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Chemokine Receptors are a class of G Protein coupled receptors (GPCRs) or transmembrane (TM) serpentine receptors present on cell membranes which act as gate keepers and signal transducers for the cells. Cellular homeostasis is maintained by GPCRs by controlling the movement of various signals and molecules from exterior to interior of the cell. Chemokine receptor subtype 3 has a critical role in homeostasis in organ systems in human body, a novel target in age-related macular degeneration disease progression, and act as co-receptor for HIV entry into cells. Their roles range from the mediation of early stage allergic and inflammatory responses, to host cell defenses and related physiological roles. Little is known about their structure - function properties at the receptor level and the downstream signaling events after the receptor is stimulated. My dissertation focuses on Chemokine receptor subtype 3 (CCR3). CCR3 expressing cell lines available to date are mortal cell line, meant for single use assay purpose with limited/transient CCR3 receptor expression. These cell lines are not a viable option for CCR3 receptor expression-purification and stabilization for biophysical and related structural studies. My work focused on developing a human endothelial kidney (HEK 293) cell line stably expressing human CCR3 using a tetracycline inducible mammalian protein expression vector. This cell line is immortal and can be propagated for cell culture scale-up for semi-preparative scale purification of CCR3. The HEK 293 CCR3 cell line was used specifically for two purposes, as elaborated in this dissertation. The

first is to overexpress the human CCR3 receptor for purification and characterization, by establishing a standard membrane protein purification method for human CCR3 membrane protein. Cellular membrane protein expression in human cells is one of the tough challenges in protein biochemistry. Membrane proteins lose their structural and functional integrity once removed from their lipid bilayer environment in cell membranes; amembrane protein is stabilized in its native biophysical environment. As a fundamental pre-requisite for maintaining the near-native conditions around the membrane protein molecule during its extraction and purification, one has to maintain its biophysical integrity and to preserve the structural and functional features of the protein during the overall extraction and purification processes. The purified human CCR3 has several uses, such as 1) in protein sequencing to identify any possible sequence variants and post translational modifications in the protein, 2) to obtain data useful in receptor modeling and structure-based drug discovery efforts of CCR3, 3) biophysical characterization of the receptor at a single molecule level and its dimer and oligomeric states, and 4) immobilizing GPCRs on surfaces for ligand/drug screening with SPRI-based methods. All the above said uses require human CCR3 receptor purified in significant quantities; micrograms to milligrams. Here we successfully characterized a limited amino acid sequence of the purified CCR3 GPCR by mass spectrometry based methods.

In the second objective, we were able to successfully adapt the laboratory developed CCR3 expressing HEK 293 cell line to stable isotope amino acid enriched DMEM supplemented with 10 % dialyzed FBS cell culture media. The C13 and N15

labeled Arginine (+10 Dalton) and Lysine (+8 Dalton) isotopic enrichment of the cell line was greater than ninety-five percent. This cell line was intended for the study of CCR3 receptor downstream signaling events by phosphoproteomics studies. Temporal phosphorylation of the signaling protein in the cell is the rationale behind global cellular phosphorylation analysis. Protein phosphorylation is the second most common post-translation modification (PTM) after acetylation. Reversible phosphorylation is critical in the functional aspects of cellular proteome and the signaling events involving biochemical pathways. The overall phosphopeptide enrichment for complex SILAC HEK 293 whole cell lysate protein digested peptide samples is developedwith titanium dioxide nanoparticles (TiO_2) as a metal ion affinity resin. The developed SILAC workflow can be applied to study temporal phosphorylation dynamics for different cellular physiological states, following CCR3 receptor stimulation.

In summary, the results emphasize overall stable cell line development of CCR3 receptor expression in HEK 293, analytical methods in CCR3 characterization, and methods inphosphopeptide enrichment from complex protein samples digests such as HEK 293 cell line.

STUDIES OF THE HUMAN CCR3 CHEMOKINE RECEPTOR. DEVELOPMENT OF A CELL LINE STABLY EXPRESSING CCR3, RECEPTOR PURIFICATION AND CHARACTERIZATION, AND PHOSPHOPEPTIDE ENRICHMENT METHODS TO STUDY THE CCR3 GPCR SIGNALING PATHWAY

by

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APPROVAL PAGE

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GLOSSARY OF TERMS

CCR3. Chemokine receptor subtype three

CCL11. Eotaxin

PTMS. Post translational modifications

HOS. High Order Structure

HEK 293-TetR. Human endothelial kidney cells - tetracycline regulatory

HEK293. Human endothelial kidney cells

SDS PAGE. Sodium dodecyl polyacrylamide gel electrophoresis

DDM. n-Dodecyl-ß-D-maltoside

CHS. Cholesteryl hemisuccinate

DTT. Dithiothreitol

BME. beta-mercaptoethanol

IMAC. Immobilized Metal Affinity Chromatography

SPE. Solid phase extraction

MWCO. Molecular weight cut off

SUMO. Glycine-Glycine linker

EDTA. Ethylene Diamine Tetra Acetic Acid

HRP. Horse Radish Peroxidase

AGC. Automatic Gain Control

CHAPTER I

CHEMOKINE RECEPTOR BACKGROUND INFORMATION

1.1 Overview of Chemokine Receptors and G-Protein Coupled Receptors

Chemokine Receptors are a class of cell membrane bound seven trans-membrane receptors (7TM). Chemokines are small peptide signaling biomolecules and the natural binding partners of chemokine receptors. Chemokines and chemokine receptor interactions, by mediating physiological effects, are pivotal in maintaining homeostasis at the organ level. These receptors play a major role in immunomodulatory-related physiologies such as chemotaxis of white blood corpuscle (WBC) components of blood during inflammatory responses and early stages of host tissue infections. Chemotactic movement in eosinophils, neutrophils, and other WBC are mediated by chemotactic chemokines and cytokine binding. These chemokines bind onto cell surface chemokine receptors and activate or stimulate the receptor. Hencethese receptors are term as chemokine receptors. Chemokine receptor stimulation produces a cascade of cellular signaling events that promote cellular migration [1]. Chemokine receptors belong to a superfamily of structurally similar G-protein coupled receptors (GPCR) with several disulfide bond linkages between their transmembrane domains [2]. These receptors fall in the subcategory of G gamma class of Rhodopsin-like GPCRs [3,4]. Chemokine receptor-expressing immune cell migration is mediated by cytokines, chemokines/chemotactic mediators and related signaling peptides [1]. To date, more than fifty chemokines have

been reported to activate chemokine receptors [5]. For instance, chemokine receptor subtype-3 (CCR3) expression in cells promotes their migration after stimulation by eotaxin1 (CCL11), a potent chemokine agonist of CCR3. Stromal cells and immune cells release the chemokine eotaxin1 (CCL11).

Chemokines and their respective cell membrane binding receptors, known as chemokine receptors, are present on the cell membrane of immune cells and play a critical role in cellular migration and cell-cell communication during early stage infection, inflammatory physiological processes, and are critical for maintenance of homeostasis. There are approximately 48 known endogenous chemokines in humans and mice and 25 chemokine receptors [3]. In these, twenty are involved in signaling and five are classified as non-signaling chemokine receptors. Several endogenous chemokines ligands stimulate the signaling classes of chemokine receptors. Chemokine receptors are further classified into CC type, CXC-type and CX3C type chemokine receptors. The terminology derives from the pattern of cysteine amino acids in the protein domains, the sulphydryl (-SH) containing amino acid cysteines facilitate disulfide bond formation (-S-S-) in the chemokine molecule. Chemokines are a diverse group of small cytokines, ranging from 8 to 10 kiloDalton (kDa) in molecular mass. Chemokines promote chemotactic movement of immune cells during infection. The hallmark feature of chemokine receptor-ligand interactions and the consequent downstream signaling mechanisms is the diverse nature of receptor-ligand specificity. This process is not limited to one or a few receptor-ligand pairs. Different chemokines interact with one or more chemokine receptors, but the selectivity in binding affinity of the interaction varies

significantly. Chemokine receptor mediated subcellular signaling mechanisms were only recently discovered and are still poorly understood in a broader subcellular systems biology level. There are important avenues to decipher the signaling mechanisms of chemokine receptor stimulation.

1.2 Role of Human CCR3, G-protein Coupled Receptor-Mediated Effects in Cellular Physiology and Biomedical Significance

Chemokine-mediated physiological effects are pro-inflammatory cellular responses, cell–cell communication in immune cell components and organ homeostasis and chemotactic movements [6,7]. CCR3 also serves as as a co-receptor for HIV entry into cells expressing the receptor. Cells of the immune system coordinate responses by chemokine receptor-based signaling during host infection.

Primary and secondary immune responses, most inflammatory diseases such as atopic dermatitis, allergic rhinitis, and asthma are mediated by chemokine signaling in cells. Close to 50 million people suffer from allergy-related diseases in the United States. Allergy-related diseases themselves burden approximately 18 billion dollars on the health care industry [8]. In addition to allergy-related ailments, chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and metabolic syndrome conditions are mediated in part through chemokine receptor mediated pathologies.

In the past decade, CCR3 expression has also been demonstrated in other cell types and tissues such as choroidal endothelial cells in the retina of the human eye during age-related macular degeneration (AMD) disease progression [9]. AMD is a retinal vascular proliferative disease, one of the leading causes of blindness affecting a vast

number of people worldwide. AMD is most prevalent in industrialized nations. In addition to VEGF-A mediated effects on progression of AMD, CCR3 mediated proliferation of choroid vasculature leads to progression of the disease. There are reports on the role of CCR3 mediated cellular mechanisms in cancer progression. Eotaxin1 (CCL11) promotes prostate cancer cell invasion via a CCR3 - ERK-mediated signaling pathway and upregulation of MMP3 expression [10]. In Osteoarthritis, CCR3 promotes activation of fibroblasts such synoviocytes and induces inflammatory responses [11]. Recently, overexpression of CCR3 was shown to be involved in the progression of prostate cancer in obese patients [12].

As illustrated by the studies mentioned above, the role of CCR3 in mediating these various clinical pathologies suggests that CCR3 antagonists could provide a strategic therapeutic intervention for some clinical conditions. Several small molecule drugs have been developed to block CCR3 receptors, in the area of allergic diseases such as allergic rhinitis, atopic dermatitis, and asthma. In the late stages of clinical development, most of these CCR3 blocking drug candidates failed to provide a significant improvement over existing therapies. Preliminary drug discovery efforts were carried out with ligand-based modeling studies as CCR3 receptor structural information like biophysical, crystallographic or NMR data lacks for structure-based drug design efforts for CCR3 antagonists.

The occurrence of chemokine receptor subtype three CCR3 was first reported separately by Daugherty et. al., 1996 and Ponathet. al., 1996. CCR3 plays a critical role

in eosinophil trafficking. Further, CCR3 is reported to express in eosinophils, basophils, Th2 lymphocytes and mast cells.

Initially discovered chemotactic movement of murine CCR3 expressed in eosinophils exhibited chemotaxis upon stimulation with CCL11 (eotaxin); functional chemotaxis assay methods, Gao et. al., 1996. Competitive binding studies have shown CCL11 has the strongest binding affinity for CCR3 with kD 0.1 nM. Functional assays such as intracellular calcium mobilization assay have shown a positive trend with chemokine stimulation. Eotaxin and RANTES (CCL5) come under beta chemokines; cysteine residues adjacent to each other -CC- type chemokines. Eotaxin also playsa significant role in the development of allergy mediates process.

The most potent peptide ligand eotaxin (CCL11) stimulating CCR3 and regulates the recruitment and activation of inflammatory leukocytes, in particular eosinophils. Eotaxin plays a fundamental role in the development of allergic responses in a diverse manner. Some of the primary cellular signal pathways like ERK, Akt, Ras and JAK/Stat are activated by chemokines receptor stimulation with their natural agonist eotaxin and RANTES.

1.3 Structural Features of G-Protein Coupled Receptors and Chemokine Receptors

Structurally chemokine receptors are classified into gamma subtype class; rhodopsin type G-protein coupled receptors (GPCR). One of the closest partner of CCR3 is CXCR4 receptor. CXCR4 protein structure was solved by structural biology and related methods by the fusion of T4 lysozyme between intracellular loop 5 and 6 of

CXCR4 also by introducing a point mutation to stabilize the GPCR in protein crystallization. Two distinct structure of CXCR4 were reported; one by stabilizing CXCR4 with a small molecule and the other by an acyclic peptide ligand CVX15 [13]. The distinctive hallmarks of chemokine receptors are the formation of homodimers and heterodimer complexes. Dimerization and oligomerization tendency of CCR3 is evident with experimental data. Affinity purified CCR3 protein analyzed by SDS PAGE gel electrophoresis.

Structural biochemistry of chemokine receptors provided some insightful details for drug discovery related efforts, Handel et. al., [14,15,16].

1.4 GPCR Structure and Functional Characteristics

G-protein coupled receptors, also termed as seven transmembranes (7TM) receptors or serpentine receptors act as signal transducers from the cell-extracellular environment to the intracellular regions of the cell. All the GPCRs have seven transmembrane domains, extracellular and intracellular domains. GPCRs are present in most primitive pathogens such as herpes virus expressing chemokine receptor, rhodopsin in light harvesting bacteria, and in highly complex eukaryotes. GPCR exist in two distinct structuralconformations; R, the inactive conformation, and R* the active conformation. R* the active conformation happen once the ligand or agonist bind on to the extracellular domains of the receptor. GPCR activation and canonical signal propagation occur with a diverse array of extracellular stimuli such as light, odorant, peptides, hormones, biogenic amines, growth factors and lipids [17]. Functional GPCRs in the human body exists with diversity with notable physiological roles. Predominantly GPCRs maintain homeostasis

at organ level physiology, and some of this class of GPCRs are chemokine, angiotensin, and beta-adrenergic, dopamine receptors. Structural homology exists in the above stated GPCRs. GPCRs can exist as monomeric, homodimer, heterodimer, and oligomeric states. These dimers have unique signaling responses for a particularcheckpoint states in GPCR biosynthetic pathway [18, 19].

Mammalian GPCRs requires the right protein confirmation and important post translational modifications (PTMs) to maintain structural integrity in the cell membrane. PTMs are variable chemical modifications on amino acid side chain that contribute to the receptor biophysical stability and functional relevance. Some the most common PTMs are acetylation, phosphorylation, and disulfide bond linkages between cysteine amino acids, glycosylations. PTMs of mammalian cellular GPCRs are critical to maintaining their higher order functional structures (HOS) [20]. The above mentioned PTMs are not possible during GPCRs biosynthesis in prokaryotic cells such as bacteria and plant based cell lines such as Wheat germ [21].

1.5 Structural Features of Chemokine Receptors

Chemokine receptors have chemokines as their natural ligands with either -CC- or -CXC- based amino acid sequence motifs, which competitively bind to their cognate chemokine receptors. Chemokines are peptides with basic characteristics with a molecular weight ranging from 8 kDa to10 kDa. Members of the -CXC- class of chemokines possess a strong chemotactic nature in neutrophil immune response, whereas members of the -CC- class of chemokines attract monocytes, a type of white blood corpuscle [22,23].

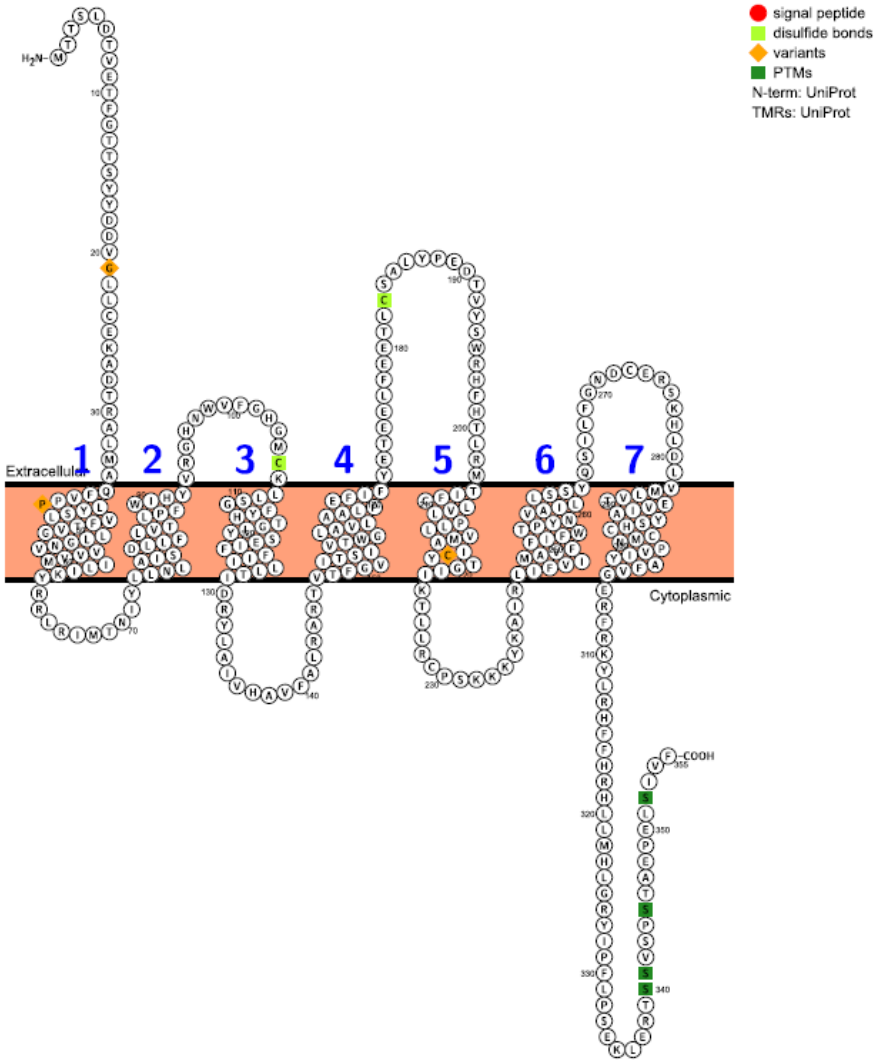

Figure 1. G-protein Coupled Receptor, a Seven Transmembrane Helix Receptor Surrounded Laterally by a Hydrophobic Phospholipid Bilayer. The Extracellular, Intracellular, Transmembrane and C-terminus domain are labeled. Protter, an open source software, was used to generate the cartoon of CCR3 with a 355 amino acid sequence.

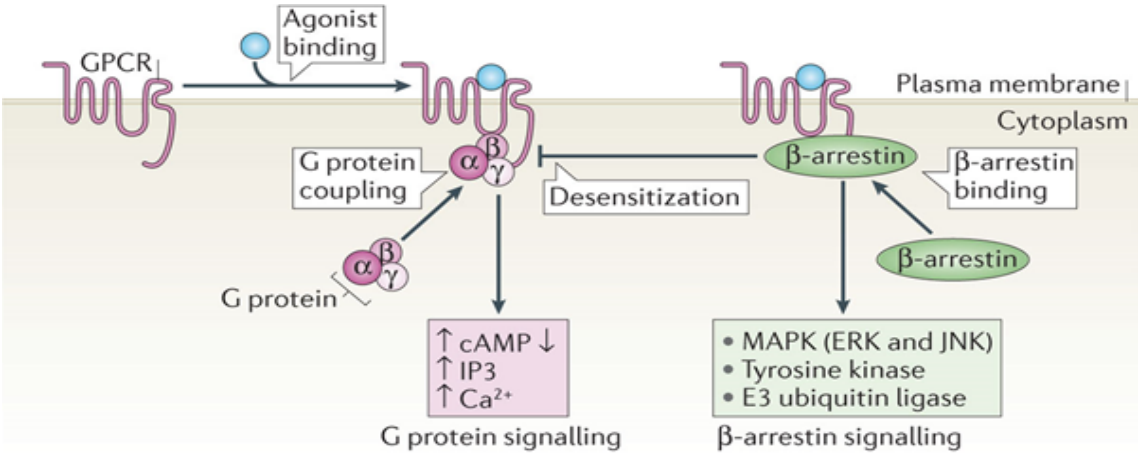

Figure 2. Functional Attribute of G-Protein Coupled Receptor and the Canonical Cascade of Downstream Signaling Events. [33].

1.6 Structural Basis of Chemokine Receptor Activation

Chemokine receptor family of GPCR has a relatively short N-terminal extracellular region/domain that has conformational flexibility. This N-terminus region is critical for initial binding with the natural ligand (which is eotaxin1 for CCR3) for receptor activation. Eotaxin1 is an 8.5kDa -CC- class basic peptide, the most potent agonist for CCR3 activation and binding to extracellular domain N-terminus of the CCR3 receptor. The binding of agonists such as Eotaxin and RANTES to CCR3 extracellular N-terminal domain leads to increased intracellular calcium (Ca^{2+}) levels [24, 25, 26]. Structural homology in observed between eotaxin1 and RANTES. Both these chemokines have affinity for CCR3.

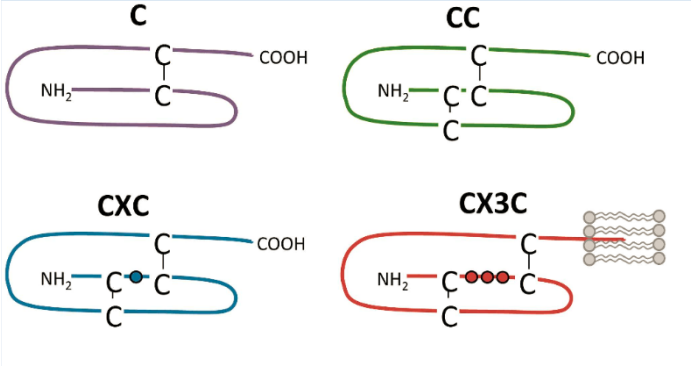

Figure 3. Structural Classes of Chemokine Receptor Ligands.

