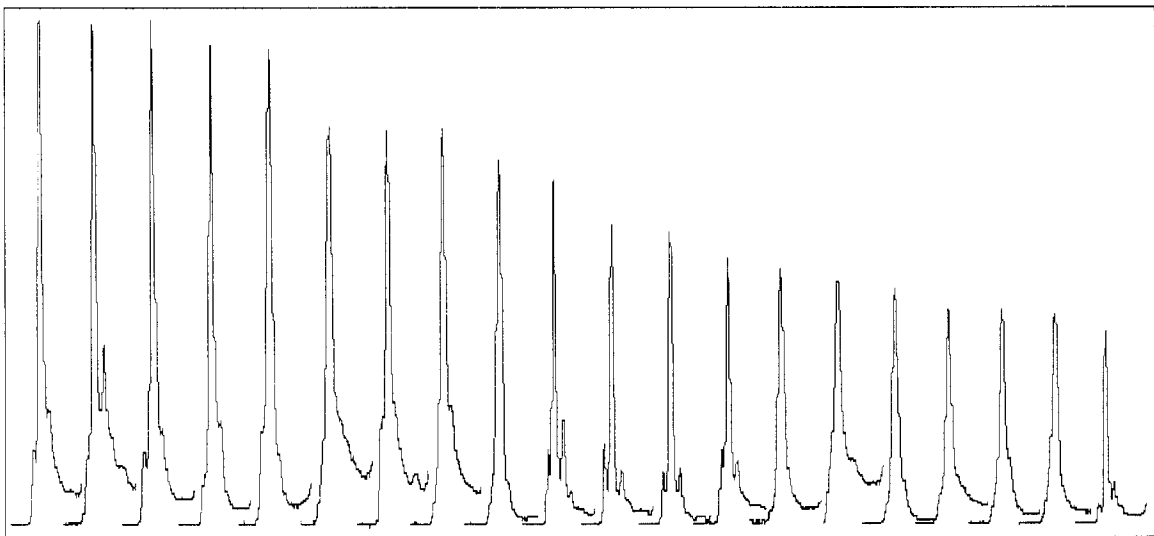
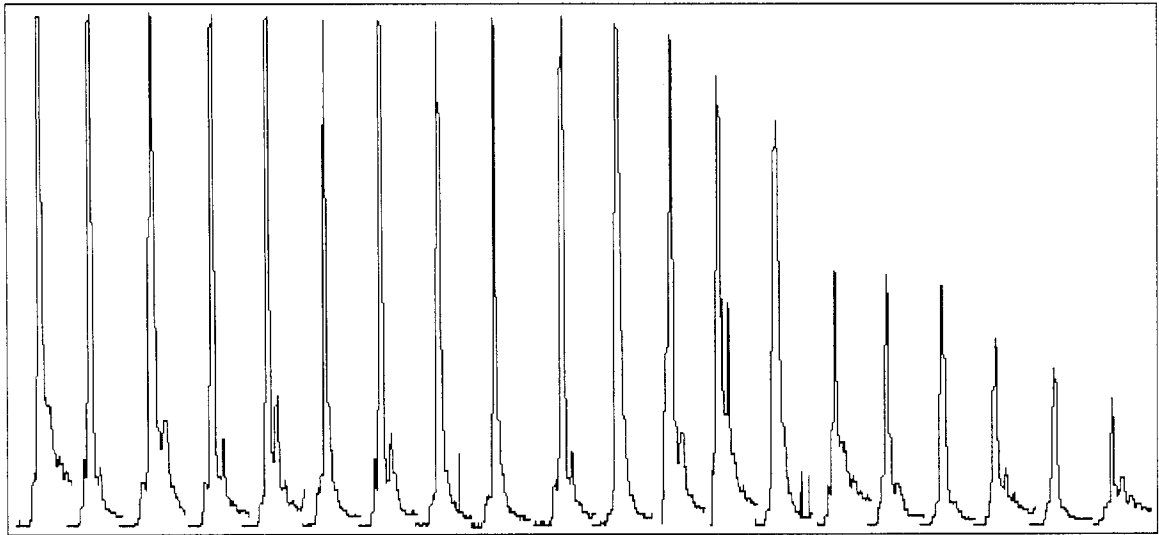


Figure 5.7: Blank CE Run from Cell Supernatant

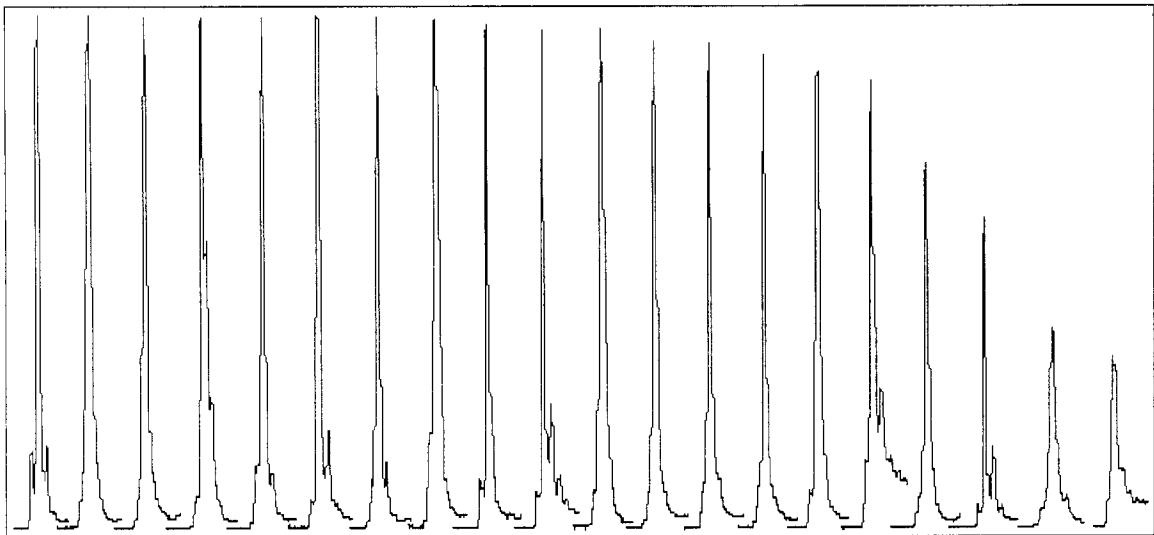
Each of the previous figures is divided into three regions of peaks as demonstrated in chapter 4 displaying the cultured SupT1 T-cell line. Each figure represents distinct variability from donor to donor as well as within the donor CD4+ T-cell population itself. Specifically, region 1 from each donor appears to be the most reproducible peak pattern. Region 2 has minor differences in protein expression, and region 3 has the most differentiation of all highlighted regions. Almost all peaks vary in intensity and migration time in the higher molecular weight region 3 of the electropherograms. These regions are further broken down in Figures 5.8-5.19 to further exemplify the variability within each donor population.