Blocking CCR3 receptor activation or stimulation results in inhibition of downstream signaling and consequent chemotactic migration in cells and associated inflammatory responses. Several small molecule drugs have been developed to block the CCR3 receptor, with potential uses in the areas of inflammatory disorders such as allergic rhinitis, atopic dermatitis, and asthma. Most of these experimental compounds proved to lack the desired efficacy in the late stages of clinical development. Pharmacological studies are based on ligand-based modeling, cell-based screening and related secondary messenger reporter assays, such as calcium influx assay. Regarding the structural basis of interaction, eotaxin1 (CCL11) is recognized by amino acid residues 8 to 23 in the sequence in CCR3. In this region, sulfation of tyrosine play a critical role, as reported recently by Millard, Christopher J., et. al., [27]. Sulfation of tyrosine residues enhances CCR3 receptor activity. Other than the above report, comprehensive structural information about CCR3 is lacking. CCR3 closest sister chemokine receptor, CXCR4, has been reported with its crystal structure and the structural basis of receptor activation were also elucidated [28,29]. Exploring the CCR3 structural and the complementary

biochemical signaling mechanism will pave the way for better drug discovery related efforts for CCR3 antagonists [30,31,32].

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CHAPTER II

SPECIFIC OBJECTIVES OF CCR3 STUDIES

In structural and receptor signaling related studies, uniform expression of chemokine receptor in cell lines is imperative. Biophysical studies of CCR3 GPCR membrane protein require a highly pure form of the receptor. Expression and purification of chemokine receptors such as CCR5, CCR3, CXCR4 and CX3CR1 GPCRs in E. coli protein expression system [1], CXCR4 [2] and CCR3 expression in HEK 293 Wang et. al [3] have been reported. Bacterial expression of chemokine receptors is a much more economical and faster process, but the produced CCR3 lacks post translational modifications (PTMs); these PTMs render proper folding and function integrity to the receptor. Wang et. al., (2013) described the overall methods of stable cell line expression and purification of CCR3, but did not provide all the necessary details for establishment of a cell line expressing the human variant of CCR3 and the required purification protocol.

2.1 First Objective: Mammalian Cell Line Development for Human CCR3 Expression

Protein expression, followed by purification of CCR3 in significant quantities is the prime objective for any structure–function studies of CCR3 in order to enable subsequent biophysical studies. Practical methods to develop a mammalian expression

system for CCR3 production is imperative in order to have the receptor with close similarity at the amino acid sequence and post translational modifications, as it occurs in wild type CCR3 observed in vivo, in human cells and tissues. Asparagine N-glycosylations, disulfide linkages, phosphorylations, etc., are all important PTMs for proper folding and functionality of the receptor in the cell membrane. Reversible phosphorylation events play an essential role in signal transduction through transmembrane GPCR phosphorylation events. The enzymes that are required to render the appropriate PTM in the cell cytoplasm are absent in prokaryotes and even in Chinese hamister ovary (CHO) cell lines [4]. HEK 293 cell line is a valid human cell line for GPCR expression and for the study of GPCR signaling pathways in heterologous expression systems. GPCRs expressed in heterologous HEK 293 have post translational modifications similar to the natural receptor higher order structure (HOS) as wild type CCR3 *in vivo*. CCR3 is a functional receptor and is present in low abundance *in vivo*. Furthermore, the expression level varies over time, depending on the physiological state of cells and tissues. With optimal extraction methods such as affinity ligand based affinity capture, pull down-extraction methods from tissue protein, the ability to isolate significant amount receptor is not sufficient as a methodology for structure-function type studies, since these methods require repeated use of purified receptor.

For research methods involved in structural biology, high microgram to milligram quantities of highly purified protein resolved in monomer or dimer states are required. So, it is a rational strategy to develop an effective insect or a mammalian-human cell line

based GPCR expression system to produce chemokine receptor subtype 3 (CCR3) in considerable quantities in a reliable manner.

2.2 Second Objective: Methods in CCR3 Purification and Characterization

The second objective is to successfully purify CCR3 receptor in microgram to milligram quantities. Membrane proteins are some of the most challenging proteins to work with, as they tend to lose their functional stability and aggregate when devoid of their biophysical environment around their transmembrane domains. It is critical to keep the receptor in the buffers during the overallextraction and purification process. Biophysical stability of membrane proteins is paramount during the overall solubilization in order to stabilize the protein [5,6]. The above criteria should be considered during all the steps of cell lysis and membrane protein solubilization. Affinity-based purification is one of the best strategies for efficient purification of a recombinant expressed membrane protein receptor [7]. The protein purification process using affinity methods requires the target protein to be tagged with a particular amino acid sequence for selective binding-elution. Affinity tags consist of amino acid sequences such as poly-histidine tag, Flag tag or a Rho tag. A complementary binding partner for this tag is immobilized on a stationary phase resin. The target recombinant protein with the affinity tag (amino acid sequence) is pulled out of the cell lysate due to its binding affinity [8]. Immobilized metal affinity chromatography (IMAC) purification is one the widely used methods for purifying recombinant proteins such as enzymes, structural and membrane proteins [9,10]. During the affinity purification process, using a mild detergent helps in maintaining the

functionality; this important criterion should be considered during membrane protein purification. n-Dodecyl-beta-D-maltopyranoside (DDM) is an oligosaccharide based nonionic detergent widely used in purification of GPCR and other membrane protein extraction-solubilization processes [11,12]. Critical micellar concentration (CMC) of the chosen detergent is a primary descriptor to be taken into account in the purification of membrane proteins [13]. Each GPCR protein molecule is surrounded by approximately 50–55 phospholipid molecules in the cell membrane. A detergent that substitutes for these phospholipids must have closely the same number of detergent molecules to form a single receptor-detergent molecular self-assembly, a micelle.

Once the CCR3 receptor is purified, the next step is the analytical characterization of the protein usking methods such as 1) absorbance 280 nm measurements (A280 assay), 2) sodium dodecyl polyacrylamide gel electrophoresis (SDS PAGE), 3) Immunofluorescence and 4) dot blot and immunoblotting (Western blotting).In addition, dynamic light scattering (DLS) methods are carried out to assess protein aggregation levels during cell lysis, extraction, and purification processes. One other important class of techniques is the bottom-up mass spectrometry based characterization methods to characterize CCR3 at the protein sequence level.

2.3 Third Objective: Phosphopeptide Enrichment Methods and Phosphoproteomics-Based Analysis of HEK 293 Whole Cell Proteome by Liquid Chromatography - Tandem Mass Spectrometry

CCR3 mediated downstream signaling involves a group of canonical subcellular events, similar to other GPCR downstream signaling pathways. In vertebrates, CCR3 is

expressed transiently in eosinophils, which are a major contributor for early stage innate immunity and chemotaxis leading to eosinophil migration to the inflammatory tissue and preliminary infective stages [14,15][1,2]. A few studies have reported that CCR3-mediated subcellular signaling events result in ERK, JAK, STAT pathway activation. In these pathways, some signaling proteins are phosphorylated in a reversible fashion by protein kinases [16,17]. Here, the primary objectives are to develop an overall method to look for global cellular and temporal phosphorylation events; this is after CCR3 receptor is expressed in HEK 293 and stimulated by its most potent natural agonist, eotaxin1 (CCL11) [18,19]. The methods employed are **S**table **I**sotope **L**abeling in **C**ell **C**ulture (SILAC). Methods involve use of the CCR3-expressing stable HEK 293 cell line; the first step of the stable isotope enrichment process requires propagation of the cell line established for CCR3 protein expression for purification purpose, followed by enrichment with SILAC media for isotope labeling the entire HEK 293 cellular proteome. This will be followed by the development of phosphoproteomics methods for selective enrichment of phosphopeptides from whole cell lysate digests of HEK 293 [20,21,22].

Protein phosphorylation is a critical post translational modification, and one of the most common PTM in the cellular proteome. Phosphorylation can alter the functional states of proteins; phosphorylation occurs on tyrosine, threonine, and serine amino acids [23,24]. Tyrosine phosphorylation is a critical phosphorylation event occurring on tyrosine residue of protein kinases; these are pivotal in signaling pathways of GPCR [25]. Methods involve HEK 293 cellular protein lysate digestion, followed by phosphopeptide enrichment methods. Immobilized Metal Affinity Chromatography (IMAC) with TiO_2

nanoparticles is the method employed for selective enrichment of only phosphopeptides over non-phosphorylated peptides [26]. We use titanium dioxide (TiO_2) based phosphopeptide enrichment for the HEK 293 CCR3 expressing stable cell line. The overall goal is to enrich phosphopeptides with subsequent analysis by high mass accuracy mass spectrometry, using a front end nanoflow reverse phase liquid chromatography separation (HPLC-MS). Nanoflow liquid chromatography - tandem mass spectrometry methods are utilized to acquire tandem mass spectrometry data of HEK 293phosphoproteome. Later, the raw phosphopeptide MS/MS data are processed with proteomics-informatics tools, in order to localize the phosphorylation sites on the corresponding phosphopeptides.

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CHAPTER III

AN OVERVIEW OF G PROTEIN-COUPLED RECEPTOR EXPRESSION SYSTEMS

Functional membrane proteins such as membrane-bound ion channels and G-protein-coupled receptors are expressed in relatively lower amounts than most other functional proteins in the cytosolic environment. At a basal expression level, cells produce low copy numbers of membrane-bound functional proteins such as ion channels, GPCRs, and transmembrane protein kinases. Over expression of these membrane proteins can lead to cellular toxicity and cell death. Except for rhodopsin in the eye, most of the signaling GPCRs over expression under in vitro conditions is a monumental challenge. GPCR expression in prokaryotic bacterial systems such as E. coli expression is relatively easier compared to eukaryotic and related mammalian cell line-based expression. Some of the eukaryotic expression systems are yeast, Pichia pistorius, insect cell systems such as baculovirus/Sf9 cell expression, and mammalian expression systems like Chinese hamster ovary (CHO) cell lines, and HEK 293 cell lines. Expression of human GPCRs in mammalian cell lines is the ideal strategy when the primary purpose is to have homogeneity at the protein higher order structural (HOS) level. Various methods of producing membrane proteins from their respective sources like heterologous expression systems are discussed here.

3.1 Natural Sources: From Tissue and Organ Preparations

Rhodopsin is a membrane protein present in retinal tissue of the eye, and is the most abundant GPCR available. It can be isolated from its natural source, is the most abundant membrane protein GPCR successfully isolated to date by analytical methods [1,2]. Rhodopsin has also been acquired from squid tissue preparations. Most of the bio-analytical based methods were carried out to study the structural aspects and for protein crystallography studies. Except for the rhodopsin class of GPCRs, all other GPCRs present in vertebrate tissue extracts are predominantly in minute concentrations. Their concentrations are on the orders of femto moles (10^{-15}) to a few picomoles (10^{-12}) in the total tissue protein extract. In structure-function relationship studies, the most widely used methods are molecular biology based amino acid sequence modifications or site-directed mutagenesis (SDM) in GPCRs. These SDMs are introduced to determine the functional attributes of a particular amino acid to the GPCR receptor function. STM methods are not possible when the GPCR is isolated from natural sources such as tissues and organs. Moreover, isolation and purification of GPCRs from their natural sources in significantly higher concentration is not practically feasible. When the receptor is in the native state, purification of a native receptor from a complex sample with a high protein dynamic range, like whole cell lysate protein, may not be possible in practice. The development of various protein expression systems as alternative means to successfully express and purify functional receptors has enabled novel approaches for GPCR structural biology studies. Some of the host cell types used in expressing GPCRs range

from bacteria, yeast, and insect cells to mammalian cells, and recently cell-free expression has also been used for in-vitro expression of GPCRs. These heterologous expression systems for GPCR production were developed to purify a functional GPCR for analytical and biophysical - structural studies of the receptor [3].

Vertebrate and mammalian GPCRs have post translational modifications (PTM), these PTMs happen on the endoplasmic reticulum after the polypeptide chain is synthesized. PTMs facilitate acquisition of the correct protein folding and organization in the cell membrane, to enable their active functionality.

A few important PTMs are glycosylation, phosphorylations and disulfide bridges between cysteine amino acids. N-linked or N-glycosylation occurs at the nitrogen of asparagine residues. N-Glycosylation is seen on the extracellular (EC loop) of GPCRs and it renders specificity for ligand and other molecular recognition mechanisms of GPCRs. Another PTM, disulfide bond linkages exist between transmembrane domains of GPCRs. Some additional significant and particular PTM modification is fatty acid Palmitoylation, this PTM aids in in proper folding of GPCR after its biosynthesis and trafficking to the cell membrane. Variability in protein phosphorylation with variability is seen on serine (S), tyrosine (Y) and threonine (T) residues. Palmitoylation and phosphorylation are present in the intracellular domains of the GPCR. Functionally, palmitoylation aids in biophysical stabilization of the GPCR in the phospholipid bilayer and phosphorylation of GPCR account to its functional attributes and phosphorylation is an important reversible regulatory PTM in GPCRs. Phosphorylation can desensitize the GPCR upon its stimulation by a natural ligand or an agonist.

3.2 Bacterial Cell Based Expression and Production of GPCRs

Expression of GPCRs in bacterial takes advantage of faster growth rate, doubling time of bacterial cultures and simple scale-up procedures of GPCR protein expression. Bacterial expression of recombinant proteins is economical, and the media is relatively cost effective over mammalian and insect cell expressions. Prokaryotes like bacteria do not have biochemical machinery to produce post translational modifications (PTMs) such as glycosylations, phosphorylations, and palmitoylations. GPCR miss folding may occur and possibly can abolish receptor functionality when these critical PTMs are deficient, but not always the case unless a functional study is carried out on GPCRs produced in bacteria, insect cell lines, and mammalian cell lines. The above stated deficiency at the PTM level is the major setback of GPCR expressed in E. coli expression systems. Several groups had successfully produced functional GPCR, such as murine neurotensin receptor (NTS1) l in E. coli system [4].

3.3 Expression of GPCRs in Yeast Cells

Pichia pastoris is a methylotrophic yeast widely used in therapeutic protein expression. The growth media employed in yeast cell culture is comparatively cheaper over media used in mammalian and insect cell line for protein production. In addition to the above, yeast also has a higher growth rate over mammalian cell culture for protein expression. With the applications of recombinant DNA methods, functional GPCRs have are produced in yeast cultures with high efficiency [5]. Yeast is a eukaryotic organism, and it provides the optimal conditions for GPCRs to have PTMs, but these PTM have subtle differences when the same GPCR is expressed in a mammalian expression system.

Further yeast cell membrane have considerably lower cholesterol and more ergosterol, a similar biomolecular constituent in the cell membrane. The evolution and folding of GPCR in yeast cell expression have some disparities over mammalian expression systems. However, there are reported works in the full functioning expression of GPCRs in P. pistorius [6,7].

3.4 Insect Cell Lines in GPCR Protein Expression

Insect cell based expression of GPCRs can yield a significantly larger amount of the target protein; a single cell culture batch can produce higher titers of the target GPCR. Further insect cell expression is one of the most widely used methods of therapeutic protein production as well. The expression level of GPCR varies greatly in insect cell line expression depending on media composition and growth condition of the cultures. It is a prudent task to screen for the levels of target protein expression before cell culture scale-up. Preliminary screening is carried out for process development efficiency and cost-benefit analysis [8]. Baculovirus having the GPCR expression construct DNA used as the transfection agents used in insect cell expression systems. The steps involved are, optimal incubation-growth of the insect cell culture, final step to killing the insect cell culture at the late stage of cell growth and harvesting the cells for cell lysis and downstream target protein purification. The main advantage of insect cell-based protein expression are, the produced target protein have post translational modification very similar to mammalian expression system, but a few PTM especially glycosylation homogeneity of the GPCR is a setback. This variability in PTMs and other associated changes leads to protein miss folding in respect to its active conformation and the overall protein functionality [9].

Insect cell based expression of GPCRs are carried out in Spodoptera frugiperda sf9; beta1 adrenergic receptor [10], neurotensin receptor, corticotropin release factor CRF1 are a few GPCRs successfully produced in insect cells with baculo virus-insect cell expression systems in the last two decades.

Polyhedrin is used in a baculovirus expression system to express mammalian proteins in sf9 insect cells. It is advisable to be cautious due to virulentnature of the virus used in infect mammalian cells. Safety and diligence are important criteria for insect cell culture expression, Jarvis et. al., 2009 [11]. Insect cell protein expression and mammalian cell cultures differ primarily in their growth conditions; insect cell culture is carried at 27 ^{o} C in CO_2 jacketed incubator, whereas the mammalian cell culture based protein expression is carried out at 37 ^{o} C. The membrane composition of insect cells differs from mammalian cells in their phospholipid composition; low levels of cholesterol in seen in insect cell lines. To compensate for this disparity and to stabilizing the mammalian GPCR, phospholipids and cholesteryl esters are incorporated in during insect cell cultures to express mammalian GPCRs. One of the primary disadvantages of insect cell expression system is their susceptibilityto contamination during protein expression.

3.5 Mammalian Cell Lines in Membrane Protein Expression

Membrane protein production using mammalian cell lines had matured significantly in the last decade, with significant improvements in methods to overexpress membrane proteins and in preventing protein heterogeneity at PTM level. In mammalian expression system heterogeneity is minimized by optimizing cell culture growth conditions and clonal selection to create stably transfected mammalian cell lines. Vital

and healthy cells are a pre-requisite for efficient expression of mammalian membrane protein production. Expression systems based on mammalian cells is a promising alternative when baclovirus-Sf9 insect cell expression system fails to yield the target protein in the desired functional state. One of the advantages of mammalian expression systems is having the enzymes necessary for the important post translational modifications and having the native biophysical environment for stabilizing in the cell membrane. The GPCR produced will possess all the N-linked glycosylations, disulfide linkages and other putative post translational modifications on the target membrane protein. A typical mammalian cell membrane composition is approximately fifty percent each for lipids and proteins. Further the additional component, cholesterol is present in the mammalian cell membrane [12]. Cholesterol with other related lipids such as glycerolipids and glycerospingolipid are critical lipid biomolecule having an important role in stabilizing the membrane protein for its functional activity.

3.6 Heterologous Expression of Membrane GPCRs in Mammalian Cell Lines

GPCR expression in mammalian systems is carried out either by a short transient transfection or with a robust, stable transfection methods with an antibiotic selection of the transiently transfected cells, this process renders different clonal establishment of the cell line. Transient transfection is carried out for expression construct confirmation and to estimate the level of efficiency in target protein expression. Transient transfection methods are a quicker means to test target proteins expressed by plasmid DNA constructs before moving to stable cell line for scale-up. Most of the heterologous expression systems were design with a primary goal overexpress significant amount of functional

protein for a semi-preparative scale production of the target protein molecule with an intent of high order structural (HOS) uniformity [13]. The target GPCR gene of interest is subcloned into a non-replicative viral genome, such as *semiliki forest virus* and *baculovirus*. This viral genomic DNA is used for transfecting the cells for protein induction.

3.7 Transient Expression

Transient transfection methods aid in producing membrane protein in a few days, the protein expressing gene is subcloned into a non-replicative viral DNA. Transfer of this plasmid DNA into cells is aided by cationic transfection reagents, such as Lipofectamine (life technologies) or by electrophoretic methods. Here the host cell genomic do not have the plasmid DNA integrate into transient transfection methods for protein expression. The major drawback in transient methods is its low efficiency in target protein expression; a unit cell culture produces low titers or low copy number of the target protein. Moreover, the target protein structure heterogeneity at the post translational modification level is a problem; the purified protein exists in multiple heterogeneous states. In the perspective of a structural membrane protein, transient expression yields a limited quantity for a significant amount of cell culture - lysate protein extract. Nonetheless, transient expression of proteins in mammalian cells is by far the quickest method of protein expression for initial screening purposes and qualitative identification of the target protein.

The principal rationale of transient expression is the lack of efficiency of viral DNA in entering host cells; the mammalian cell systems. These viral DNA have strong

promoter regions, in the case of insect cell expression, baculoviral DNA has polyhedrin promoter region, TMV tobacco virus promoter region is used for protein expression in mammalian cells. Proteins can be successfully expressed, with the target gene of interest sub-cloned successfully into viral plasmid DNA as the initial step. Mammalian cell lines predominantly used in GPCR expression are Chinese Hamster Ovary (CHO) and Human Kidney Endothelial (HEK 293) cells. CHO cell lines do not possess all the glycosylation enzymes and a few specific post translational modifications seen in human proteins [14,15].

Moreover, the target protein expression levels or titers are comparatively lower in CHO cell lines over HEK 293 cells. With these remarkable facts, HEK 293 cell type is the widely used cell line in producing proteins that require posttranslational modifications similar to in vivo expressed GPCR, critical in structure-function relationship [16,17].

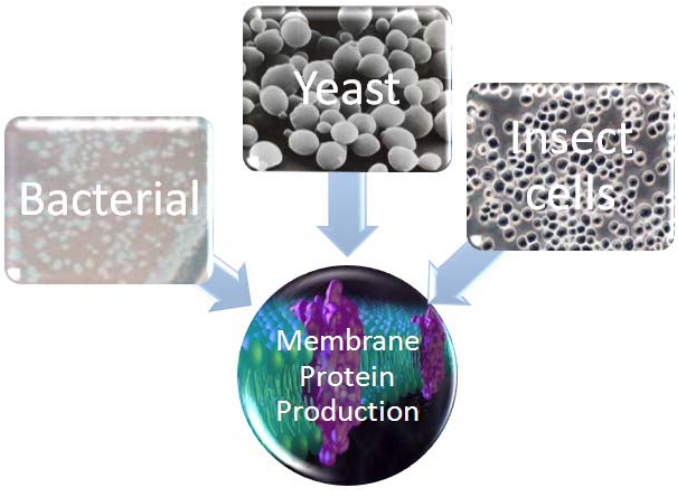

Figure 4. Expression Systems Used in Membrane Protein Production and Purification. Note: Mammalian and cell-free expression systems are not represented.