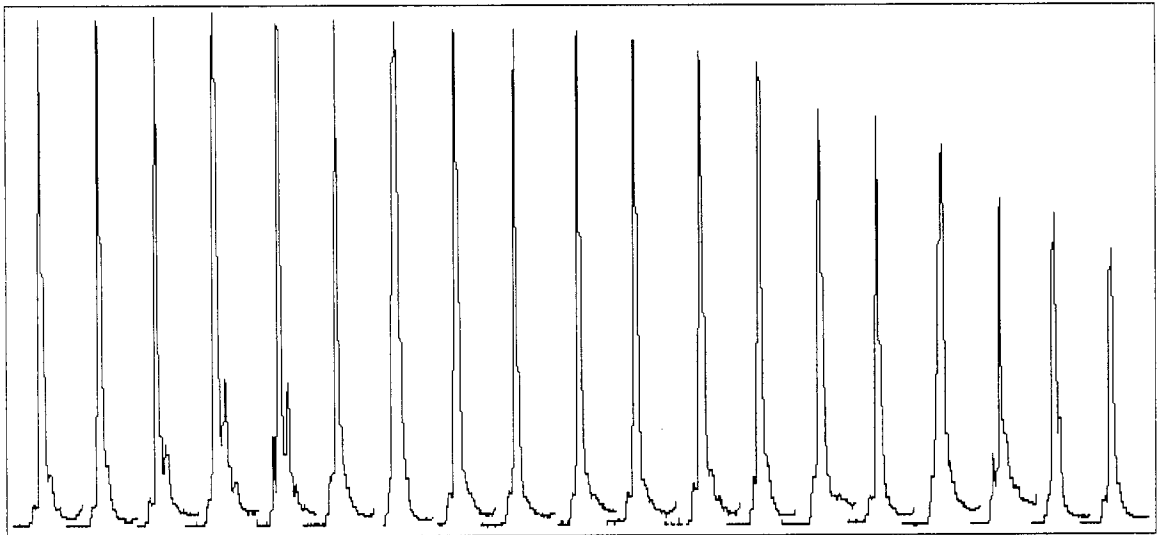
**Figure 5.8:** Compiled Electropherograms from Region 1-Blood Donor #1



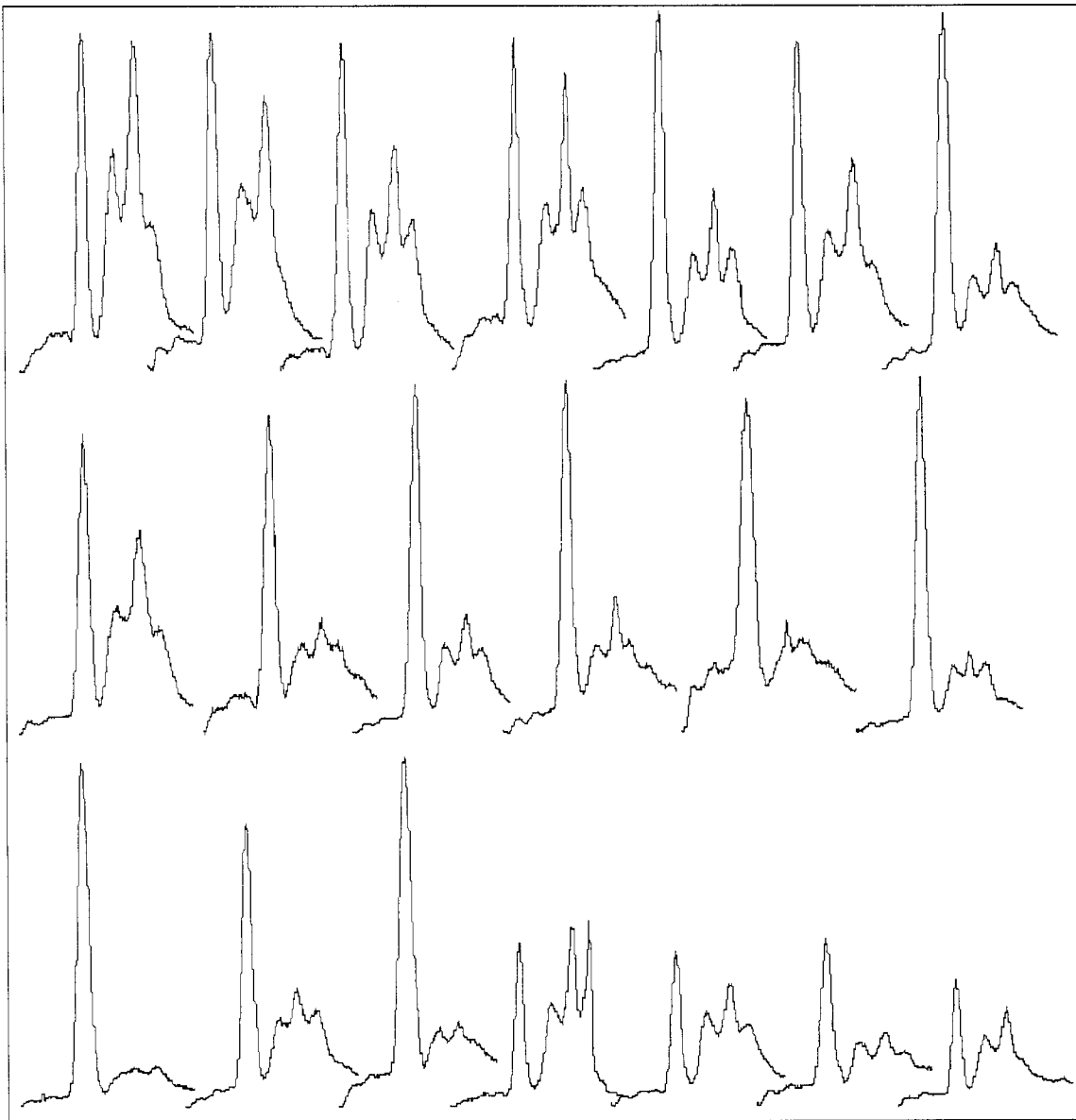
**Figure 5.9:** Compiled Electropherograms from Region 1-Blood Donor #2