3.8 Stable Cell-line Development for GPCR Expression Uniformity

Stable cell line expression is quite similar to transient expression; the only difference, the plasmid DNA has an additional antibiotic resistance cassette in the expression construct to isolate and culture the transfected cells. This antibiotic resistance aids in selecting the transfected cells for propagation and stable clone establishment. In the stable cell line development process, the plasmid DNA is integrated into the host cell genomic DNA. Once the transient transfection is complete, the selection antibiotic is constituted in the cell culture media for selective isolation of the transfected cells. These clones are capable of expressing the target protein expression at different levelsof structural quality; protein high order structural features. Estimation of protein titer is a fundamental element for cell line development and expression optimization. The selected antibiotic resistant clones are picked for clonal expanded to validate for target protein

expression. Analytical biochemistry and related assays, such as immunoblotting, immunofluorescence, and fluorescent assisted cell sorting (FACS) are standardmethods to estimate protein titer in methods of stable cell line development. The above mentioned clonal selection establishment is carried out to preclude untransfected and to prevent the unwanted clonal growth in the culture media. In some cases, the target protein expression level dropsslowly during cell culture propagation; this is due to phenotypic changes and reduction of protein expression in the stable cell line. The primary cultures are used for long-term storage in liquid nitrogen for future use to maintain uniformity in the protein expression. Invitrogen-Life Technologies propriety Flp-In and TetR systems are a few a few mammalian expression plasmid systems to generate stable cell for human protein production. One of the hallmark featuresof stably transfected cell line is the expression construct plasmid DNA is successfully and completelyintegrated into the host cell genomic DNA, and a CMV promoter region controls the protein expression.

3.9 Tetracycline Inducible Expression System. TetR System [18, 19, 20, 21].

In tetracycline regulatory system of inducible gene expression, the protein is tightly by tetracycline repressor and tetracycline operator plasmid system. The TetR protein expression system constitutes of inducible expression vector-operator (TO vector) and a regulatory vector-repressor (TR vector). The gene of interest is subcloned into multiple cloning site of the tetracycline inducible/operator (TO) plasmid. This inducible expression plasmid is under tight control by a strong human cytomegalovirus immediately early to the CMV promoter and two tetracycline operator two sites (TeO_2).

Tetracycline regulatory plasmid, the TR vector encodes the Tet repressor (TetR) is also under control of human CMV promoter.

In the absence of tetracycline, the TR vector always represses the particular gene expression. The target protein production is carried out by the addition of trace amounts of tetracycline in the culture media. Some traces of leaky target protein expression is observed due to inevitable presence of tetracycline that might be carried in with DMEM, 10 % fetal bovine serum albumin (FBS). A trace amount of tetracycline is incorporated in the culture during the protein expression phase. The tetracycline repressor vector expresses the Tet repressor, this Tet repressor protein molecules in produced in excess and they bind to two $TetO_2$ binding domains of the tetracycline inducible-operator vector (TO vector), preventing the expression of the gene of interest.

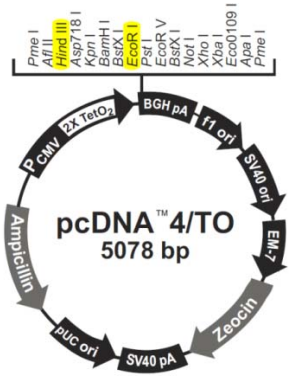

Figure 5. pcDNA4/TO Vector with the Multiple Cloning Site, adapted from Life Technologies T-REx Protein Expression Manual.

3.10 Cell-Free Expression Systems for Membrane Protein Production

Cell-free expression (CF) of membrane proteins were successfully carried out in E. coli, insect cells as well as in mammalian cell lines. The basic concept of protein translation happens *in vitro* with the DNA plasmid product in the CF reaction media. Cell-free expression of membrane protein is one of the most efficient and faster methods to produce a protein which is difficult to express by conventional methods *in vivo*, in cell lines. Further, the throughput attained by CF systems is necessary to mention. CF methods are the cost effect ways to screen mammalian expression plasmids. Cell lysate homogenate extracts of E. coli, insect cells, and mammalian cell are used in protein expression by CF methods [22]. Synthetic lipids and associated phospholipid are used as membrane lipid ingredients the CF media for correct folding in the lipid self-assembly or micelle to render the protein fully functional and facilitate easier purification. The translated protein assembled directly in micelle with the lipids present in the cell-free media [23]. With a limited and user-defined volumes of the reactionmixture, a high level of protein expression may be obtained in a short span of time. The principal disadvantage of CF expression system is their prohibitively higher expenses for any larger scale expressions.

Membrane proteins such as GPCRs and cell membrane ion channels primarily function in maintaining cellular homeostasis. Their expression is tight control by genomic and cellular protein expression turnover for the membrane. Excessive expression of membrane proteins renders cytotoxicity and eventual cell death. The above stated reason

is the primary factor for membrane protein expression in heterologous systems in significantlybulk is a quite challenging factor.

The cell-free expression reaction mixture is highly versatile, the ways in which the ingredients for protein translation and assembling and proper folding of the membrane protein in a functional state can be tighly controlled, unlike cell line based expression systems. The above factor is an advantage when the membrane protein forms biphasic self-assemblies nanoscale structures such as micelle, bicelli, liposomes, etc.

Selection of the most appropriate lipid favored environment is imperative for proper assembly of the membrane protein after protein biosynthesis in the reaction mixture of a cell-free expression systems.

With the above stated overview, cell-free systems in membrane protein expression is an effective alternative to express a protein in a shorter time interval. Cell-free methods is a promising option for high throughput means of membrane protein production.

3.11 Solubilizing and Extraction of GPCRs and Other Membrane Proteins from Cell Membranes

Efficient extraction of cellular proteome and target protein solubilization is imperative for a higher titer. After the GPCR is expressed in the respective cellular systems such as E. coli, P. pichorus, insect cells or mammalian cell lines, efficient extraction of the membrane protein is necessary without abolishing its functionality. When the membrane proteins are devoid of their native environment, the phospholipid-cholesterol constituents of the cell membrane, transmembrane proteins tend to aggregate

and crash out of homogeneous phase. Mammalian and insect cells do not possess a rigid cell wall. Instead, their cytoplasm and subcellular components are held by a flexible lipid bilayer cell membrane which can be disrupted easily. Membrane protein solubilization is carried out with a mild lysis buffer under low concentration of surfactant such as SDS, Triton-X. E. coli, bacteria, and yeast possess a sturdy and rigid cell wall, mechanical disruption with a homogenizer or with glass bead is necessary to break their rigid cell wall for efficient protein extraction. Further, one of the most important physicochemical factor to be considered is temperature elevation during cell lysis and protein extraction. GPCRs are unstable when perturbed with elevated temperature, also when are devoid of the native lipid membrane biophysical environment. Membrane proteins tend to aggregate at high temperature, leading to insoluble and non-functional protein by itself. The primary criterion for solubilizing and stabilizing membrane proteins are ionic strength/salt strength of the buffer employed, surfactant type and concentration, glycerol, pH, and temperature of the buffer used in the solubilization process. From the initial steps of cell lysis to the very end of purification step and membrane protein suspension in the buffer, care should be exercised not to elevate the temperature, especially during cell lysis and sonication. Mechanical disruptions tend to generate heat; always it is advisable to sonicate cells on ice under very mild conditions for membrane protein preparation for purification efforts.

Nonspecific proteolysis during cell lysis - membrane extractions tend to occur, to overcome this undesired effect, protease inhibitors are incorporated in the lysis buffer. Phosphatase inhibitors are used in lysis buffers when putative reversible phosphorylation

site in the intracellular GPCR domains are to be determined by analytical methods. During GPCR receptor stimulation/activation some of the serine, threonine, and tyrosine of the transmembrane GPCR amino acid moieties undergo reversibly phosphorylated and mediate downstream subcellular signal propagation, but not necessarily in all GPCR canonical signaling cascades.

3.12 Detergents Used in Solubilizing GPCR and Related Membrane Proteins

GPCRs are solubilization with a mild surface active agent or surfactant/detergent. Detergents are amphiphilic molecules, have both hydrophilic and lipophilic groups in their molecules at opposite ends. Detergents aid in creating self-assembled biphasic systems, by dissolving protein in aqueous based buffers. At a concentration higher than their critical solubility, individual detergent molecules undergo self-assembly to form a micelle. Micelle a molecular self-assembly of the surfactant molecules, a few nanometer in size. The concentrate at which micelle formation occur in the aqueous phase is termed as critical micellar concentration (CMC). CMC is specific and a unique descriptor for a detergent. For a given surfactant, CMC and their association-dissociation stability also depend on the ionic strength of the aqueous media, pH, and temperature of the aqueous-based buffer media.

The rationale for efficient solubilization of membrane proteins such as GPCRs is by the formation of micelle when the concentration of detergent is slightly higher than its CMC. Micelle formation aids in solubilizing individual or oligomeric states of the membrane protein molecules in the buffer. Usually, two to three times the CMC of a surfactant is ideal for membrane protein solubilization.

Micelle in solution is a biphasic system, here the protein-detergent molecular self-assembly in aqueous media is a biophysical environment that mimic phospholipid membrane of the cell [24]. The hydrophobic tails of the detergent molecule align towards the hydrophobic amino acids moieties and the hydrophilic group of the detergent molecule towards the aqueous phase.

One of the typical features chosen in membrane protein solubilization is the detergent selected must be robust enough to disrupt the membrane protein and efficiently solubilize the membrane GPCR from the cell membrane into the lysis buffer. The detergent must not show any deteriorating effects on the structure – function aspects to the membrane proteins, this is in respect to the native conformation and its biophysical functionality. Membrane proteome disruption with simultaneous solubilization-stabilizing in the buffer employed in its near native confirmation is one of the toughest challenges in membrane protein chemistry.

A universal detergent is not a practical solution for membrane protein extraction and solubilization efforts. Membrane protein solubilization is an empirical process, and the overall method employed is to validate an ideal surfactant for the particular membrane protein chosen [25]. Several surfactants are to be evaluated for their effectiveness is solubilizing membrane protein and its associated biophysical functional effects to the membrane protein during the process. These surfactants range from harsh zwitterionic CHAPS, anionic Sodium dodecyl sulfate (SDS) to moderate ones such as Triton X-100, Brij. Mild nonionic detergents such as FOS choline, Digoxin, Dodecyl-beta-D maltopyranoside (DDM). Surfactant screening methods are a rational strategy for

the specific membrane protein solubilization methods. Sometimes background literature can provide a plethora of information that can save significant time in the methods development process for efficient membrane protein extraction. Lysis-buffer composition with a molar ratio of two or more detergents is also a rational choice in solubilizing membrane proteins when using a single surfactant is not efficient enough. Most of these surfactants chosen in lysis buffer preparation are in concentrations of two to four times the critical micellar concentration (CMC). In most cases, they range from 1 % W/V to a maximum of 4 % W/V in the effective protocols employed. Excess detergent concentration is to be avoided as they tend to form micelle devoid of the target protein. Further, can interfere with the analytical methods used in the later stages of protein characterization. DDM is an oligosaccharide based nonionic detergent discovered during the 1980s, since then it was used widely in several membrane protein solubilization studies [26, 27]. In practical considerations, DDM is used in 0.5 % to 2 % W/V in 1X PBS based lysis buffer with a trace amount of EDTA in membrane preparation methods [28]. EDTA is a metal chelating agent, and if non-compatible with the downstream purification process should be avoided in the buffers. Detergents used in the preparation of these buffers are recommended to be in a highly pure state; this is meant to prevent any trace metal chelating effects on the membrane protein precipitation.

During membrane disruption process, these detergent tend to froth, so mechanical intervention such as vigorous mixing, vortexing, homogenization must be gently and care must be taken to prevent elevation in temperature during the process. Most of the solubilization methods are carried out at 4 C or on the ice. Elevated temperature is

unfavorable for GPCRs, due to its effects on misfolded and aggregation tendency on the GPCR. These effects can be detrimental and can eventually lead to a non-functional state for the GPCR, further can precipitate the protein out of solution.

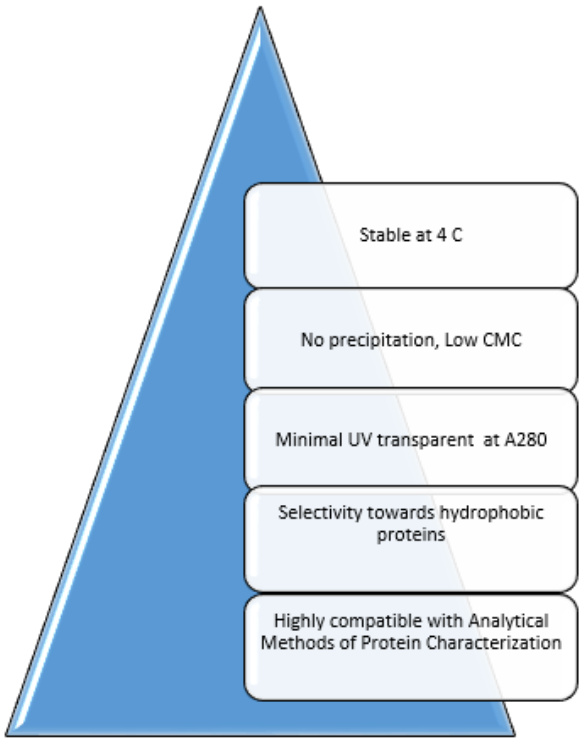

Figure 6. Criteria for Detergent Selection for Membrane Protein Solubilization-Stabilization. (Note. image correction, highly UV transparent at A280 nm).

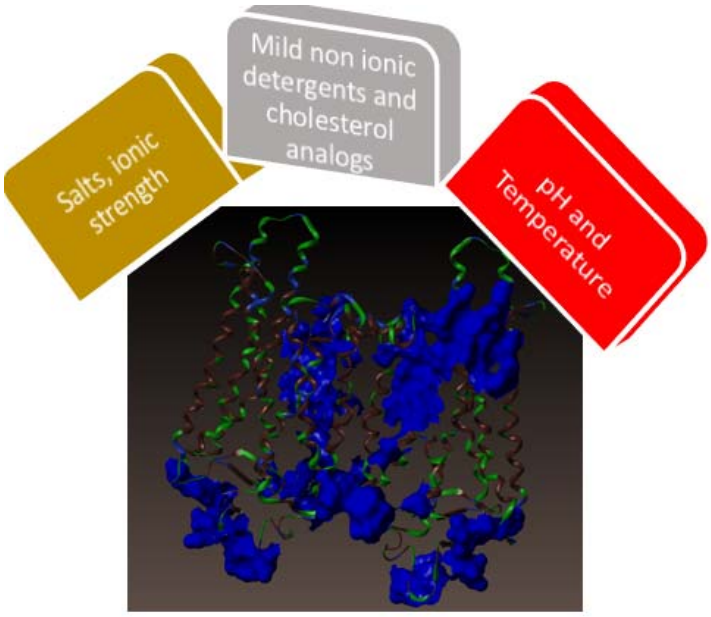

Figure 7. Factors Affecting Protein Stability and Solubility. Blue. Solvent Accessible regions of GPCR Dimer. Brown color contours indicates Lipophilic Potential. Structure view in SybylX 1.2. PDB.3CAP.

Table 1. Overview of Membrane Protein Expression in Various Expression Systems and Their Specific Attributes.

Factors	**E. Coli**	**Insect cells**	**Mammalian cells**	**Cell-free expression**
Host system	Prokaryotic bacteria	Modified insect cells	Mammalian host cell derived cell lines	Host specific CF reaction mixture
Cost	Low	medium	high	high
Time	fast	medium	long	fast
Yield	High	high	Low	high
Phosphorylation	No	yes	Yes	Host cell-specific
Glycosylation	No	Core glycosylation	Mammalian specific	Cell line specific

3.13 Affinity Tagging of GPCRs Expressed in Different Protein Expression Systems

Protein expression is the first part in protein production, the next is an effective purification of the protein, without protein degradation nor abolishing its functional attributes during the process. Native protein purification is a challenging task and not a realistic option in the realm of research unless it is a therapeutic biologic or protein product. The attainable goal of native protein purity of 90 % or more in purity takes a minimum of five or more orthogonal purification steps involved from the first phase from crude cell lysate stage.

On the other hand, affinity purification is a comparatively feasible process, never more than three steps in purification from crude purification to the final polishing step to attain a highly pure protein. Affinity purification can be successfully carried out with limited protein purification resources; it is based upon tagging the membrane protein to be expressed with an ideal affinity tag either at the N-terminus or C-terminus regions of the protein. Some of the widely used epitope affinity tags are; Polyhistidine Tag, Myc-Tag, Flag Tag (amino acid sequence -YKDDDDK tag), Rho-tag, Streptavidin tag. The selection of the Tag depends on the several factors that can range from cloning, target protein expression efficiency, yield, and purity of the final protein. These tags have highbinding affinity for their respective complementary binding partner. The binding partners are chelation metal ions such as Nickel and Cobalt ions for poly histidine or 6X His-Tagged protein purification. Polyhistidine Tag based affinity purification in not compatible when reducing agents such as Dithiothreitol (DTT), if used in the lysis buffer during protein extraction and in the membrane extract-preparation supernatant [29]. DTT

is a reducing agent incorporated in cell lysate supernatant to reduced disulfide bonds in proteins and opening up the protein for better solvent accessibility and facilitating polyhistidine tag binding to the metal ion immobilized on the resin. Biotin is a complementary binding stationary phase for streptavidin-tagged proteins, an Anti-DYKDDDDK antibody for Flag-tag purification and Anti-Rho antibody for Rho-tag on the target protein sequence. The binding partner is immobilized on a solid resin that forms the stationary phase of the purification protocol employed [30]. This affinity based selective binding-pulldown of affinity tagged proteins may be successfully carried out with higher confidence in most case when the affinity epitope tag is accessible to the resin. With a higher purity target, the affinity purified protein is further polished with additional purification steps again to remove any trace amount of contaminant proteins in solution and also nonspecific tightly bound biomolecules to the target protein sample.

Two orthogonal affinity amino acid sequence tags are presented, one on either terminus, C-terminus or N-terminus make to express on the protein. This helps to increase the purity levels by two different affinity purification steps in tandem. The affinity tag can be removed by having a protease cleavage site. After the targeted membrane protein is subsequently purified, the amino acid (AA) sequence tag is cleaved with a protease enzyme. For example, TEV protease is used when thrombin sequence is usedas an amino acid spacer between the protein sequence and the affinity tag. Finally, the amino acid tag sequence is removed from the bulk of the purified protein by biomolecule size/molecular weight cut-off filtration (MWCO) process such or dialysis.

The final step is the polishing step of purifying the protein; this is carried out by proteins hydrodynamic radius-size. A suitable size exclusion chromatography (SEC) or gel filtration chromatography is carried out for selectively fractionate the target protein from its oligomeric and protein-protein interaction biomolecular states.

Protein Sequence Spacer Affinity tag

Figure 8. Amino Acid Affinity Tags on to Either N Terminus or C Terminus of Recombinant Protein, the Complementary Immobilized Binding Partners Employed in Protein Purification Process.

Table 2. Overview of Some Commonly Used Affinity Epitope Tags and Their Binding Partners in Recombinant Protein Expression – Purification [31].

Common widely used affinity tags for purification of recombinant proteins.

Affinity tag	**Length (aa)**	**Size (kDa)**	**Matrix**
Hexahistidine (6x His)	6 (generally)	0.84	Metal ions (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+})
Glutathione S-transferase (GST)	211	26	Glutathione
FLAG	8	1.01	Anti-FLAG mAb
Streptavidin-binding peptide (SBP)	38	4.3	Streptavidin
Strep II	8	1.06	Strep-Tactin (modified streptavidin)
Maltose-binding protein (MBP)	396	42	Amylose
Calmodulin-binding peptide (CBP)	26	2.96	Calmodulin
Chitin-binding domain (CBD)	51	5.59	Chitin
S	15	1.75	S-protein of RNase A
HA	9	1.1	Anti-HA epitope mAb
c-Myc	11	1.2	Anti-Myc epitope mAb

Table 3. Epitope Tags in Protein Research

Affinity Amino acid Sequence Tag	**Complementary Binding Resins**
PolyhistidineTag	Metal ion affinity (Cobalt, Nickel)
Flag-tag (DYKDDDDK)	Anti-flag antibody
Streptavidin tag	Biotin
Rho-Tag	Anti-Rho antibody
c-Myc-tag (EQKLISEEDL)	Anti-Myc antibody

3.14 References

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CHAPTER IV

HUMAN CCR3 EXPRESSION: TETRACYCLINE REGULATORY (TETR) HEK 293 STABLE CELL LINE DEVELOPMENT

Chemokine receptor subtype three is a membrane-stabilized G protein-coupled receptor. GPCR receptors act as signal transducers from theextracellular environment to intracellular regions. They play a vital role in various critical physiological processes. GPCR are one of the importantmembranes, acts as functional cellular receptors, and the expression levels of the receptor is tightly controlled in by the cell type expressing the receptor. There are close to 500 genes expressing GPCRs in humans [1]. In prokaryotic and eukaryotic organisms protein biosynthesis of GPCR membrane proteins happens at two individual steps. First the synthesis of polypeptide amino acid chain and later the critical function, proper folding and insertion of membrane protein in the cell membrane by evolutionarily conserved domains by the endoplasmic reticulum Sec61 translocon [2, 3, 12, 13]. There is a constant discovery of GPCR adds to the existing list. From the bacterial rhodopsin to highly heterogeneous mammalian GPCRs, there is some conserved homology of the protein that attributes to their biophysical and structural level [4, 5]. GPCRs play a pivotal role in cellular physiology, and the protein translation rate is tightly regulated. Basal human CCR3 protein expression ranges in pictogram (10^{-12} grams) per milligram of protein extracted from cell lysate [6]. GPCR are promising targets in drug discovery efforts; more than 40 % of drugs target GPCRs [7]. With the above rationale,

expressing and purifying GPCRs for structural and biophysical studies has a profound impact in the scientific and pharmaceutical drug discovery community. Low levels of membrane protein expression, poor solubility and their biophysical instability create a monumental task for membrane protein expression and overall purification process development.