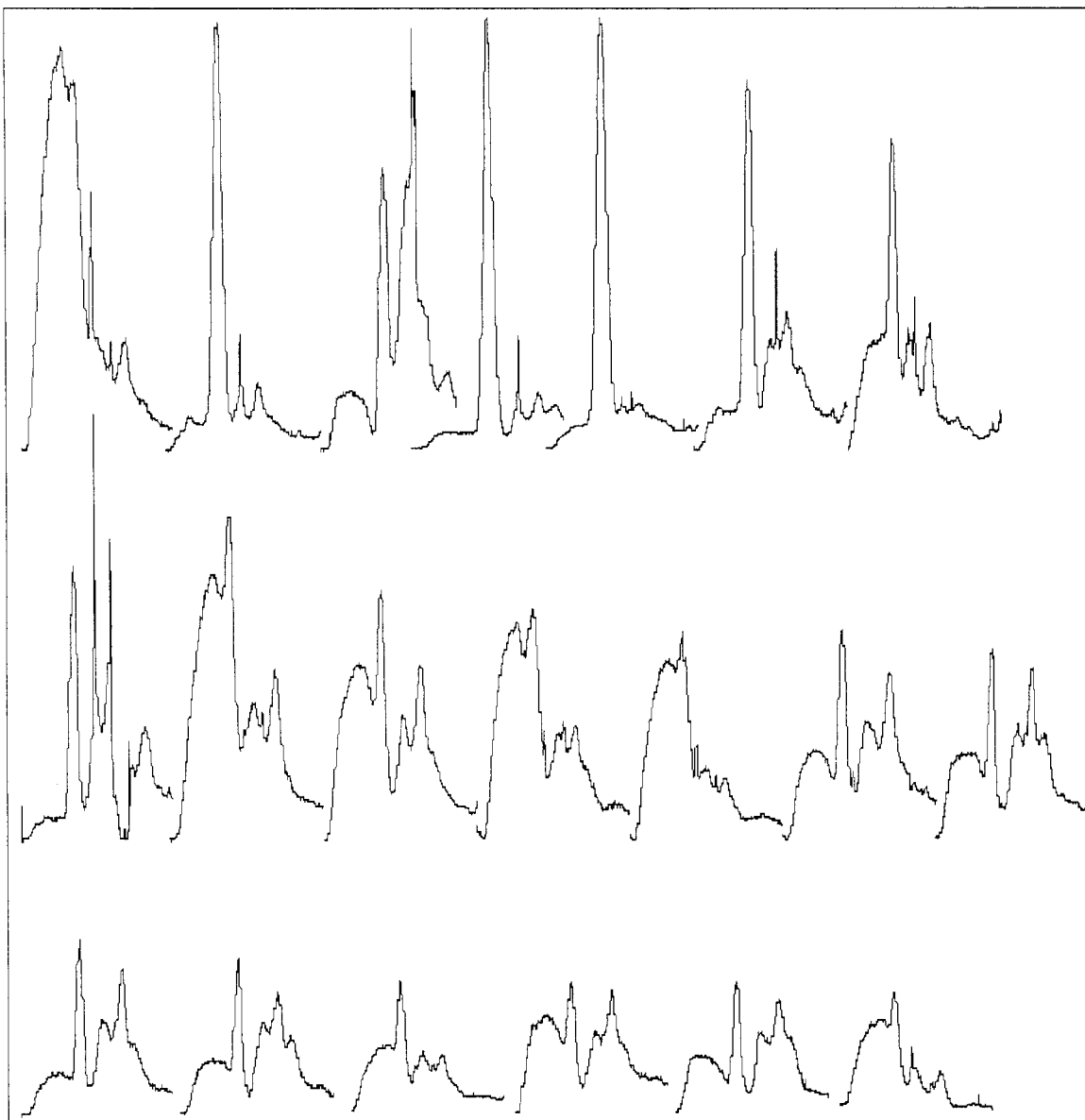
**Figure 5.10:** Compiled Electropherograms from Region 1-Blood Donor #3



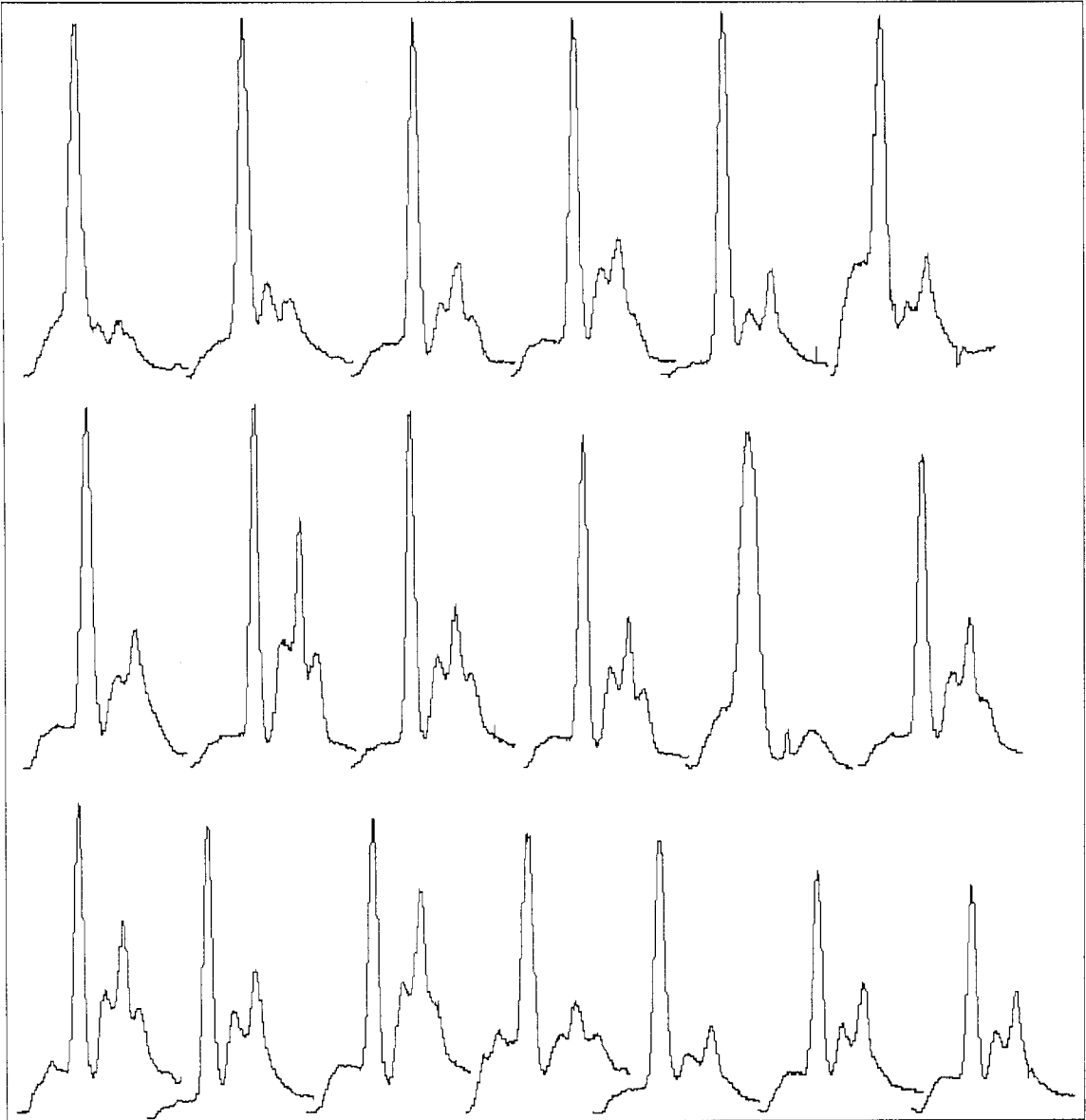
**Figure 5.11:** Compiled Electropherograms from Region 1-Blood Donor #4