Heterologous expression of GPCRs in bacteria lacks post translational modification such as glycosylation, phosphorylation, and fatty acid acylation, palmitoylation. These PTMs are important for right folding-translocation and assembled in the cell membrane, especially for mammalian GPCRs. Further, PTMs are critical for proper folding of the translated protein and structure-function relationship attributes.

4.1 Overview of Tetracycline Regulatory Expression System

Tetracycline regulatory expression system is a mammalian cell line based protein expression system, primarlyacting by repression-derepression mechanism of control in protein expression, as discussed in the earlier chapter. Expression of the protein is regulatory by elements of E. coli Tn10 encoding tetracycline operon; TetR, the tetracycline repressor gene. The de-repression of target protein expression is controlled by human CMV promoter region and is in-turn controlled by a robust human cytomegalovirus.

In the TetR regulatory protein expression system, the expression of the protein is held back by TetR repression TR vector, the pcDNA 6/TR, controlled by human CMV promoter region. Target protein induction is initiated by the addition of trace amount of

tetracycline at concentrations of 1 to 5 ug/ml in the culture media for a transient period to induce target protein expression by the pcDNA4/TO plasmid.

To establishing a stable cell line for human protein expression, the first step of transient transfection by a dual vector system. The recommended concentration ratio of tetracycline operator (TO) to tetracycline repressor (TR) vector is 1.6 (Life Technologies manufacturer recommendation) during co-transfection. High ratio of TR vector is meant to hold by protein expression all times, until tetracycline is added to media.

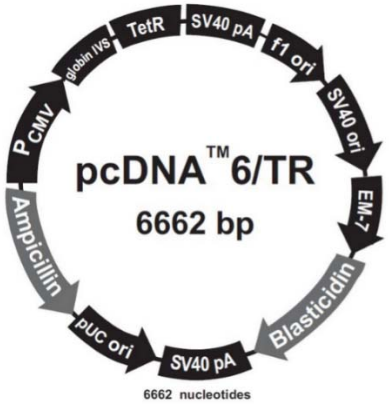


Figure 9. pcDNA 4/TR Vector, Tetracycline Repressor Vector. Adapted from Life Technologies T-REx Manual.

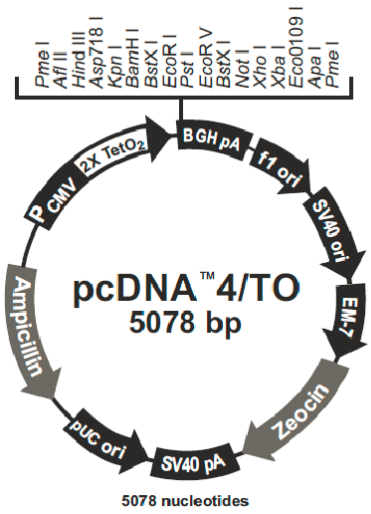


Figure 10. pcDNA4/TO Vector; Tetracycline Operator Vector, the Target Protein to be Expressed in Cloned into the pcDNA4/TO Vector. The plasmid used in establishing the Human CCR3 Expressing HEK 293 Stable Cell Line is sub-cloned with Hind III and EcoR I. CCR3 Expression Plasmid is provided by Wang et. al. 2013 PLOS. Reference. Adapted from Life Technologies T REx HEK 293 Cell Line Protein Expression Manual.

CCR3 protein induction is carried out by incorporating a trace concentration of tetracycline in the cell culture media; working range of 1 to 5 ug/ml. Further optimizing the tetracycline concentration in the DMEM culture media to prevent excess cell death after addition of tetracycline

By incorporating a trace amount of tetracycline in the cell culture media for target protein induction, the tetracycline molecule binds to TetR repressor protein expressed by the pcDNA4/TR vector. Tetracycline induces a conformational change by forming an inactive conformer of TetR and can no longer bind to TetR binding site on plasmid DNA; induces de-repression. At this step, the Tet operator (TO) having the gene of interest, expresses the target protein, in this case human CCR3 GPCR.

4.2 Experiments Methods: pcDNA4/TO Human CCR3 Expression Plasmid

Human CCR3 expression plasmid pcDNA4/TO has an antibiotic resistance sequence for zeocin and ampicillin antibiotics. The expression plasmid amplification may be carried out in LB media either with zeocin or ampicillin as the selection antibiotic. pcDNA4/TO CCR3 expression plasmid is amplified in E. coli (chemically competent BL21 DE3, from New England Biolabs) grown in LB with 100 ug/ml Ampicillin overnight at 37 ºC, at 150 rpm. Later the cells were spun at 14000 g for 20 minutes to pellet the bacterial cells. Plasmid DNA is extracted with the Promega Maxiprep kit with manufacturer instructions (Promega Maxiprep). Finally, the bound plasmid DNA is eluted with RNAse free water. Plasmid DNA purity of pcDNA4/TO CCR3 and concentration was estimated with Thermo Nanodrop 2000, UV spectrophotometer. The purified plasmid DNA concentration is estimated to be 712 nanogram/uL. This plasmid DNA is aliquoted and stored at -20 C for future HEK 293 transfection experiments.

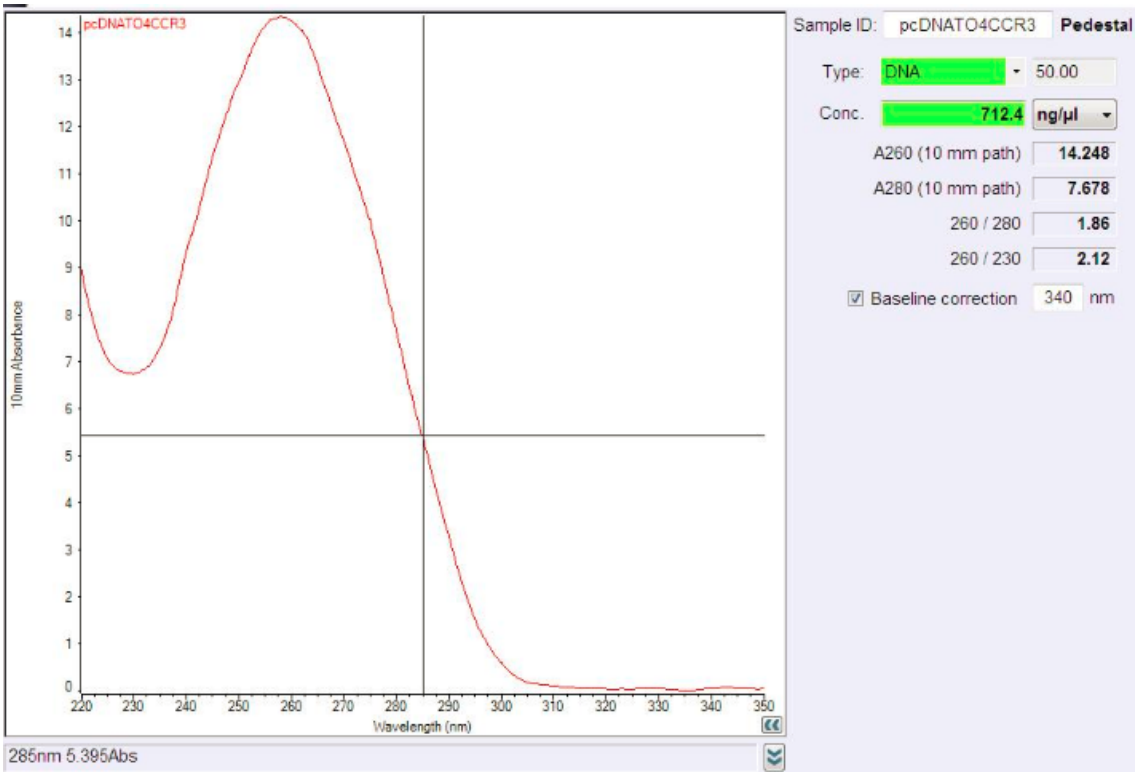


Figure 11. pcDNA4/TO CCR3 Purified Plasmid DNA Concentration Estimation by UV Spectrometry. Nanodrop UV 2000 Spectrophotometer Measurement yield 712.4 ng/uL Concentration. Promega Maxiprep protocol is followed in Plasmid DNA Extraction Purification.

4.3 pcDNA4/TO CCR3 Mammalian Expression Plasmid Restriction Digestion and Analysis

1 % Agarose gel was casted using TBE buffer and agarose with final addition of ethidium bromide, DNA staining dye. pcDNA4/TO CCR3 plasmid restriction digestion is carried out with EcoR I and Hind III restriction digestion enzymes, digested is carried for 3 hours at 37 ºC, loading dye is added to the sample after digestion. The digested plasmid DNA sample is separated on the gel. 20 kb DNA ladder is used as the reference to compare bands. Electrophoresis was carried out with Thermo Fisher gel electrophoresis

tank with power source, 90 volts current is applied to separate the digested DNA fragments. The agarose gel is imaged under UV light to visualize the plasmid DNA digestion fragment bands.

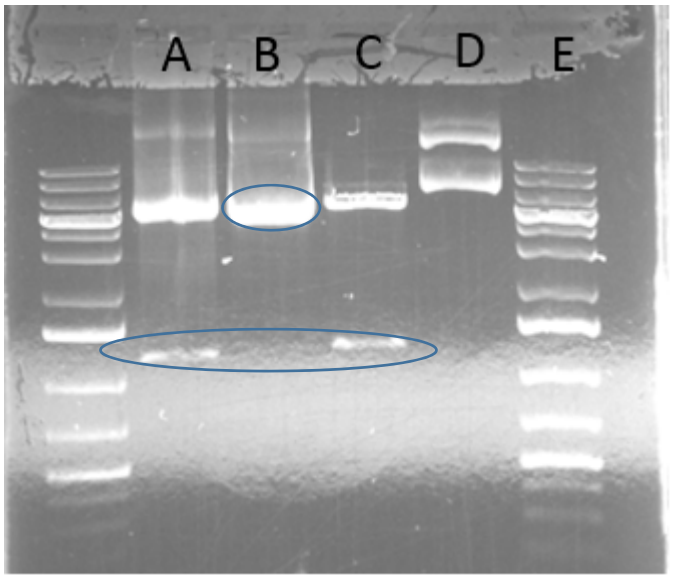


Figure 12. pcDNA4/TO CCR3 Expression Plasmid Restriction Digestion and Analysis. Restriction Digestion of the Plasmid with Hind III and EcoRI,. Precast 1 % Agarose gel is used in separating Plasmid DNA uncut and digested. DNA Preparation 1. A – Digested plasmid DNA, B – uncut plasmid DNA. DNA preparation 2. C – Digested plasmid DNA, D – uncut DNA. E – 12 kb ruler. The uncut DNA and 1280 bp CCR3-10X His tag cut DNA were marked in blue circle.

4.4 HEK293T-REx™ Cell Line Transfection

Healthy HEK293 T-REx™ cell line culture is sused in stable transfection cell line establishment protocol (Thermo Scientific catalog # 71007). Healthy cells with a confluency of 80 to 90 % is used in transient transfection experiment. This HEK 293 TetR cell line has a pcDNA4/TR, tetracycline repressor stably integrated with blasticidin resistance cassette. As per the vendor recommended protocol, 5ug/ml blasticidinis used in

the DMEM, 10 % FBS growth media during initial cell culture propagation and expansion. Life Technologies Lipofectamine 2000 (catalog number # 11668027), a cationic transfection agent is used from initial transient transfection experiments. HEK 293 TetR cell viability was verifed before seeding cells for transfection experiments. Lipofectamine 2000 to plasmid DNA concentration ratio is optimized for maximal transfection efficiency, confocal data show in later part. After repeated transfection experiments transiently transfected HEK 293 TetR cells with pcDNA/TO4 CCR3 was finally successful.

Transient transfection: 20 micrograms of plasmid DNA, pcDNA4/TO CCR3 is mixed with 114 ul of Opti-MEM media, a reduced serum medium (Gibco catalog # 31985062). The mixture is incubated at room temperature for 10 minutes. 20 uL of Lipofectamine 2000 ismixed with 130 uL of Opti-MEM media, incubated at room temperature for 20 minutes**.** The above two solutions are combined and mixed gently for 5 minutes at room temperature then added to the 90 % confluent cells having a fresh media without blasticidin antibiotic. The cells are incubated for 6 to 8 hours in a CO_2 incubator followed by fresh culture media change.

4.5 Immunofluorescence

Immunofluorescence experiment is carried out to validate CCR3 expression. Sterile coverslips is used to seed the transiently transfected cells. The protocol developed for adherent cells is described below.

Poly-L-Lysine Coating of Cover Slips: ¾ inch coverslipswere washed with ethanol and air dried and finally rinsed with sterile water. Later the coverslips are soaked

in 0.01 % poly- L-lysine dilute with sterile water, coverslipswere gently mixedwith 0.01 % poly-L-lysine solution for 30 minutes, then removed from 0.01 % poly-L-lysine solution and air dried. Finally,coverslips were washed with sterile DI water for five times and dried. Poly-L-Lysine solution is toxic to cells, when not cleaned thoroughly. The coverslips were washed thoroughly with DI water to remove any traces of poly-L-Lysine. These poly lysine coated coverslips were used to seed and fix cells and was carried out in a sterile cell culture hood.

Seeding HEK 293 cells: The coverslipswere washed with 1X PBS followed by drying. Six well sterile culture plate was used to seed cells. The HEK 293 CCR3 stable cell line maintenance media consisting of DMEM, 10 % FBS, 30 ug/ml Zeocin media.1 ml maintenance medium is utilized in each well having one cover slip.

Cells were let to grow to reach 70 % to 80 % confluency followed by CCR3 is induction with tetracycline and sodium butyrate, 2 uL of stock tetracycline in each test well and 50 uLs sodium butyrate in each well with 36 hours of CCR3 induction. Later the coverslips were removed carefully and washed with 1X PBS and proceed for paraformaldehyde fixing.

Fixing adherent HEK 293 cell on sterile coverslip for antibody probing, Freshly prepared 4 % paraformaldehyde in sterile DI water (Fisher Scientific # 30525-89-4) is used to fixing the cells on coverslips for 30 minutes. Later the coverslips were washed three times with 1X PBS. Care was taken to prevent dislodging of the adherent cell from coverslip. The cells were kept always in the hydrated in 1X PBS and never let

to dry.The coverslips were incubated or blocked in 1X PBST (1X PBS, 1% BSA, 0.1 % Tween 20) for 30 minutes to prevent nonspecific binding.

Primary antibody incubation with mouse monoclonal anti-histag primary antibody: The expressed CCR3 has a 10X polyhistidine tag at the C-terminus, so an anti-histag primary antibody is chosen in the immunofluorescence protocol. 6x-His Epitope Tag Antibody (Thermo Fisher catalog # MA1-21315) was the primary antibody, a mouse anti-histag monoclonal antibody. The primary antibody solution at a dilution of 1.500 dilution in 1X PBST is used with incubated for 30 minutes at 4ºC. Later wash three times for five minutes each with 1X PBS.

Secondary Antibody Incubation: Alexa 488 conjugated anti-mouse secondary antibody in a dilution of 1.2000 dilution in 1X PBS is used. Alexa 488 conjugated antibody solution is used to incubate for 30 minutes.

At last, the coverslipswere washed three times with 1X PBS. Hoechst 33342, DNA staining dye is used in staining nuclei for eukaryotic cells. Hoechst is used in 1.5000 dilution and washed for 5 minutes. Hoechst has an excitation wavelength at 350 nm and emission spectra at 460-490 nm. Later the coverslipwere submerged in 1X PBS until further mounting and confocal imaging.

Mounting slide: The coverslipswere carefully removed from the six-well plate. 200 microliters of polymount solution was used to fix the cells with another sterile coverslip, and the fixed coverslip was storedin the dark at 4 ºC for confocal imaging. Note: While mounting the coverslip care was take to prevent air trapping or bubbles in the polymount solution.

4.6 Confocal Imaging of Paraformaldehyde fixed CCR3 Expressed HEK 293 Cover Slip

Immunofluorescence Imaging methods by confocal microscopy are acquired with Carl Zeiss spinning disc confocal microscope with 10X and 100X (oil immersion) magnification.

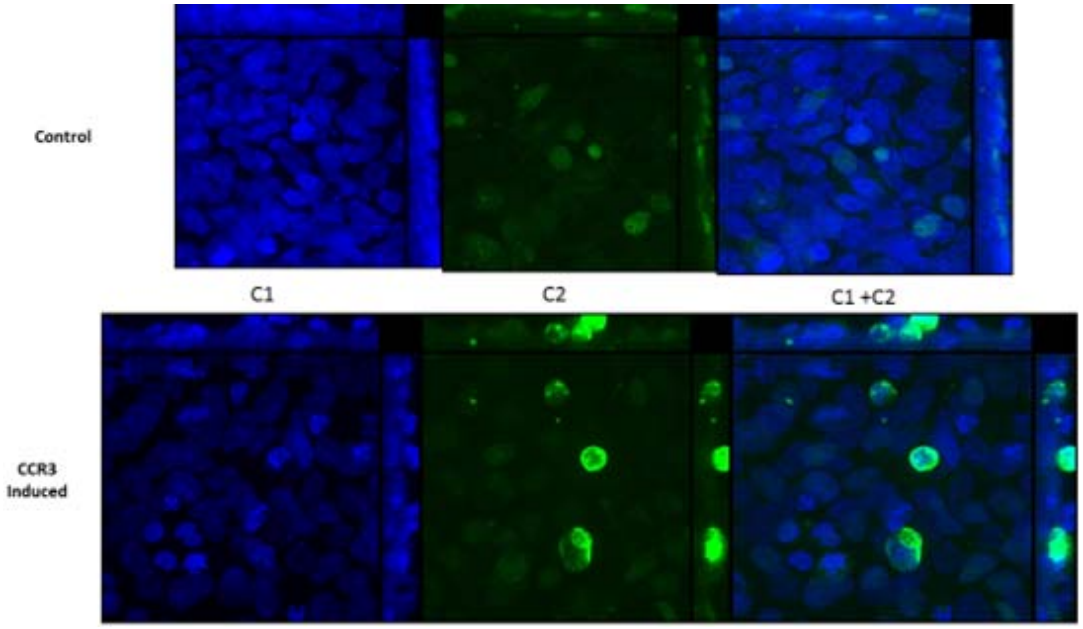


Figure 13. Immunofluorescence Images of HEK 293 Control (top) and Transient Expression of CCR3 by Tetracycline Induction.

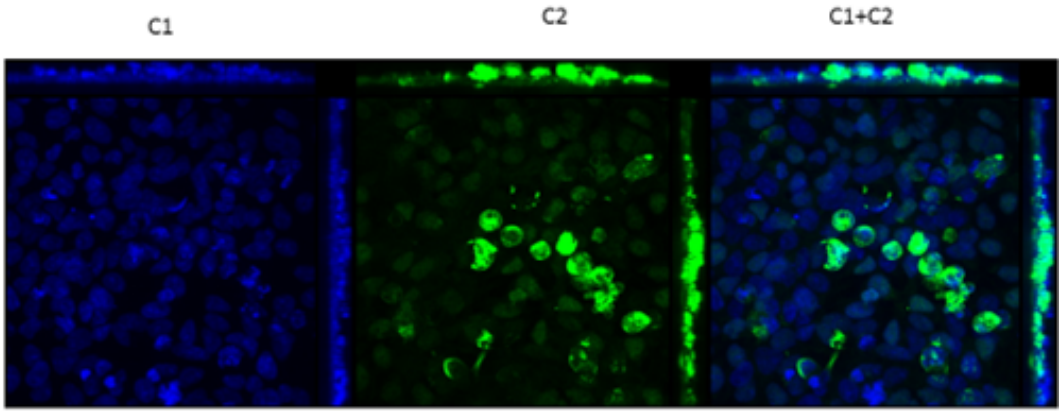


Figure 14. Immunofluorescence Image of Transient CCR3 Expression in HEK 293. Tetracycline-Induced Transient Transfected HEK293 Cells Expressing CCR3. Antibodies used in probing the fixed cells on Sterile Coverslip: Primary antibody used is Anti 6X His-Tag Mouse Monoclonal Antibody (Thermo Pierce product # MA1-21315). Green-Alexa 488 conjugated Anti-Mouse Secondary Antibody.