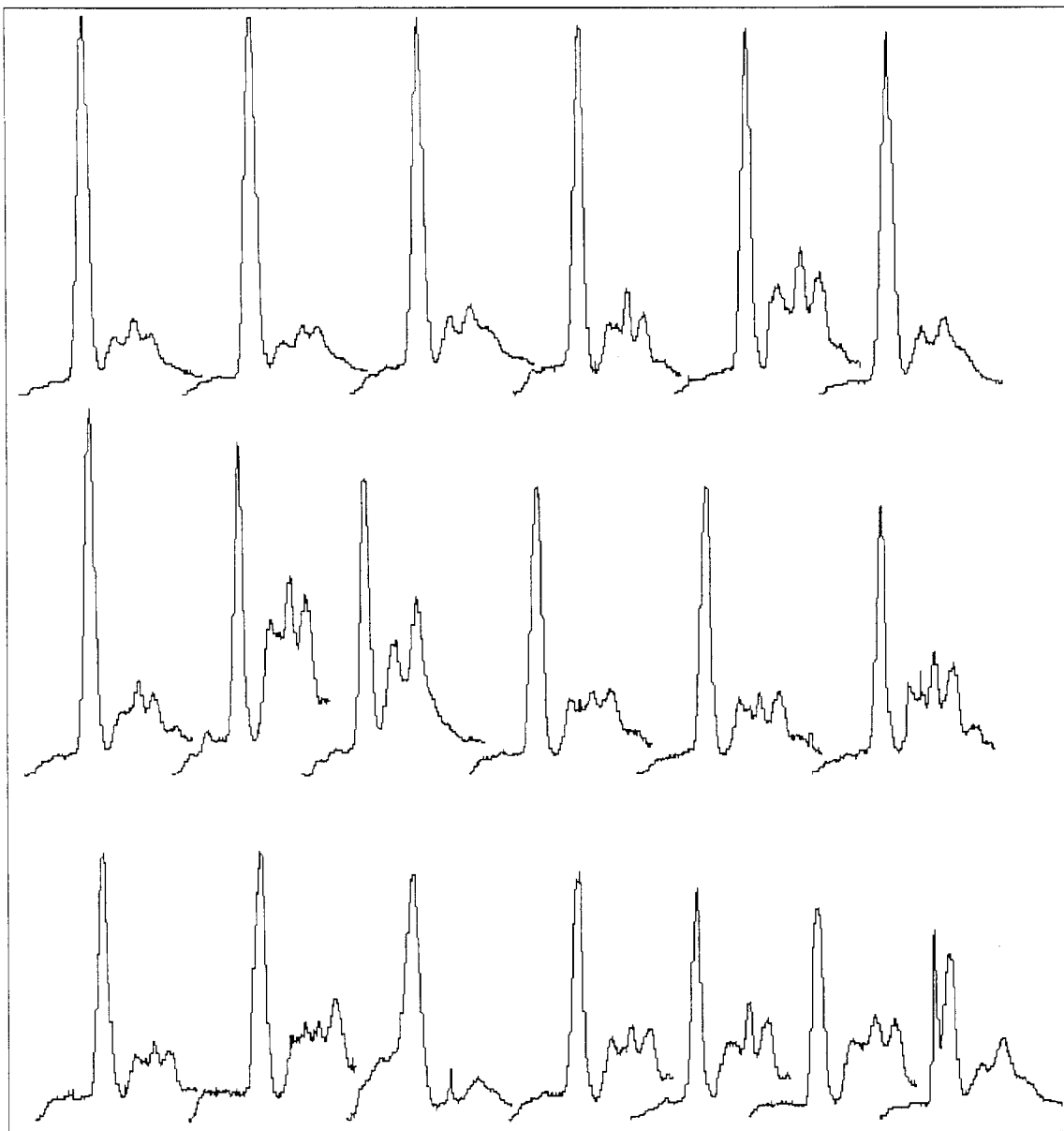
**Figure 5.12:** Compiled Electropherograms from Region 2-Blood Donor #1



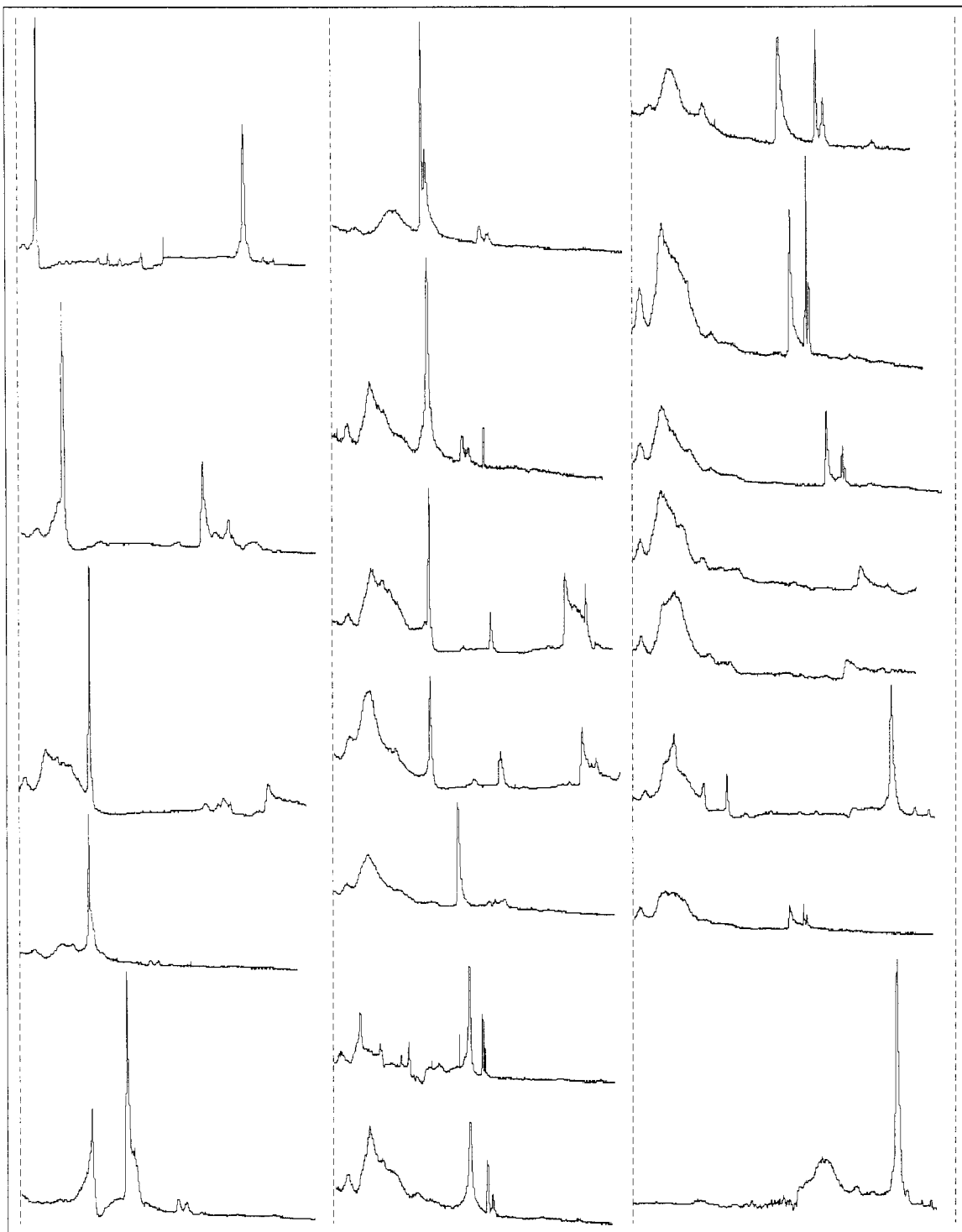
**Figure 5.13:** Compiled Electropherograms from Region 2-Blood Donor #2



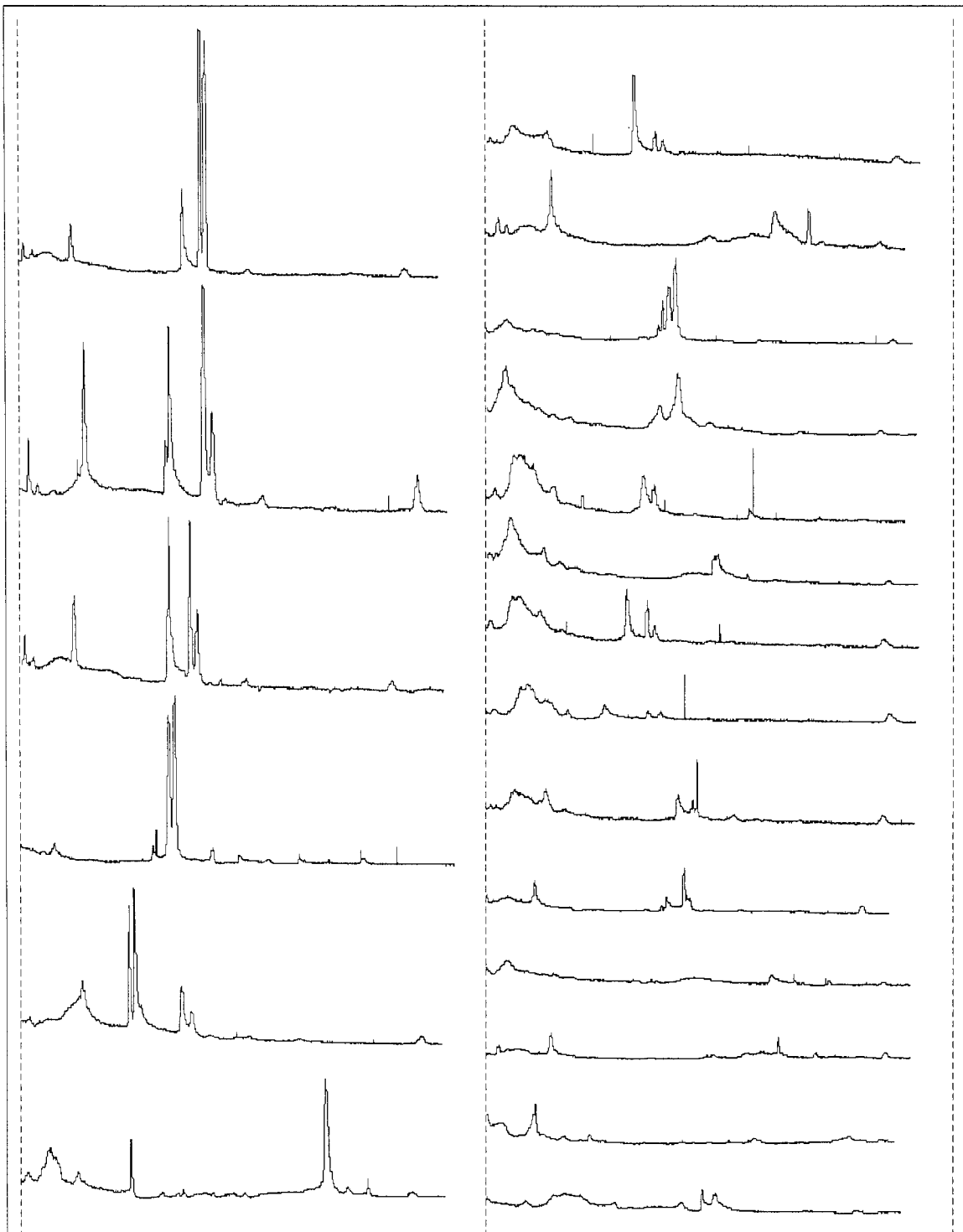
**Figure 5.14:** Compiled Electropherograms from Region 2-Blood Donor #3