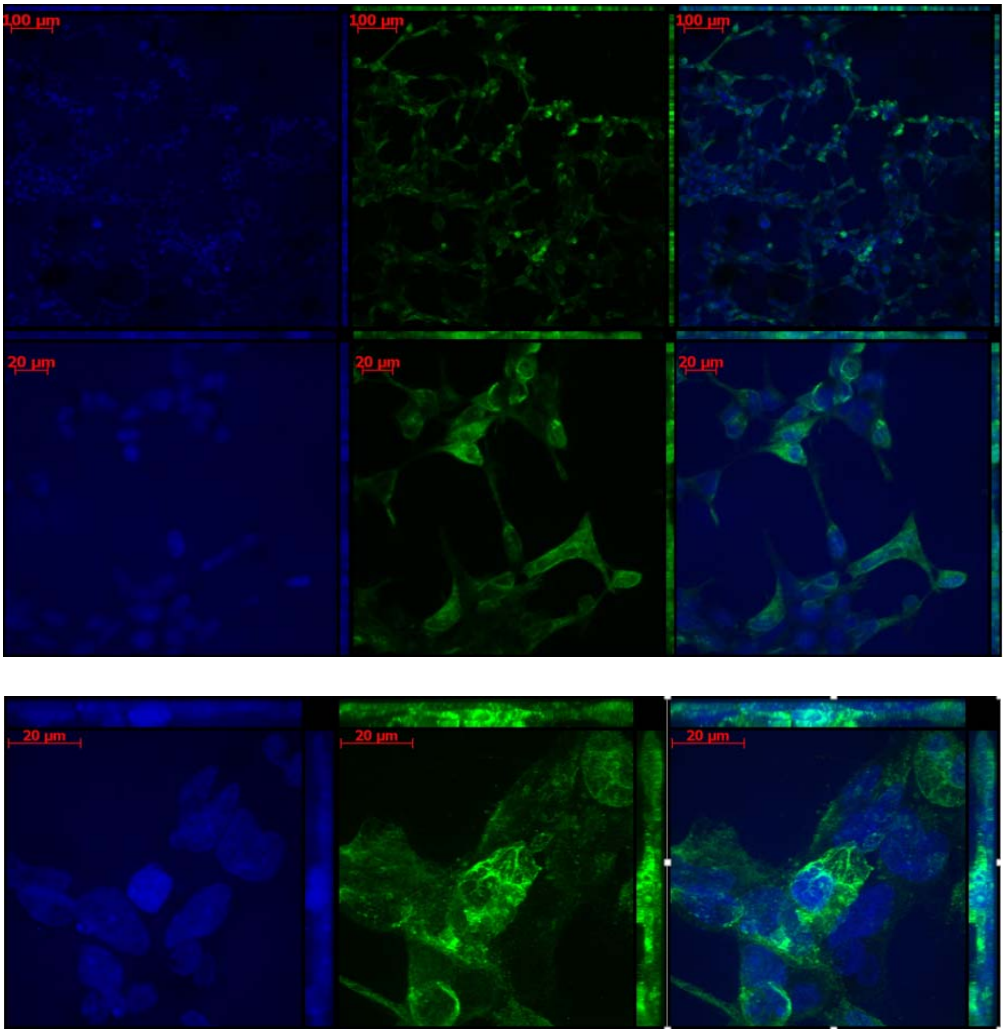
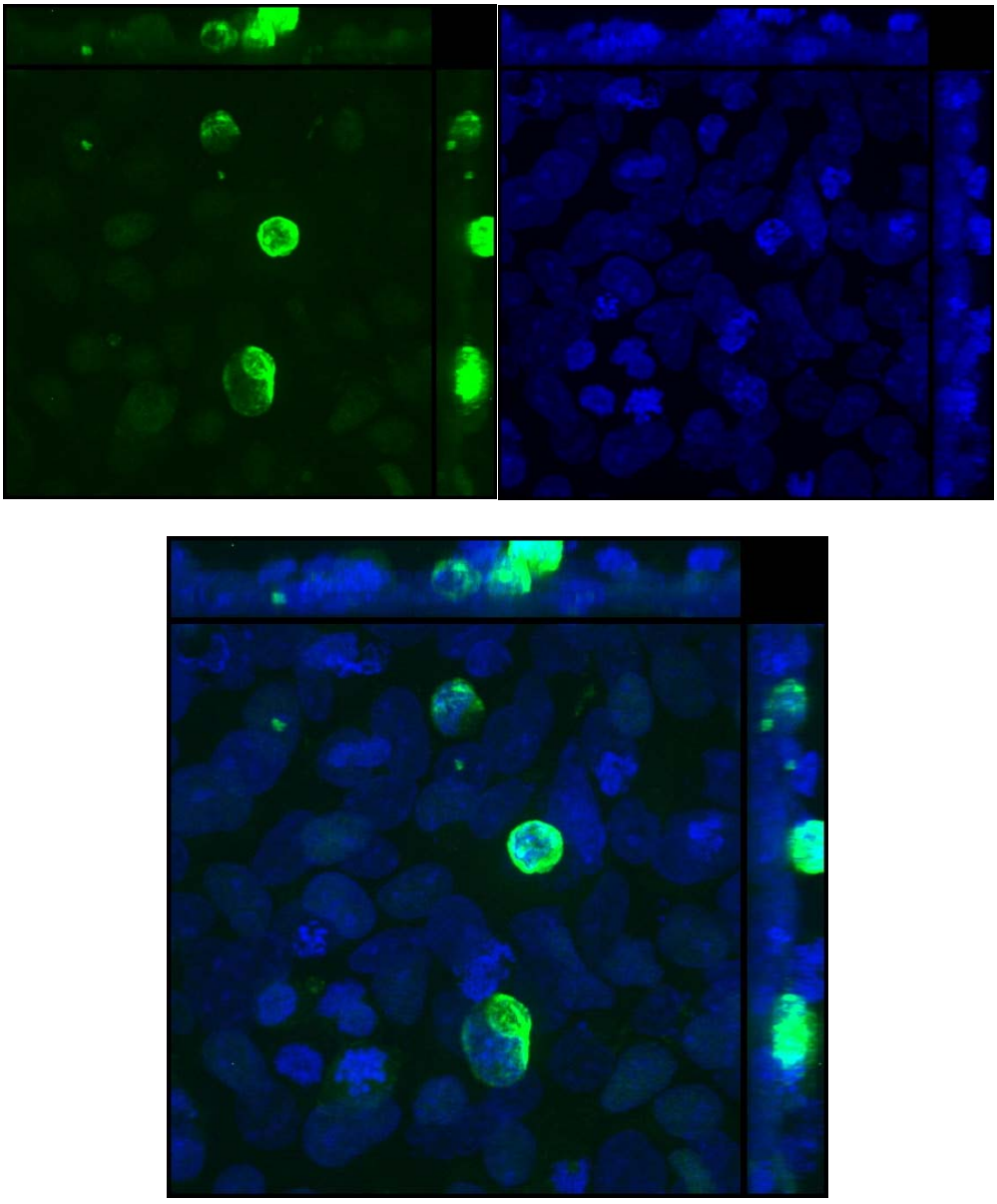


Figure 15. Immunofluorescence Imaging of HEK 293 Stable Cell Line Expressing CCR3 Expression; Low Magnification (top) and High Magnification (bottom).



Figures 16. CCR3 Expression HEK 293 after Transient Transfection. Confocal Images with Z-stack. Lipofectamine 18 uL and standard concentration of pcDNA4/TO CCR3 Plasmid DNA.

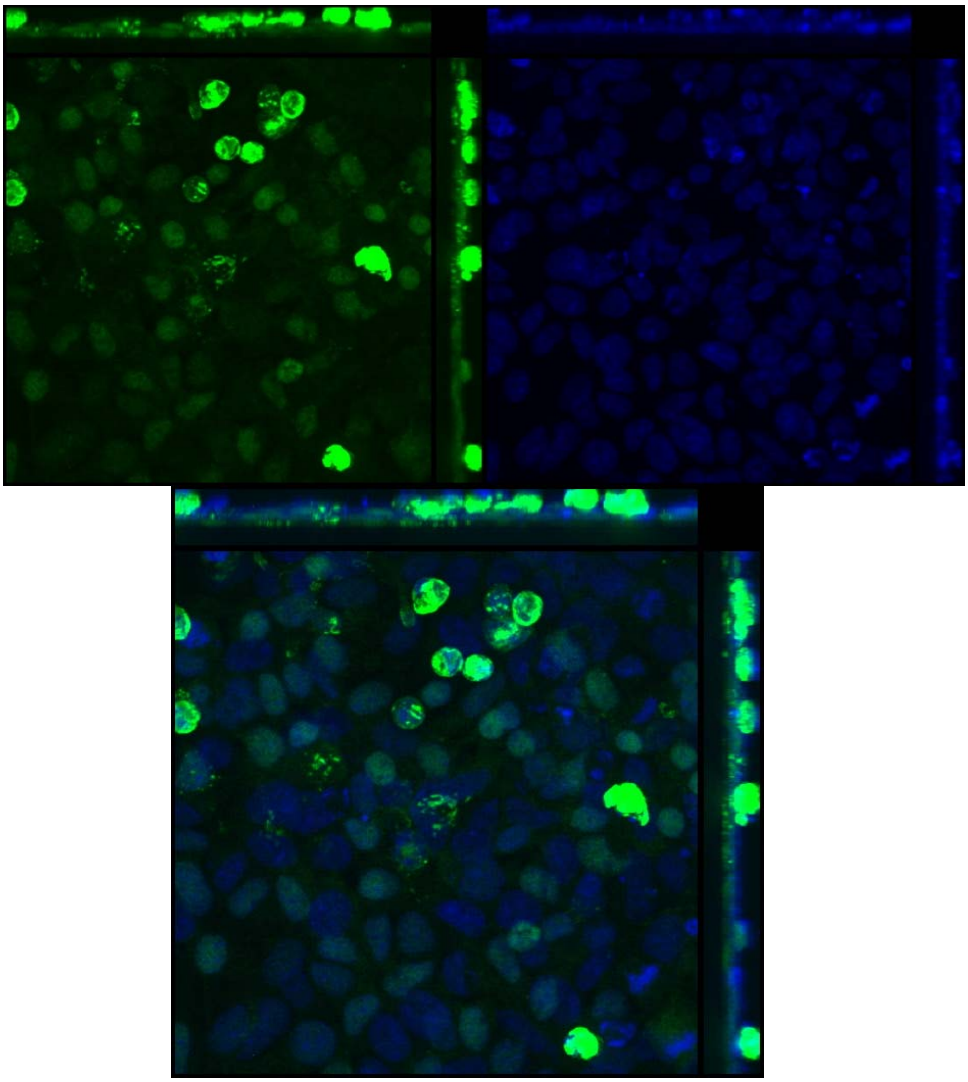


Figure 17. Additional Images of CCR3 Expression HEK 293 after Transient Transfection. Confocal Images with Z-stack, Lipofectamine 30 uL and standard concentration of pcDNA4/TO CCR3 Plasmid DNA.

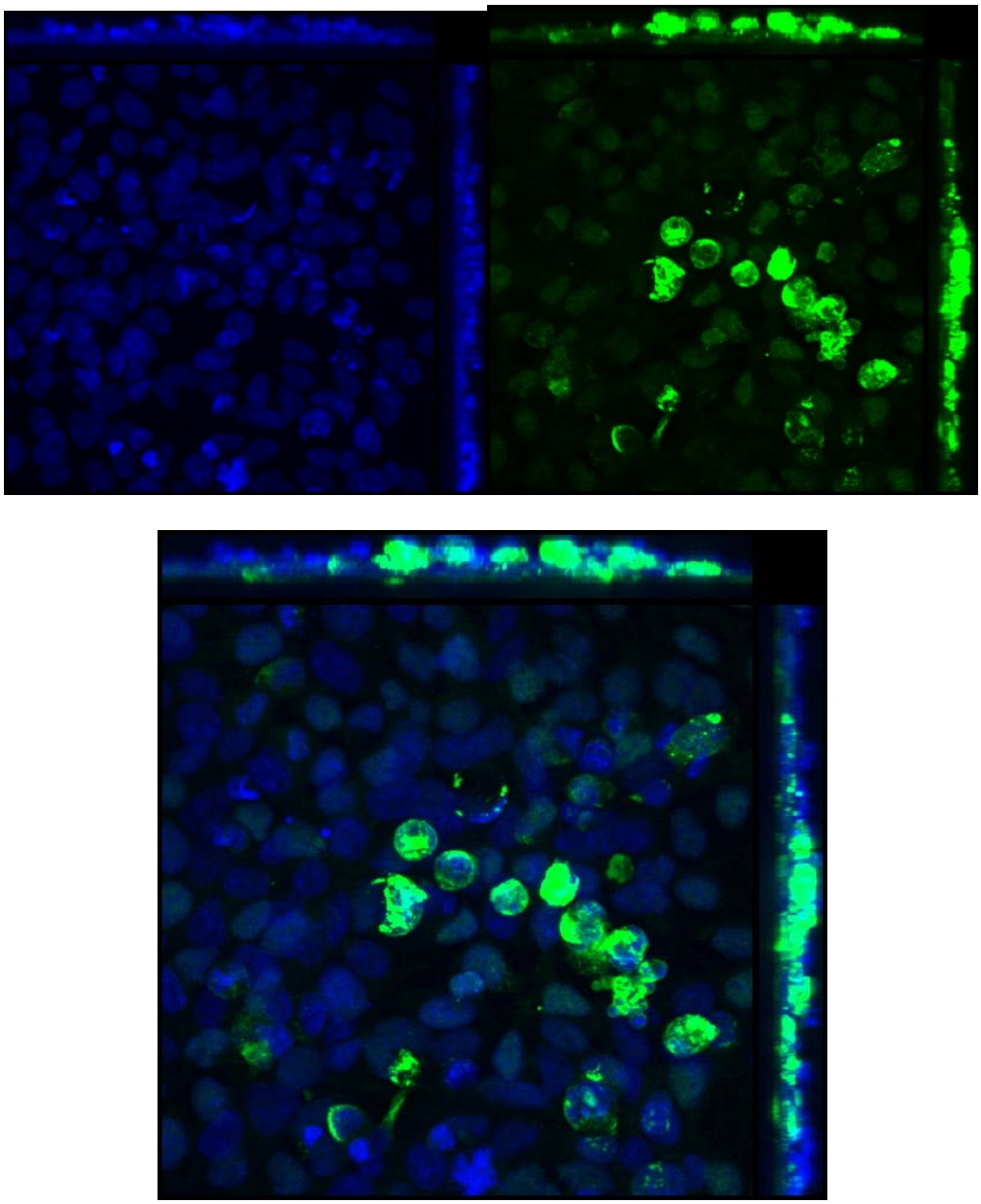
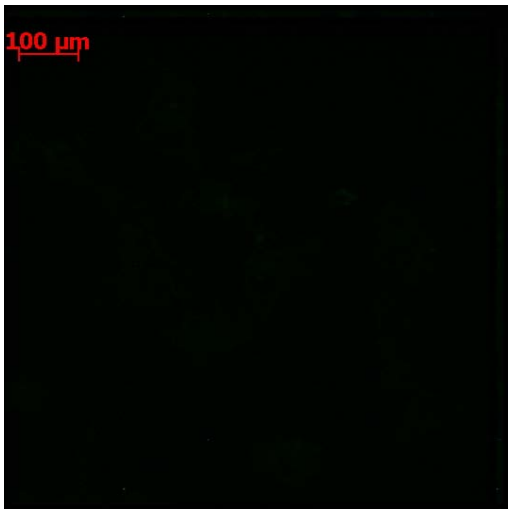


Figure 18. Alternate Field of View, Lipofectamine 30. Confocal images with Z-stack, Lipofectamine 30 uL and standard concentration of pcDNA4/TO CCR3 Plasmid DNA.



Control Channel (C1)

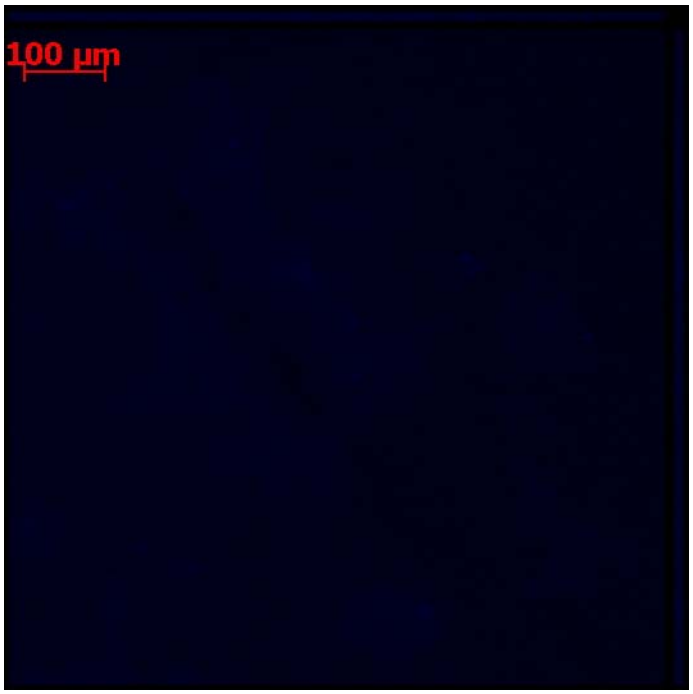


Figure 19. (Top) HEK 293 Control-C1, (Bottom) HEK 293 Control-C2; no Tetracycline Induction.

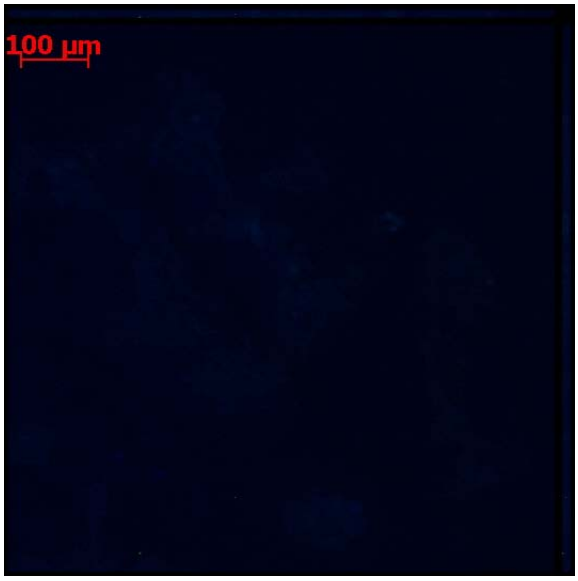


Figure 20. HEK 293 Control C1+ C2

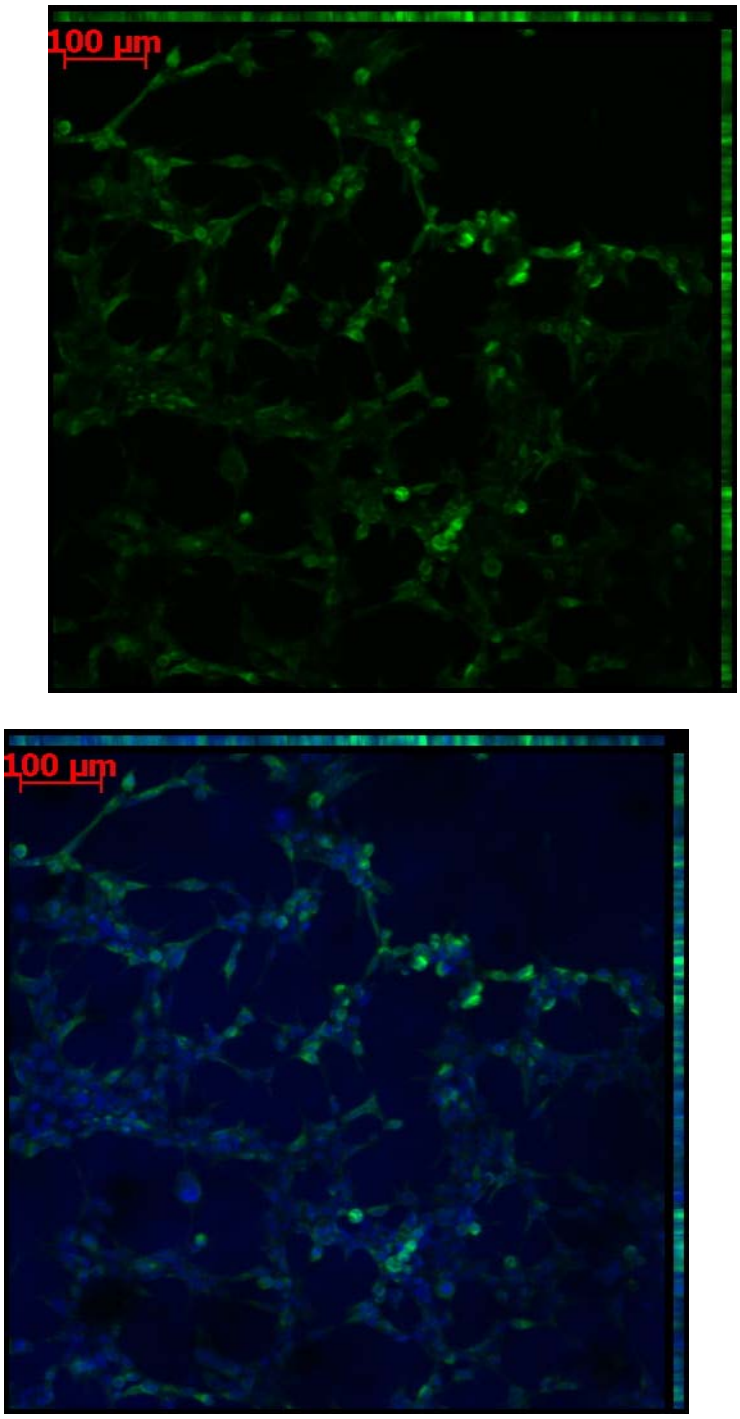


Figure 21. Stable Cell Line Expressing CCR3, Alternate Field of View. Imaging channels C1+C2.