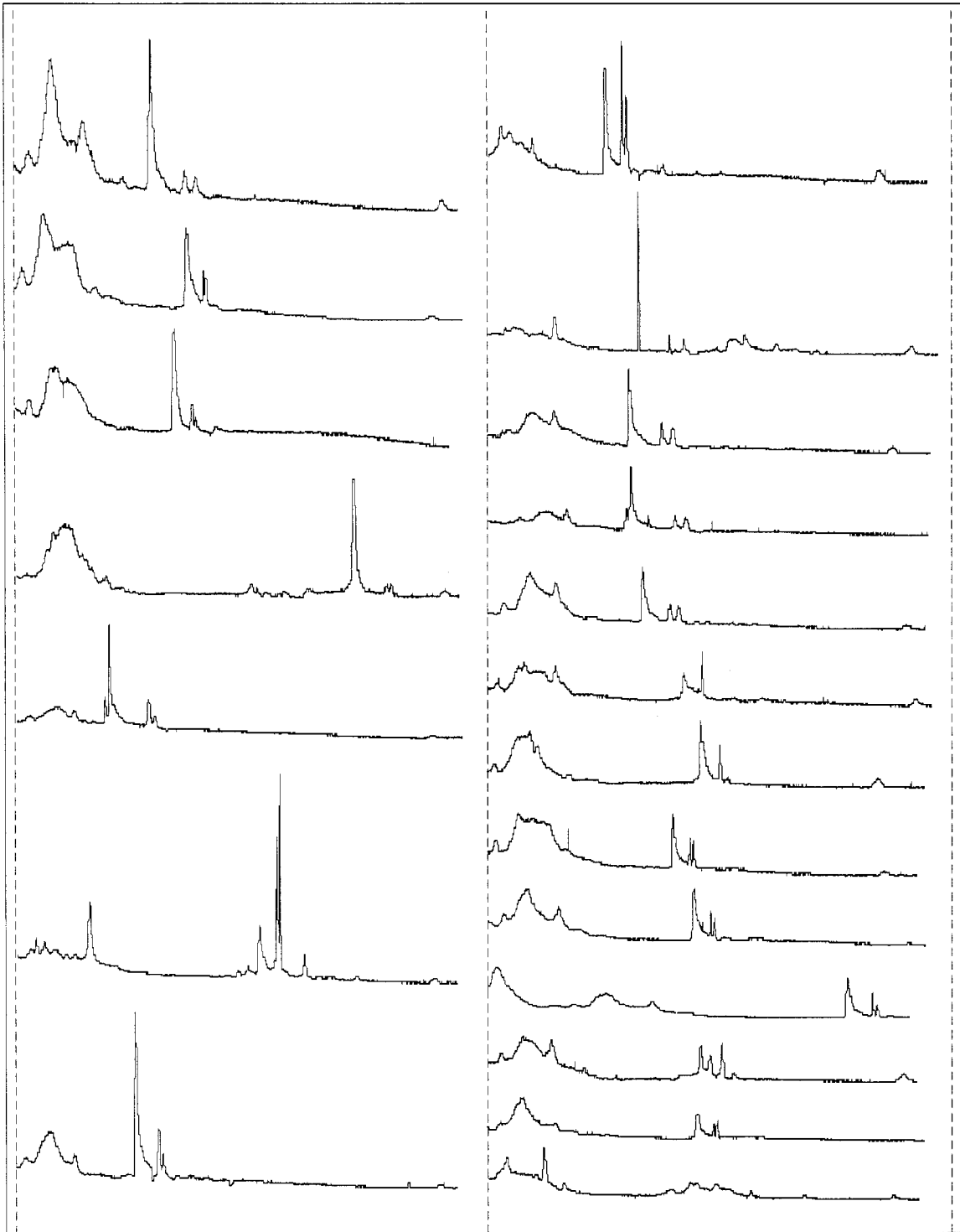
**Figure 5.15:** Compiled Electropherograms from Region 2-Blood Donor #4



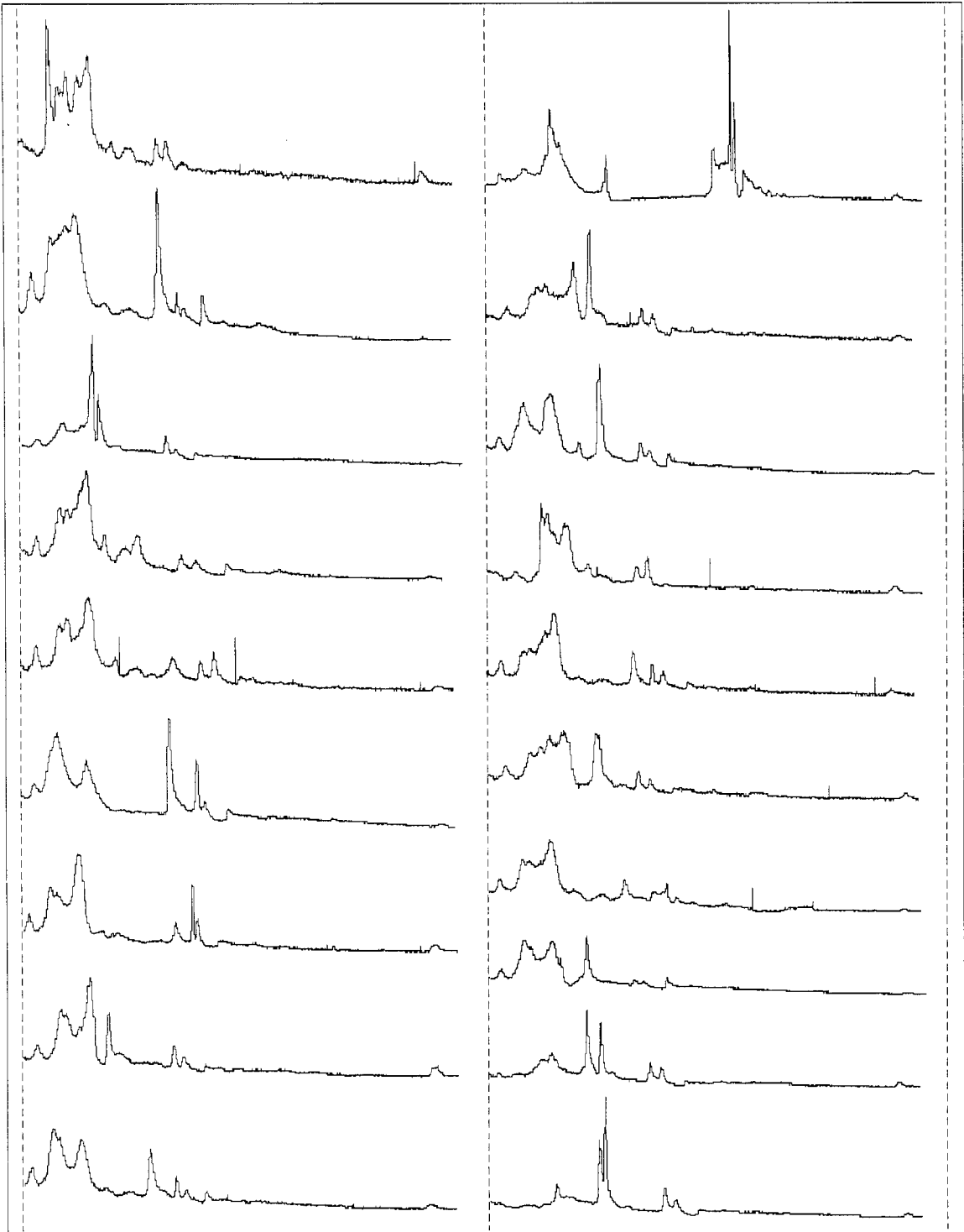
**Figure 5.16:** Compiled Electropherograms from Region 3-Blood Donor #1



**Figure 5.17:** Compiled Electropherograms from Region 3-Blood Donor #2



**Figure 5.18:** Compiled Electropherograms from Region 3-Blood Donor #3



**Figure 5.19:** Compiled Electropherograms from Region 3-Blood Donor #4

Specifically analyzing region #1 from each donor, it is evident that the lower molecular weight proteins are not as dominant in donor #1 compared to the other three donors. Intensities in this first region of peaks are significantly lower for donor #1. However, donor #1 has the most triplet state peaks in this first region. Donors #3 and #4 maintain the highest intensity peaks for this low molecular weight region, but are predominantly singlet peaks. Donor #2 is an even mix of all other donors in relation to peak intensity and resolution.

Intensity profiles for region #2 are all similar with the exception of donor #2. For this region, donor #2 is an anomaly in that it contains a broad envelope prior to the first intense peak within the region. This can possibly be attributed to a high amount of protein-protein interactions for this donor in the middle molecular weight region which can cause deterioration in resolution. Donors #1, #3, and #4 all contain an intense first peak followed by a triplet, doublet, or singlet profile at the end of the region. Primarily, this set of peaks is represented as a triplet, with the middle peak being the most intense.

The high molecular weight region #3 is the most variable of all regions. Intensity, number of peaks, and migration time of all peaks differs from run to run and donor to donor. Donor #2 appears to have the highest number of intense, sharp peak profiles in region #3. However, this holds true for only 30% of the single cell runs. Donor #4 has the least protein expression in region #3. Overall, high variability in the protein expression profile is noted within each donor population of cells and in comparison between different blood donors. While variability has been shown, identification work is

still necessary to determine which specific protein or proteins are undergoing the majority of changes.

### Notes to Chapter 5

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## **Chapter 6 –Conclusions and Future Directions**

### **Section 1: Conclusions**

The question remains—what is unique about the less than 1% of CD4+ T-cells that become infected by HIV compared to the population?

This thesis has investigated the HIV-1 infection on CD4+ T-cells employing classic analytical techniques. Two-dimensional gel electrophoresis displays the most possible information in regards to the complete proteome of CD4+ T-cells. Combining this method with mass spectrometry has led to the identification of many novel proteins present at any given time. Whether or not these up or down regulated proteins pertain to the HIV infection remains to be investigated.

In order to speed up the analytical process, the Dovichi laboratory has developed capillary electrophoresis instruments with exquisite sensitivity and separation capability with which to detect and analyze proteins from a myriad of samples. Combining the advantages of CE with that of a fractionating LC has led to a more complete homogenate protein profile of CD4+ T-cells in two dimensions, comparable to that of two-dimensional gels.

The core of the CD4+ T-cell investigation relies and will continue to rely on single cell analysis. Considering such a small percentage of T-cells actually become infected within an HIV-1 positive individual, single cell analysis allows one to discern anomalies from specific trends in the protein profile. This thesis has uncovered high variability in protein expression within the T-cell population of healthy individuals,

despite the fact that many more samples need to be analyzed to fully understand the dynamics of the proteome in single uninfected T-cells.

## **Section 2: Future Directions**

The future of this project contains many avenues. Identification of proteins within a capillary electrophoresis experiment must be performed in order to determine which proteins are responsible for certain anomalies in the protein profile in homogenates as well as single cells. Extraction of proteins from two-dimensional gels has proven to be a difficult task in regards to quantity and purity in the extraction. Analytical techniques such as ICAT and other co-migration efforts need to be explored to identify peaks within electropherograms.

Along with uninfected, healthy T-cell variability, the HIV-1 infection needs to be further studied as well. The Dovichi laboratory currently does not have the facilities necessary to manually infect T-cells with HIV. In collaboration with the microbiology department, cells can be infected at their facility and then transported to the chemistry department. However, the virus will need to be inactivated in order to transport samples. This is performed via ethanol fixation.<sup>1,2</sup> By fixing a cell or group of cells in a 70% ethanol solution, the HIV virus is rendered ineffective and the cellular contents remain unchanged. Preliminary experiments employing ethanol fixation have been performed on T-cell homogenates with two-dimensional gel electrophoresis. Preliminary results are shown in Figure 6.1. After fixation, the sample is resolubilized in lysis buffer and run in

an identical fashion as other two-dimensional gel experiments. The sample appears to show no visible deterioration in protein expression, and this process would prove extremely useful in the analysis of HIV-1 infected single cells.

The next logical progression would lead to real time infections in live samples. This could perhaps take place in mice. Once real samples are analyzed with the HIV infection, a greater understanding of the hematopoietic system will be obtained. This can lead to modulated pathway studies via drug interactions, environment, etc. Improvements to CE separations such as 5-capillary simultaneous experiments are currently being explored as well to improve turnaround.



**Figure 6.1:** Ethanol Fixed SupT1 Homogenate 2D-gel

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## **Vita**

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