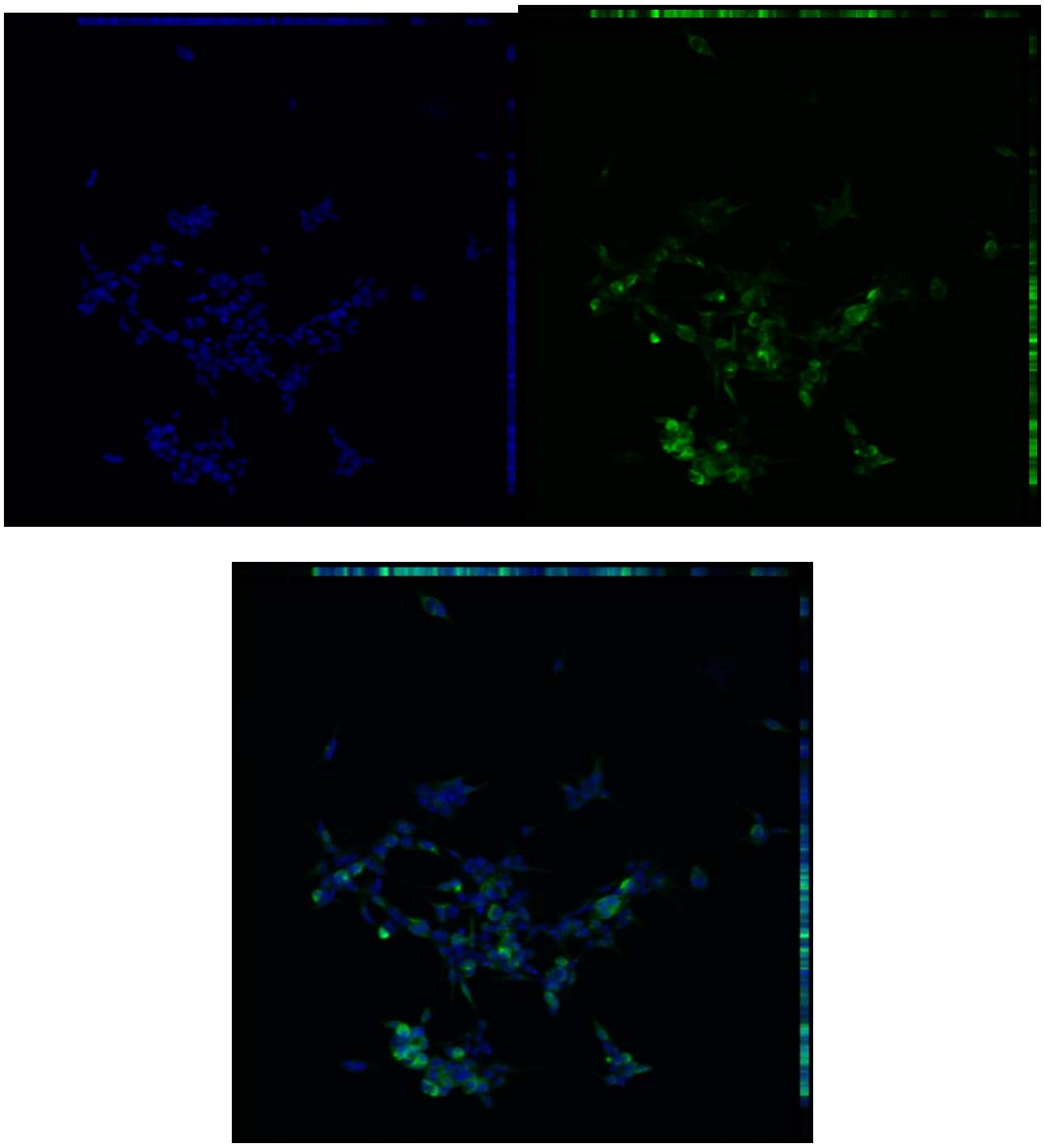


Figure 22. Alternate Field of Viewing at Low Magnification, Stable Cell Line Expressing Human CCR3 upon Induction.

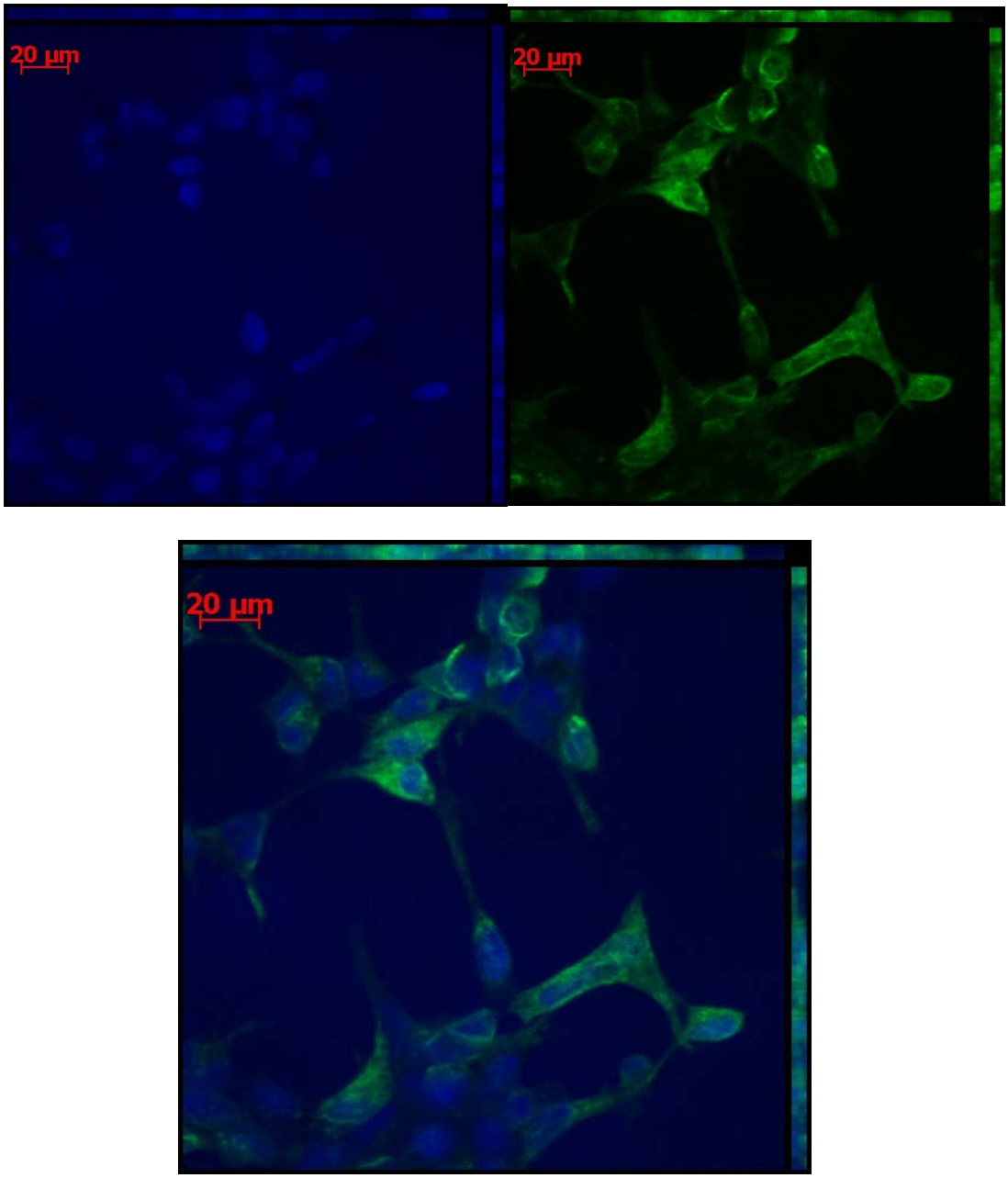


Figure 23. Stable Cell Line Expression of CCR3, Imaging at 10X Magnification.

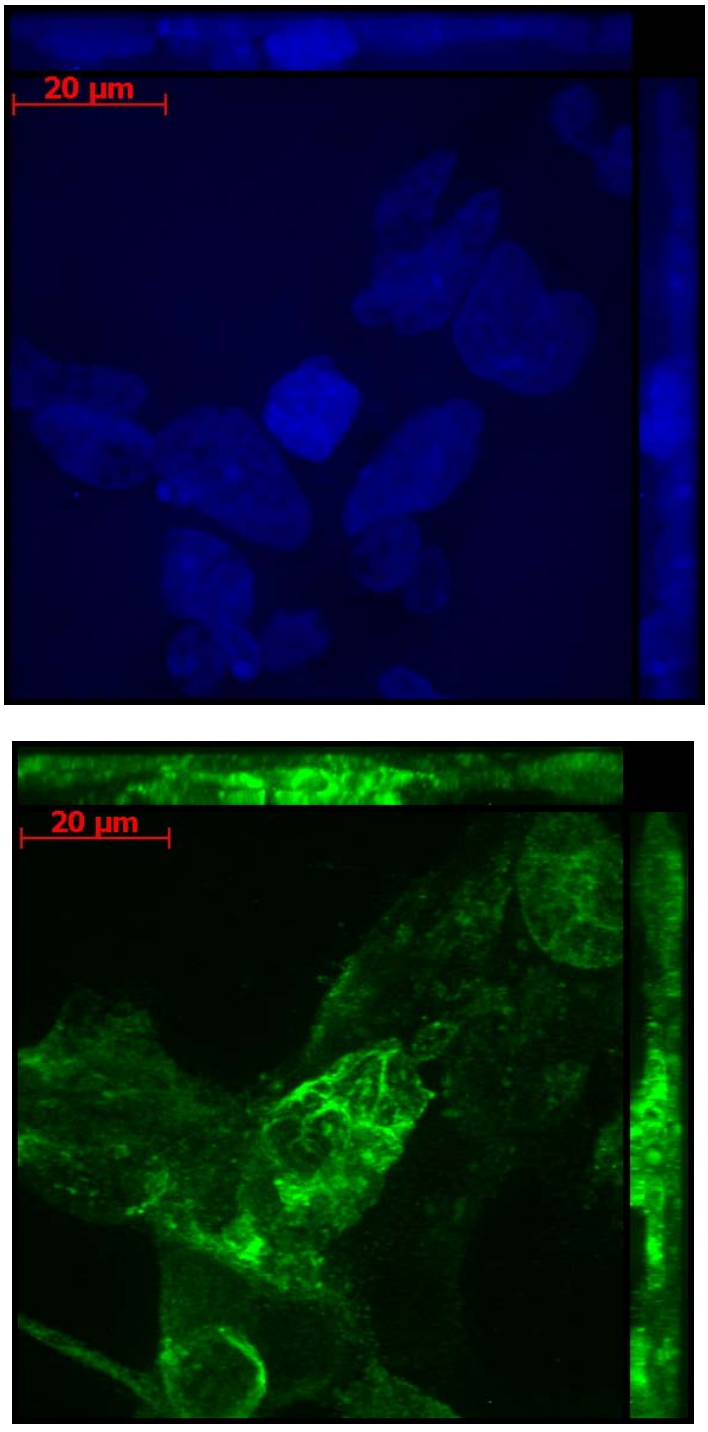


Figure 24. Stable Cell Line Expression of CCR3, Imaging at 100X Magnification, Channels C1 (top) and C2 (bottom).

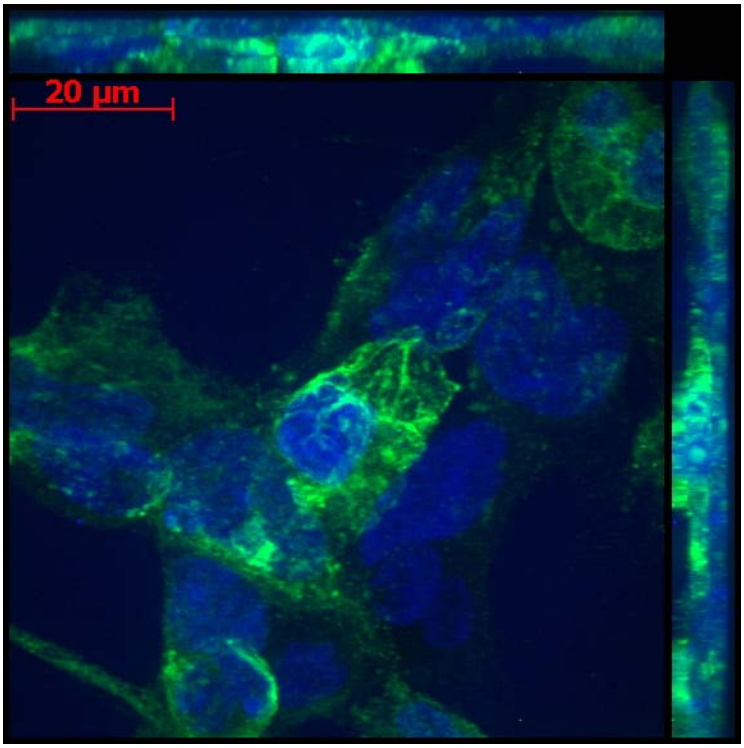


Figure 25. Stable Cell Line Expression of CCR3, Imaging at 100X Magnification, Channels C1 + C2.

4.7 HEK 293 CCR3 Stable Cell Line Development with Zeocin Antibiotic Based Clonal Selection

Zeocin is a selection antibiotic to select stably transfected HEK 293 cells with pcDNA4/TO CCR3 plasmid. The first step is to determine HEK293 T-REx^{TM} cell line sensitivity and associated viability for zeocin antibiotic. Increasing concentration of zeocin in DMEM supplemented with 10 % FBS, 2mM L-Glutamine, 5ug/ml blasticidin in culture media were prepared. Zeocin stock solutions are prepared in the incremental strengths as following 125 ug/ml, 250 ug/ml, 500 ug/ml, 750 ug/ml, 1000 ug/ml. After the zeocin sensitivity was established for the untransfected cells. Zeocin sensitivity was observed at 125 ug/ml concentration. Transient transfected CCR3 cell line are seeded in a twenty-four well culture plate, and zeocin concentration stock DMEM/10 % FBS culture media is added to individual wells. The media is replenished every three days. The initial observation is a gradually HEK 293 TetR cell death, over a period of three to four weeks. Later the zeocin-resistant HEK 293 TetR colonies start to grow in cell culture plate with retarded growth. Later gradual increase in the HEK 293 growth is seen in culture plate. Once a significantly sized colony start to appear, approximately 15 to 20 ug/ml zeocin resistant HEK TetR clones appeared. The clones are carefully picked with a sterile micropipette tip for individual clonal expansion-propagation. These cells are taken into new culture plate. Clones were labeled, and the cultures were further expanded. These stably transfected HEK 293 clones are expanded in their respective zeocin concentration containing DMEM/10 % FBS culture media. Individual clones were used for CCR3 expression scale up with tetracycline induction in the later stage. Further more, these

clones are prepared for long-term storage in liquid nitrogen vapor (-195 Celsius) according to sellers instructions with freezing medium consisting of 45 % of fresh DMEM/10 % FBS culture media, 45 % of used, from culture DMEM/10 % FBS culture media and 10 % sterile DMSO.

During the first two overall experimental methods to establish the stable cell line, cell culture contamination was observed with mycoplasma or yeast in the laboratory and the overall HEK 293 stable cell line process was repeated. It is worth noting to be vigilant due as mammalian cultures are prone to microbial contamination.

In CCR3 receptor expression, cells were cultured at 20 ug/ml Zeocin concentration in DMEM/10 % FBS/ 5ug/ml blasticidin media. The increase in zeocin concentration led to retarded grow of the stably transfected cells.

A few of these HEK 293 clones were verified for CCR3 expression by immunoblotting with ECL plus chemiluminescence reagent as the blot developing agent. Anti-human CCR3 and Anti-Histag primary antibodies are used in confirming CCR3 expression.

4.8 CCR3 Expressing in HEK 293 Verification with Dot Blot Protocol

Dot blot protocol is carried out with anti-histag primary, HRP conjugated secondary antibody, DAB substrate incubation-imaging protocol

Fisher brand nitrocellulose membrane was taken and wetted with 1X PBS, 5 uL of CCR3 induced HEK TetR cell lysate supernatants in added to the marked regions of the nitrocellulose membrane, blocked with 5 % non-fat milk in 1X PBST as the blocking buffer and probed with anti-histag primary antibody and later with goat anti-mouse HRP

conjugated secondary antibody. The nitrocellulose membrane is finally developed with DAB substrate solution (Thermo Pierce DAB substrate, catalog # 43002).

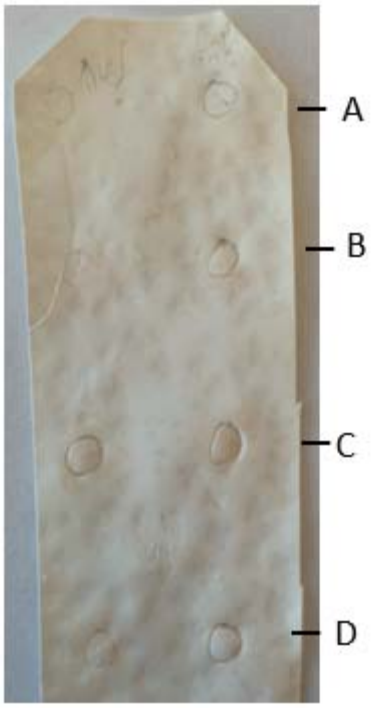


Figure 26. Dot Blot of CCR3 Expressed HEK293 Cell Line Clones; Immunoblot of Whole Cell Lysate Supernatant. A – Polyhistidine epitope positive Control.B, C, D - Cell Lysate Supernatant probed with Anti-Histidine Tag Primary Antibody, developed with HRP Secondary Antibody and DAB Substrate.

4.9 CCR3 Receptor Induction and HEK 293 Cell Pellet

Cell viability is assessed first upon addition of increasing concentration of tetracycline in DMEM culture media. 1ug/ml of tetracycline was chosen to be optimal without excess loss of viable cell after CCR3 receptor induction and cell pellet harvesting. With the above-stated rationale, 1ug/ml tetracycline and 50 mM sodium butyrate was used in the final DMEM with 10 % FBS media for 36 hours to stimulate CCR3 production. Tetracycline is a protein inducing agent and sodium butyrate is a histone decyclase inhibitor [8,9,10]. Cell death is observed to some extent after addition

of tetracycline to the culture media. The protein induction time was kept to 36 hours to prevent excessive loss of viable cells, due to tetracycline toxicity. After 36 hours of protein induction, the cell was washed with cold PBS twice and harvest by scraping with a sterile cell scraper. Cells were pelleted at 5000 g for 20 minutes at 4 Celsius, the supernatant 1X PBS was removed. Cell pellet was finally stored at -80 ^{o}C for later downstream affinity purification process.

4.10 HEK 293 Cell Lysis

Cell lysis was carried out with base buffer with DDM and CHS (1.5 % DDM and 0.05 % CHS) in 50 mM Tris base with 1X Protease Inhibitor cocktail, 2 mM $CaCl_2$, 5 mM KCl, 5 mM $MgCl_2$, 1.5 % DDM, 0.05 % CHS , final buffer pH 7.4. To approximately 500 mg of the harvested cell pellet gradual dropwise addition of 1.5 ml cell lysis-membrane extraction buffer with simultaneous mixing (base buffer with 1.5 % DDM with 0.05 % CHS in 1X protease inhibitor cocktail). The cell pellet was slowly dissolved with mild homogenization process; a hand held micro homogenizer (Manufacturer-Whitman Glass) is used for mild disruption of the pellet and re-suspend in the buffer. After intermittent homogenization, the cell pellet was re-suspended thoroughly. The homogenization process was mild and gentle to prevent foaming in a microcentrifuge tube and is carried over ice at 4^{o} C. Membrane proteins tend to aggregate even under slightly elevated temperatures, mechanical homogenization tends to elevate sample temperature. Later the lysate was centrifuged at 15000 g for 30 minutes at 4^{o}C to sediment the cell debris. The supernatant was collected. CCR3 protein expression

in confirmed in the extract cell lysate supernatant. Dot blot and Western blot analysis were carried out to verify CCR3 expression.

4.11 SDS PAGE Gel Electrophoresis and Western Blotting

Bio-rad 10 % SDS PAGE with 0.75 mm thickness pre-cast gel is prepared for CCR3 expression analysis of the cell lysate supernatants. Five samples of DTT-reduced, one non-reduced cell lysate supernatants, and a negative control of HEK 293 lysate supernatant were used. The reduced 0.75 mm thickness samples were incubated for 1.5 hours at room temperature with intermittent vortexing. DDT reduce disulfide bonds and facilitate opening of the protein for LDS binding and promote migration of the protein in an applied voltage potential. Membrane proteins tend to aggregate during elevated temperatures, so the cell lysate is not heating to 100^{o} C for 5 minutes by conventional SDS sample preparation protocol. Bio-rad protein molecular weight ladder is used for protein migration comparison. The gel was run with at 125 volts for approximately 90 minutes, till the sample dye front reaches the bottom of the gel. Membrane proteins migrate in the range of 70 % to 85 % of their true molecular weight; this is due improper unfolding of membrane protein and excess binding of LDS molecules to membrane proteins during sample preparation [11].

Transfer of Proteins to Nitrocellulose Membrane by Wet Transfer Method for Immunoblotting: The electrophoretically separated proteins were later transferred from SDS PAGE Gel to the nitrocellulose membrane transfer by wet transfer method with the transfer buffer lasting for 1 hour.

Note: All of the following incubations and washes are performed at 4^{o} C

The membrane is immediately blocked with blocking buffer consisting of 5 % bovine serum albumin in PBST buffer for 1 hour, followed by three washes with 1X PBST (1X phosphate buffer saline in 0.1 % Tween 20).

Primary antibody probing with mouse monoclonal anti-human CCR3: Mouse monoclonal anti-human CCR3 antibody wasacquired through NIH AIDS Reagent Program (catalog # 4923) and used as the primary antibody. The blot is incubated at 1.700 ratio of primary antibody in 10 % blocking buffer in PBST overnight at 4^{o} C with slow shaking. The followed morning 3X washes with PBST was carried out and proceeded to secondary Antibody incubated at room temperature. Goat anti-mouse horseradish peroxidase conjugated (HRP) antibody was used in 1.1500 ratio of 10 % blocking buffer for 1 hour at room temperature followed by final three washes with 1X PBST for 5 minutes each.

ECL plus Western blot substrate (Thermo Scientific catalog # 32132) 5 ml of substrate solution a mix of 1.1 ratio of reagent solution A and reagent solution B (2.5 ml solution A + 2.5 ml solution B) is added, and the blot was incubated for 5 minutes-air dried for a few minutes and development by X-ray film with exposure of 15 minutes and later and developer with a development machine.

Western blot Image: HEK 293 CCR3 cell lysate protein extract supernatant of different stable cell line clones. Blot developed with ECL plus reagent. Chemiluminescence.

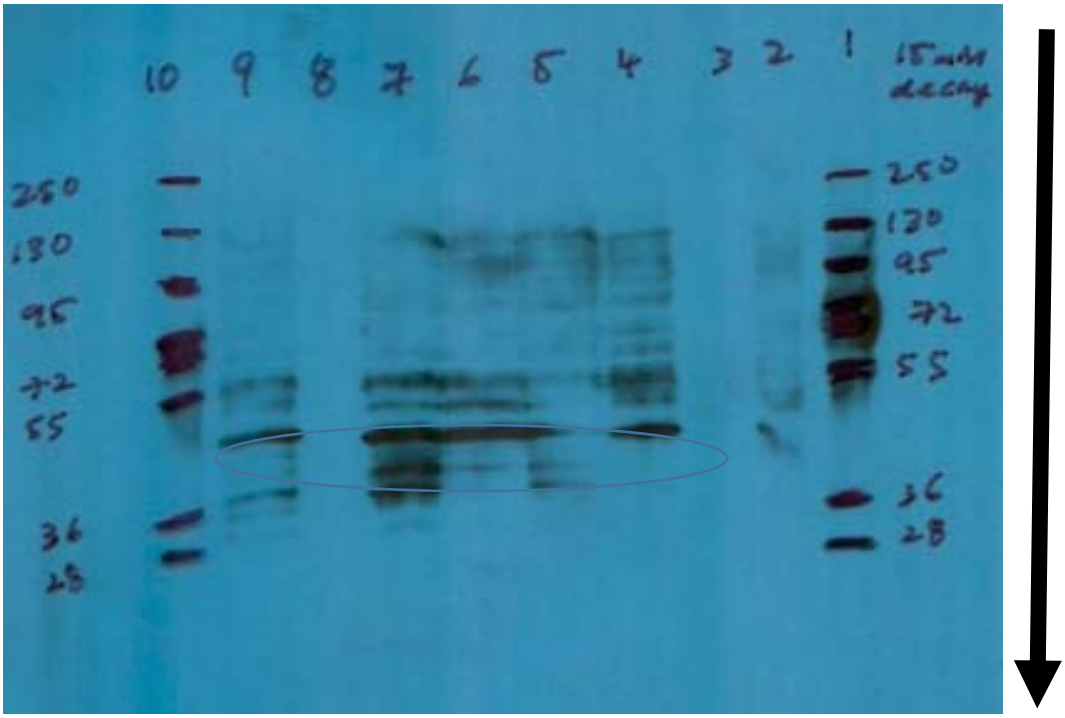


Figure 27. Immunoblotting of CCR3 Expressing HEK 293 Cell Line Clones. Anti CCR3 Primary Antibody Probing.1, 10 - protein molecular weight markers, 2 - HEK 293 Negative Control (do not express CCR3). 3,8 - blank lanes, 9 - non-reduced CCR3 Expressed HEK 293 Cell Lysate Supernatant. 4, 5, 6, 7 - Reduced CCR3 Expressed HEK 293 Cell Lysate Supernatants of several batches of Protein Extractions.

Note: CCR3 Migrates approximately at 38 kDa, according to the Labeled Protein Marker.

4.12 In-Gel Digestion Method for Mass Spectrometry Analysis

Preliminary mass spectrometry identify CCR3 expression with in-gel digestion followed by nanoflow HPLC-MS Analysis

The cell lysate supernatant is reduced with NuPAGE 4X LDS loading buffer with 10mM DTT at room temperature with shaking. The NuPAGE samples were not heated,

heating the samples leads to membrane protein aggregation. 20 uLs of the sample is separated on a 4-12 % NuPAGE Bis-Tris gel followed by stain with Thermo Gel code blue simple staining. The gel is imaged, and the band corresponding to 34 kDa to 42 kDa was excised and cut into 1 mm pieces. The gel plugs are destained with 70 % Acetonitrile in 50 mM Ammonium bicarbonate (AMBIC), this step is repeated twice and finally the gel plugs were shrunk with 80 % acetonitrile and proceeded with standard In-gel digestion protocol. First reduction with 50 mM dithiothreitol (DTT) for 20 minutes in the dark at 45 C, followed by alkylation with 55 mM iodoacetamide for 45 minutes in the dark at 40 C temperature.

Fresh stock of sequencing grade trypsin (Thermo Scientific # 90057) is prepared with 50 mM AMBIC and digested at 12.5 nanograms/uL concentration of trypsin. Digested was carried out overnight in a thermomixer (Eppendorf) at 37 C. Later the digestion was quenched with 10 % formic acid peptides were extracted with 70 % ACN and the extracted peptides were dried to completion in thermo savant speed vacuum apparatus.

C18 solid phase extraction, cleaning of digested peptides: The In-gel digested and dried peptides sample has salt and surfactant contaminants in them. C18 based affinity desalting of peptide is a microscale affinity method to desalt in-gel and in-solution digest protein samples with a C18 stationary phase resin. This C18 clean-up/desalting is carried out with Thermo Scientific 200 uL C18 micropipette tip.

The cleaned peptide were eluted with 50 % ACN /0.1 % formic acid from the C18 tip. These cleaned peptide sample is re-suspended in 10 uL of 5 % ACN/0.1%/95 % H_20 (nanoflow HPLC mobile phase A).

4.13 Peptides Identified by Liquid Chromatography Tandem Mass Spectrometer

LC-ESI-MS Analysis with Thermo Orbitrap Mass Spectrometer 6 uLs of the above C18 cleaned peptide sample was analyzed with Dionex nanoflow Ultimate 3000 HPLC coupled to Thermo Scientific Orbitrap Velos Mass Spectrometer. Peptides were separated with 80-minute gradient on the nanoflow 75 micro ID 15 centimeters C18 column and sprayed into the mass spectrometer with Thermo nanospray ion source. Top six precursor masses are selected for MS/MS fragmentation in the Orbitrap Mass Spectrometer.

Data Analysis. The raw MS file is searched against Proteome Discoverer 1.4 peptide tandem MS search software with Mascot server at a precursor mass tolerance of 10 ppm and fragment ion mass tolerance of 0.01 Dalton.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	
2	P51677	C-C chemokine receptor type 3 OS=Homo sapiens GN=CCR3	1.67	2.54	1	1	1	1	355	41.0	8.22	
3		A2	Sequence	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	ΔCn	XCorr	Charge	MH+ [Da]
4		High	YIPFLPSEK	1	1	1	P51677		0.0000	1.67	2	1093.59526

Figure 28.Tryptic Peptide YIPFLPSEK from CCR3 Digest. The peptide was identified with Proteome Discoverer 1.4 search of the raw MS spectra.

YIPFLPSEK is the peptide identified by a preliminary run of a whole cell lysate supernatant. MH+ (Da) **1093.59526**, with a charge state of +2, highlighted in blue font in the CCR3 sequence map below

Chemokine Receptor Subtype 3 Amino Acid Sequence:

Note. Highlighted amino acids are cysteines in various domains of the 355 amino acid protein sequence and the two cysteines adjacent to each other -CC- is the basis for CCR3 receptor naming term. Total amino acid length. 1 – 355, Disulfide bonding is a post. translational modification exists between cysteine AAs at 106 – 183 positon:

>sp|P51677|1-355

MTTSLDTVETFGTTSYYDDVGLLCEKADTRALMAQFVPPLYSLVFTVGLLGNVV

VVMILIKYRRLRIMTNIYLLNLAISDLLFLVTLPFWIHYVRGHNWVFGHGMCKLL

SGFYHTGLYSEIFFIILLTIDRYLAIVHAVFALRARTVTFGVITSIVTWGLAVLAAL

PEFIFYETEELFEETLCSALYPEDTVYSWRHFHTLRMTIFCLVLPLLVMAICYTGII

KTLLRCPSKKKYKAIRLIFVIMAVFFIFWTPYNVAILLSSYQSILFGNDCERSKHLD

LVMLVTEVIAYSHCCMNPVIYAFVGERFRKYLRHFFHRHLLMHLGR**YIPFLPSE**

KLERTSSVSPSTAEPELSIVF

Note. The location of cysteine residues in various domains and the two cysteines adjacent to each other -CC- is the basis for CCR3 receptor naming term.

HEK 293 T-REx culture media and selection antibiotics used to establish stably transfected clones:

HEK T REx media

DMEM + 10 % FBS + 2 mM Glutamine + 5 ug/ml Blasticidin (pcDNA4/TR plasmid selection antibiotic)

To 450 ml Media add 50 ml FBS + 5 ml of 200 mM L - Glutamine

Liquid nitrogen storage media a freezing media for long term storage of CCR3 expressing stable cell line developed in the laboratory:

45 % - DMEM Media

45 % - Conditioning Media (media decanted from earlier generation of HEK culture)

10 % - sterile DMSO

The above freezing media is used to store the stable cell line expressing CCR3 in T-REx HEK 293 cells developed in the lab. The freezing media is mix thoroughly and chilled before using the cell for long term freezing. 1.5 ml of the cells suspended in freezer vials are let to stand for 30 minutes to cool it down. The HEK 293 cell pellet is mix in freezing media. Five million to 10 million cells were stored in each vial for long term storage.

The freezer vials were first store at -20 C for 2 hours later transfer to -80 C freezer for overnight night storage. Finally transferred into liquid nitrogen vapor canister in nitrogen vapors for long-term storage. The working concentrations of zeocin antibiotic stocks for (antibiotic for pcDNA4/TO CCR3 plasmid).10 mg/ml of Zeocin stock is used in preparing the stock solutions.

1	1000 ug/ml
2	750 ug /ml
3	500 ug/ml
4	250 ug/ml
5	250 ug/ml
6	125 ug/ ml

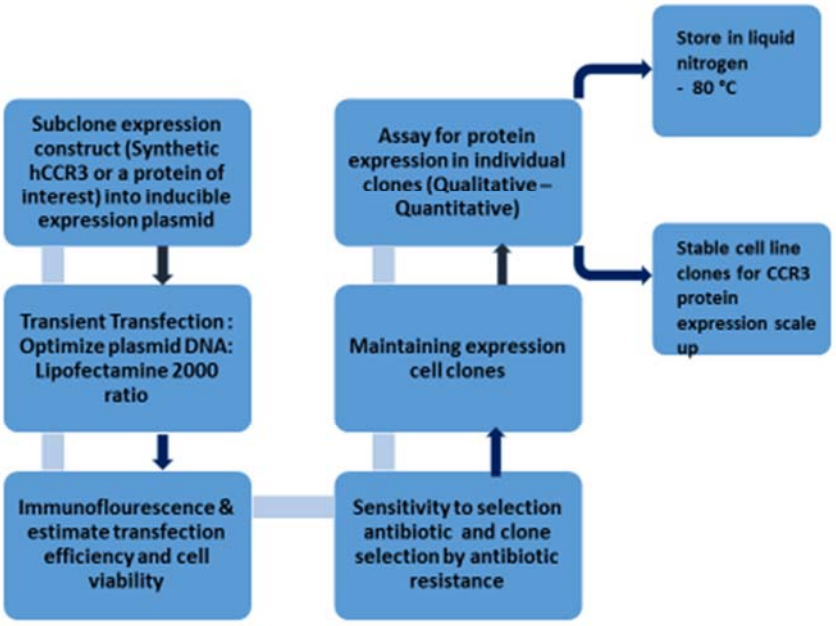


Figure 29. Human CCR3 expressing HEK293 stable cell line process development.

4.14 Conclusions and Future Work

We have successfully developed a mammalian HEK 293 stable cell line expression system to produce CCR3 with tetracycline induction. At individual

experimental stages, we validated the expression of CCR3 by confocal immunofluorescence microscopy, immunoblotting, and preliminary mass spectrometry to confirm the expression of CCR3. Zeocin antibiotic sensitivity for untransfected cells isobserved at 250 micrograms/ml, and the stable cell line growth was carried out at 30 ug/ml. With final conclusions, tetracycline based stable expression system was successfully developed to express human CCR3 in a robust manner by repeated batches of CCR3 expressions. This HEK 293 CCR3 expression cell line can be used in scale-up experiment for semi-preparative and preparative scale purification of CCR3 for any molecular biology, structural biology, and related biophysical studies. The stable cell line may also be used for several functional studies associated with the receptor and for downstream signaling events.

4.15 References

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CHAPTER V

HUMAN CCR3 PURIFICATION AND CHARACTERIZATION

5.1 HEK 293 Culture Scale-Up for Semi-preparative Scale Protein Expression: Experimental Methods

HEK 293 TetR stable clones developed were taken from liquid nitrogen canister for culture expansion and scale up with DMEM supplemented with 10 % FBS and the respective selection antibiotics. After the culture attainsconfluency of 70 to 80 %, CCR3 membrane protein induction was carried out with 2ug/ml tetracycline and 50 mM sodium butyrate for 36 hours. Sodium butyrate, a histone decyclase inhibitor is reported to enhance tetracycline-mediated expression of GPCRs [1,2]. A stock concentration of 1 mg/ml tetracycline is prepared by dissolving in 70 % ethanol and sterile filtered with a 0.22-micron membrane filter. Similarly for sodium butyrate stocks were made as well. Freshly prepared cell culture constituents are described below. Autoclave sterile DI water is used making stock solutions, and the final solutions were sterile filtered.

Table 4. DMEM with 10 % FBS Culture Media used in CCR3 Expression.

HEK 293 Stable cell line culture media
DMEM with low glucose constitute 10 % FBS, 5 ug/ml blasticidin, 10 mg/ml zeocin antibiotic stock (30 ug/ml media concentration) 1 mg/ml tetracycline (2 ug/ml media concentration) 500 mM sodium butyrate stock solution, 50 mM as the final concentration in DMEM cell culture media

Linear concentrations of tetracycline solutions was used in DMEM culture media for CCR3 protein induction. An optimal concentration was chosen to avoid excessive cell death after addition of tetracycline to the culture media. Beyond the maximal induction time of 36 hours, significant decline in cell viability is noticed, dead cells start to appear to detach from the flask and float in DMEM culture media.CCR3 protein induction was carried out for 36 hours at 37 Celsius, 5 % CO_2 in a water-jacketed incubator (Thermo Scientific). Later the cells were washed twice with chilled 4 C 1X sterile PBS twice gently without dislodging the adherent cells. The cells were harvested with a cell scraper in 1X PBS and spun at 8000 g for 15 minutes at 4 C. The supernatant PBS was discarded gently, HEK 293 CCR3 cell pelletwas stored at - 80 celsius for later steps of affinity purification.

During tetracycline induction of CCR3 in the cell culture with tetracycline some extent of HEK 293 cell death was observed. After repeated batches of CCR3 expression, beyond thirty-six hours cell death was rapid. On a caution note, HEK 293 cell are harvested at thirty-six hours after tetracycline induction to prevent excess loss of CCR3 induced HEK 293 cells at harvest.

5.2 HEK 293 Cell Lysis and Membrane Protein Solubilization

N-Dodecyl-β-D-maltoside (DDM) is one of the most widely used nonionic mild solubilizing agent or detergent for membrane protein solubilization such as diverse GPCRs and mammalian cell membrane ion channel protein [3,4,5].DDM is a nonionic surfactant with a low critical micellar concentration, widely used in the last decade in various applications such as biophysical and structural studies, protein sequencing, and hydrogen-deuterium (HDX) exchange mass spectrometry methods HDX mass spectrometry is structural mass spectrometry method used to study solvent accessible regions of protein. One of the significantadvantages of DDM is its wider application in the massspectrometry-based application; characterizing membrane protein studies. Unlike most other detergents, DDM do not interfere with the ionization efficiency of the peptide in the ion source of the mass spectrometer, nor contribute to any signaling suppression in nanoflow LC-MS platforms of peptide mass spectrometry [4]. Further, if a trace amount of DDM remains bound to reverse phase C18 LC column, it elutes at a very high organic phase. Almost all peptides bound to the C18 column have eluted be this point; most peptides elute from 20 % organic mobile phase composition and up to 70 % organic in a reverse phase nanoflow HPLC gradient.

5.3 Critical Micellar Concentration (CMC): Role in Membrane Protein Solubilization

CMC is defined as the minimum concentration at which the surfactant molecules start to form stableself-assembly structures termed micelle. CMC of a detergent is a critical factor to be chosen rationally for extraction and solubilizing a membrane protein [6]. In most cases, two to five times the CMC of a particular surfactant is adequate to solubilize membrane protein with gentle cell pellet disruption. Efficient extraction of membrane protein fraction is feasible at these CMC without affecting downstream purification incompatibility in LC-MS based protein chemistry and analytical studies. Further in a reverse phase LC - MS/MS protein digest sample run, DDM comes out very late in the gradient at high organic; most of the digested peptide ions, even the hydrophobic peptides have eluted by that point [4]. On the contrary, with ionic surfactants like SDS, CHAPS, and Triton X-100, significant MS signal suppression will be observed [7]. These ionic surfactants form adduct ions or elute as positively charges molecular ions at low acidic pH of the reverse phase HPLC mobile phases leading to signal suppression of the peptides, the analytes intended for MS based characterization.

5.4 Immobilized Metal Affinity Chromatography. Ni^{2+} (II)NTA Resin Affinity Purification of CCR3

The expressed CCR3 receptor has a fused deca histidine (10X) peptide tag present in the intracellular C-terminus domain. This 10X poly-histidine affinity tag facilitates CCR3 purification with the help of Ni-NTA resin column. The chemokine receptor CCR3 has disulfide (-S-S-) bonds between two transmembrane domains. For IMAC

purification of recombinant proteins with Ni-NTA resin, the polyhistidine epitope do not require any particular conformation, this facilitated purification even under a denaturing condition such as 6M guanidine or 8M urea. 8M urea is preferred as it is compatible with SDS PAGE analysis; guanidine precipitated during sample preparation in the presence of SDS [8,9]. With repeated batch purification of CCR3, the 10X poly-histidine affinity tag is tightly bound and not accessible to facilitate affinity based CCR3 10X polyhistidine purification. When immunoblotting is carried out on CCR3 expressed cell lysate in two conditions, non-reduced supernatant and reduced supernatant with 20 mM beta-mercaptoethanol (BME is a reducing agent of disulfide bond linkages in protein and unfolds the protein) and probed with the anti-Histag primary antibody the immunoblot ECL signal intensity of non-reduced is significantly lower than the intensity of the reduced sample.

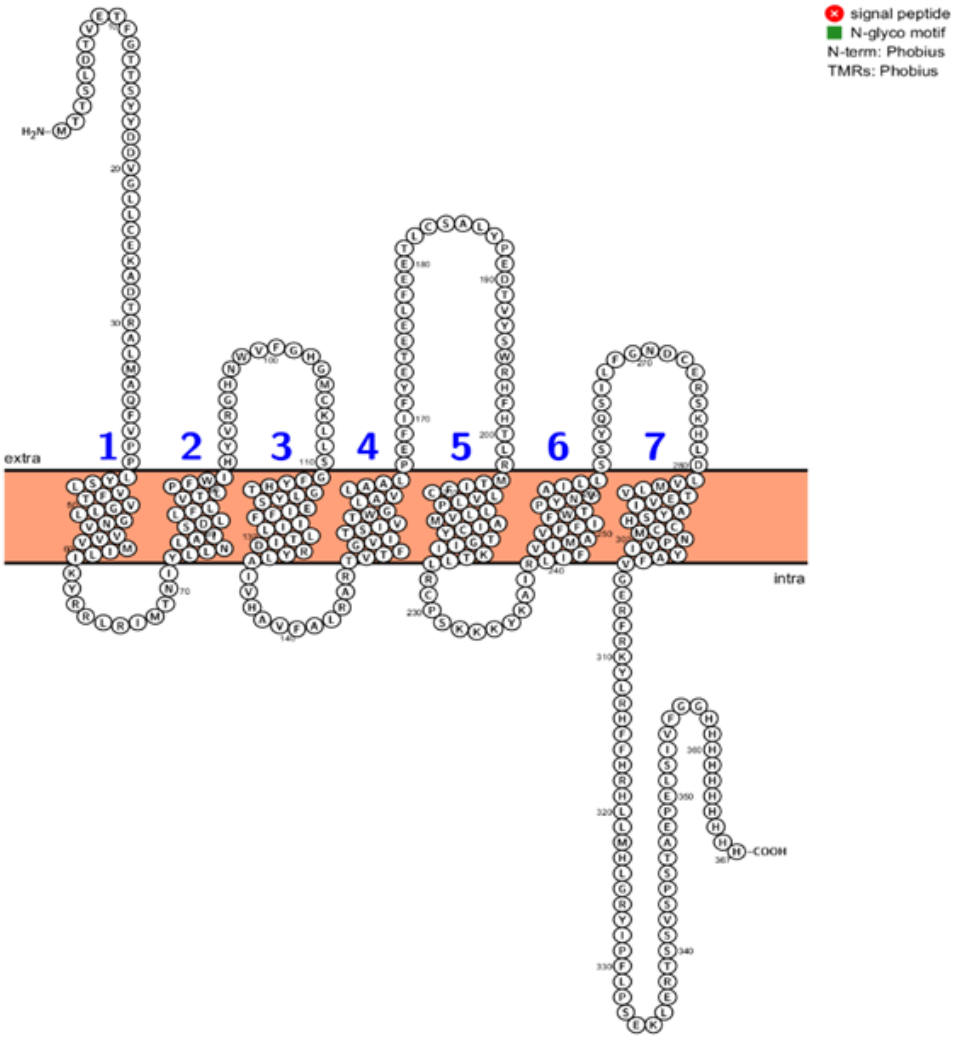


Figure 30. Human CCR3 Extracellular N-terminus Sequence, the 7 TM Domains and Intracellular with a SUMO –Glycine-Glycine-10X Polyhistidine Affinity Tag. Image developed in Protter, an open source software to visualize Proteoforms. Reference. Omasits et al., Bioinformatics. 2013 Nov 21.

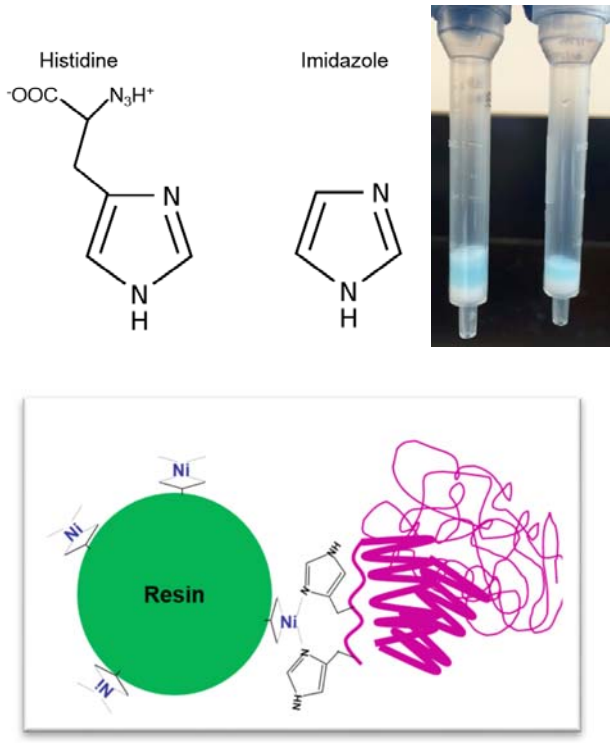


Figure 31. Polyhistidine-Tagged Protein binding to Ni NTA Resin.

Histidine and Imidazole similar affinity for Ni^{2+} immobilized on to nitriloacetic acid (Ni^{2+} NTA) resin. At higher concentration of imidazole, the 10X Histidine tag affinity bound CCR3is selectively eluted from the Ni^{2+} NTA resin.

Note: Ten histidine amino acid tag (deca histidine tag peptide affinity tag) has a high binding affinity with nickel Ni^{2+}. The recommendation concentration is 250 mM Imidazole for most poly-histidine tagged proteins. Repeated attempts were unsucessful, by increasing the imidazole concentration to 300 mM, the target CCR3-10X polyhistidine affinity tag membrane receptor was finally purified.

B. Self packed Ni-NTA resin in 250 microliters and 500 microliters in a 2 ml gravity flow plastic column shell.

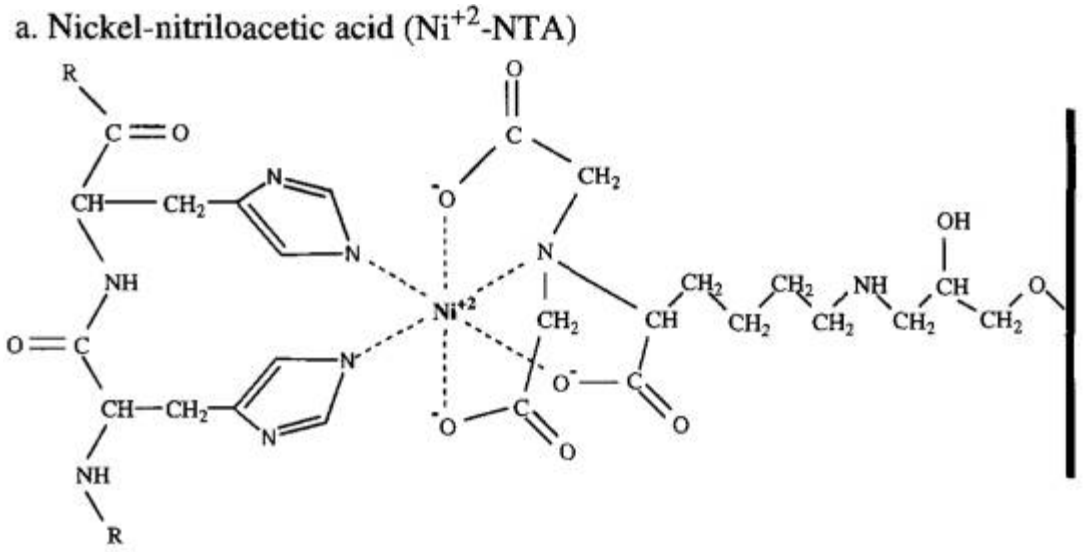


Figure 32. Mechanism of Histidine Tagged Protein Binding to Ni^{2+} Atoms Immobilized on the Resin. The Electron Donor of the Heterocyclic Imidazole ring forms co-ordinates bonding with Ni^{2+} ion Immobilized onto Resin. Image reference [32].

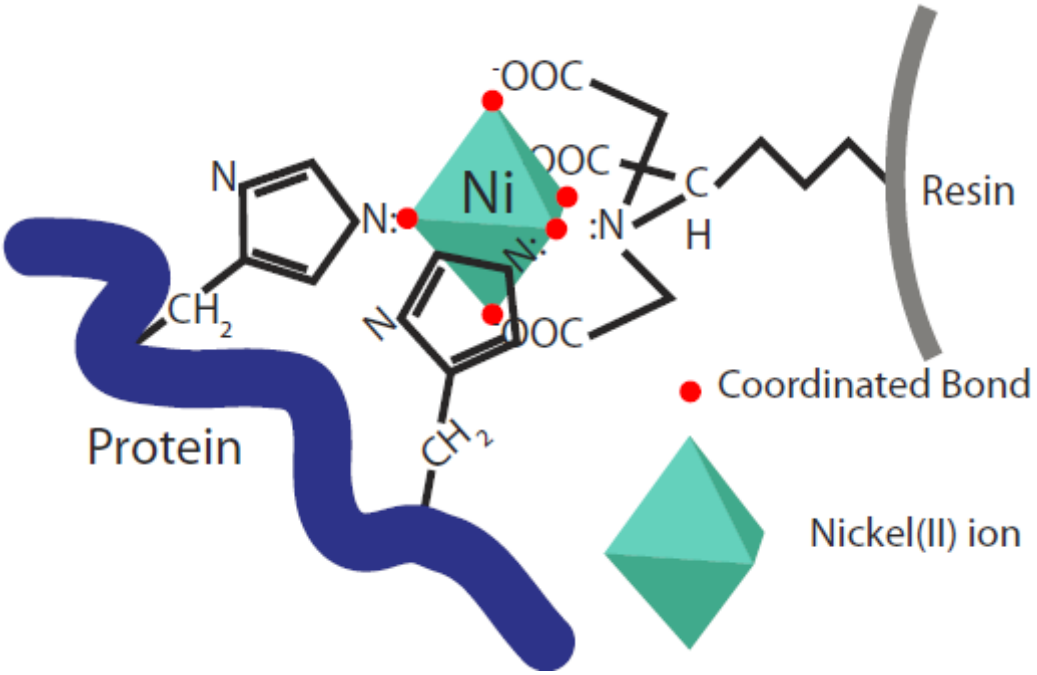


Figure 33. Histidine Amino Repeats Binding to Ni $^{2+}$(II) via Co-ordinate Bonding, Nickel is Immobilizedonto Resin. Reference: Sigma-Aldrich, Epitope tags in protein research reference manual.

5.5 HEK 293 Lysate Supernatant Reduction: C-Terminus 10X Polyhistidine Tag Accessibility of CCR3 Purification

Note. Non-Reduced HEK lysate was used to purify with Ni-NTA and eluted with 250 mM Imidazole followed by another 300 mM Imidazole Elution. The Western blot clearly validates the affinity purification was not working, due to inaccessibility of the 10X His Tag to bind to the Ni-NTA resin.

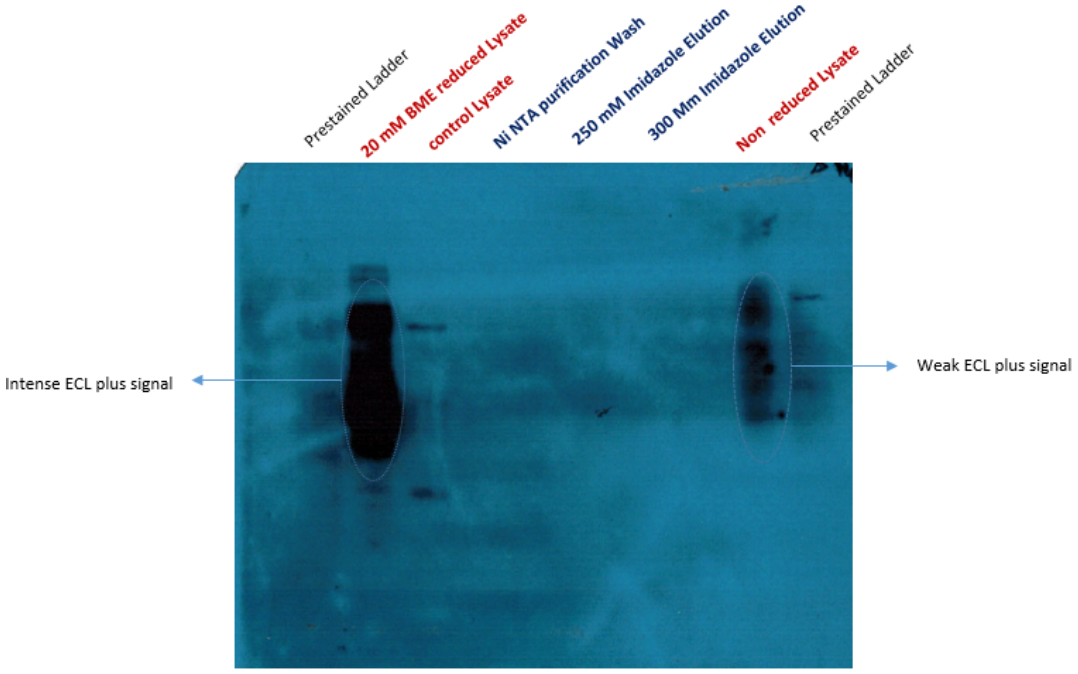


Figure 34. Immunoblot of 20 Mm Beta-Mercaptoethanol Reduced and Non-Reduced HEK 293 Cell Lysate Supernatant.

In efforts to unfold CCR3 and improve accessibility of 10X His Tag of CCR3, 20 mM BME is used in reducing the cell lysate at room temperature for 1hour. Non-reduced and BME reduced HEK 293 lysate supernatant samples and all the non-reduced Ni-NTA affinity purified samples are separated on a 4 -12 % NuPAGE gel and probed with Anti 6X His primary tag antibody in 1.2000 dilution. Horseradish peroxidase (HRP) secondary antibody in 1.5000 dilution. Incubated for 5 minutes in ECL plus substrate and the blot-x-ray film is developed for 15 minutes.

Human CCR3-Glycine-Glycine-10X Poly Histidine tag Amino Acid Sequence. -Gly-Gly- is a SUMO cleavage site:

>spCCR3_HUMAN C-C chemokine receptor type 3 GG 10X Histag OS=Homo sapiens GN=CCR3 PE=1 SV=1

MTTSLDTVETFGTTSYYDDVGLLCEKADTRALMAQFVPPLYSLVFTVGLLGNVV
VVMILIKYRRLRIMTNIYLLNLAISDLLFLVTLPFWIHYVRGHNWVFGHGMCKLL
SGFYHTGLYSEIFFIILLTIDRYLAIVHAVFALRARTVTFGVITSIVTWGLAVLAAL
PEFIFYETEELFEETLCSALYPEDTVYSWRHFHTLRMTIFCLVLPLLVMAICYTGII
KTLLRCPSKKKYKAIRLIFVIMAVFFIFWTPYNVAILLSSYQSILFGNDCERSKHLD
LVMLVTEVIAYSHCCMNPVIYAFVGERFRKYLRHFFHRHLLMHLGRYIPFLPSEK
LERTSSVSPSTAEPELSIVFGGHHHHHHHHHH

5.6 Membrane Protein Base Buffer Preparation and Composition

Initially HEK 293 cell lysis and membrane protein solubilization was carried out with 1X PBS based buffer with 1 % DDM to disrupt and solubilize the cell pellet and extract the membrane protein fraction. On visual observed, the cell pellet is not completely solubilized. Following literature reviews and planned experimentation, CCR3 membrane protein solubilizing buffer composition is optimized for efficient disruption of the cell pellet.

The solubilizing base buffer is meticulously incorporated to stabilize the membrane proteins during solubilization process. Stevens et. al. [10] worked out an optimal GPCR membrane protein solubilizing base buffer with the final step of incorporating the required surfactant, DDM. This base buffer composition is reportedwidely by several group working on GPCR solubilization and structural - function related biophysical works.

5.7 CCR3 Expressed HEK 293 Cell Pellet Lysis and Solubilization

Buffer composition with 1X protease inhibition cocktail used for cell lysis and membrane protein extraction:

Base buffer with DDM and CHS (1.5 % DDM and 0.05 % CHS)

50 mM Tris.HCl

2 mMCalcium Chloride ($CaCL_2$)

5 mM Potassium Chloride (KCL)

5 mM Magnesium Chloride (MgCL2)

4 mM EDTA

10 % Glycerol

1.5 % DDM(n-Dodecyl-β-D-maltoside)

0.25 % CHS (Cholestryl Hemi Succinate)

pH 7.4.

Ultrapure DDM $C_{24}H_{46}O_{11}$(Arcos Chemicals # 329370010) with 98.5% high pure was used in preparing the lysis base buffer. A highly pure form of detergent is imperative, any trace amount of metal ions can bind and denature-precipitate protein out of the solubilizing buffer. Dodecyl β - D - maltopyranoside (DDM), $C_{24}H_{46}O_{11}$ Critical Micellar Concentration (CMC) of DDM is 0.16 mM.

Cholesteryl hemisuccinate (Anatrace catalog # 50-109-0966) is a cholesterol analog, aids in stabilizing mammalian membrane protein stabilization in functional state during cell lysis and downstream solubilization.

Approximately 100 mg of HEK 293 cell pellet is obtained from ten T-25 culture flasks; this cell pellet is used in affinity-based purification of the CCR3 receptor. The cell pellets were thawed on ice, to facilitate mechanical disruption by low power output hand held mechanical homogenizer.

The above prepared lysis buffer is added in a dropwise manner with simultaneous gently - intermittent vortexing until most of the cell pellet is re-suspended. Once the cell pellet is re-suspended, micro homogenization is performed with WITMAN handheld micro homogenizer. Short strokes of five seconds each for three times were carried out to resuspend the pellet completely. There are literature reports of GPCR having a tendency to aggregate even at slightly elevated temperatures, as discussed in earlier chapters. Once the protein molecule starts aggregating, they tend to crash out, render insoluble state of the homogeneous aqueous lysate buffer. Micro homogenization was carried out on ice at 4 C to prevent CCR3 denaturation and aggregation.

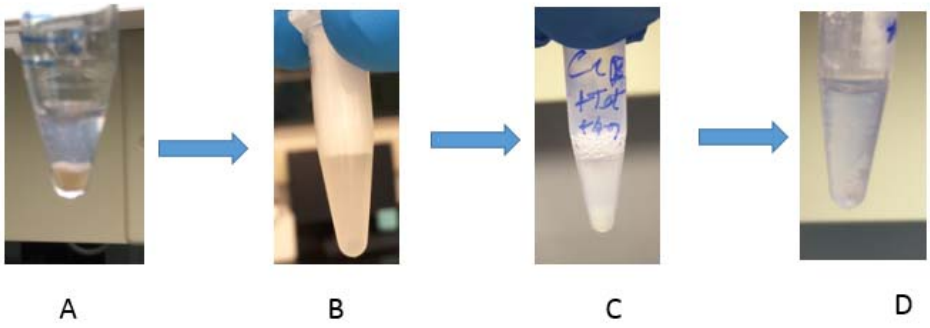


Figure 35. HEK 293 Cell Pellets Solubilizing Process for CCR3 Purification.

A - Harvested cell pellet in lysis buffer

B - Homogenized cell pellet

C - Solubilized cell pellet with cell lysate debris

D - 20,000 spinned cell lysate supernatant with sediment cell debris

Completely solubilized cell pellet was spinned at 15,000 g for 30 minutes at 4 Celsius, the clear supernatant is the membrane fraction having CCR3 - 10X Histidine tags, this clarified supernatant was used for IMAC Ni^{2+} NTA affinity purification of CCR3 receptor.

Removal of cell debris is carried out by spinning the lysate at 15000 g for 30 minutes at 4 C to clarify the lysate. The supernatant is collect and proceeded for affinity purification by Ni $^{2+}$NTA resin purification step.

To prevent any possibility of protein aggregation on storage, the cell lysate supernatant is taken to the next step of Ni $^{2+}$NTA purification, the same day of lysis and membrane solubilization.

5.8 Immobilized Metal Affinity Chromatography. Ni $^{2+}$NTA Resin Based Affinity Purification of CCR3

Ni $^{2+}$NTA purification is carried out based on selective binding affinity of CCR3 - C terminus 10X histidine tag to the Ni $^{2+}$NTA agarose resin. Rigorous washing with a low concentration of 25 mM Imidazole buffer and a final wash with 300 mM NaCl to remove any traces of nonspecifically bound proteins on the column. Finally, sequential elution of CCR3 with 300 mM imidazole.

All the Ni $^{2+}$NTA buffers were prepared freshly with 0.05 % DDM (critical micellar concentration of DDM is 0.17 mM). The Ni NTA resin is equilibrated with 0.01

% DDM before the purification steps were started. DDM is incorporated in all the Ni-affinity purification buffers at 0.01 % to maintain the CCR3 micelle and preclude any membrane protein adsorption in the resin or precipitation during the purificationprocess.Further, to avoid any changes in solvent strength that might lead to disruption of CCR3 micelle.

5.9 CCR3 IMAC Purification under Native and Denaturation Conditions

CCR3 has a disulfide bond between cysteine residues C106 - C183 of two transmembrane domains and tightly bound protein can hinder free accessibility of 10X polyhistidine tag to the Ni^{2+}NTA; Observed with immunoblotting experiment with reduced and non-reduced CCR3 expressed HEK 293 cell lysate supernatant, western blot in figure 3. Finally the cell lysate with reducing agent with 20 mM BME a disulfide bond breakers.

Ni $^{2+}$NTA resin is conjugated to agarose beads, Ni^{2+}NTA resin can tolerate beta-mercaptoethanol, a reducing agent. So it was chosen at low concentration to reduce the CCR3 lysate supernatant. 20 mM final BME concentration of the lysate with intermittent vortexing and kept on ice for one hour. Later the lysate is loaded in the Ni $^{2+}$NTA. The cell lysate supernatant mixed Ni-NTA resin slurry at 4 Celsius for 4 hours.

The affinity purification process is done under native and denaturation conditions, 6M Guanidine. Denaturation facility increased accessibility of the 10X polyhistidine tag and also disrupt any aggregates of CCR3 protein.

Table 5. Buffers used in Ni-NTA Based IMAC Purification.

Buffer used in Purification	**Composition**
Equilibration buffer	20mM Sod. Phosphate, 300 mM sodium chloride, 10mM Imidazole,0.05 % DDM, 10 % Glycerol **pH 7.4**
Wash buffer	PBS with 25 mM Imidazole, 0.05 % DDM, 10 % Glycerol, **pH 7.4,**
Elution Buffer	Base buffer, 300 mM Imidazole, base buffer with 500 mM imidazole,10 % Glycerol. 0.05 % DDM
Column regeneration buffer	20mM MES salt, 0.1 M (100 mM) NaCl , **pH 5.0**

Note: All the buffers used in Ni $^{2+}$ NTA purification are incorporated with 0.05 % DDM as the final concentration. To prevent disruption of membrane CCR3 protein micelle.

Steps in IMAC Ni $^{2+}$ NTA Affinity Purification of Human CCR3

All the purification steps were carried at 4-degreeCelsius

1. Thermo Fisher brand gravity flow and centrifugal spin column, 2 ml volume is used to pack 600 uL of Ni-NTA resin. The storage buffer was removed and spinned at 700 g for two minutes to remove storage into a retain centrifuge tube holder.
2. Two resin bed volumes of equilibrating buffer is let to seep through bed, gently shaken to pass through the resin, later centrifuged at 700 g for 2 minutes to remove the equilibration buffer

3. Ni-NTA spin column is prepared for protein binding; bottom plug is put back. BME reduced CCR3 lysate extract supernatant is added with gently mixing with a pipetting. The columns were put on to rotator shaker for 4 hours in cold room, at 4 C. In this step CCR3 binding Ni-NTA resin happens
4. The bottom cap of resin column was removed and spin at 700 g for 2 minutes to collect flow through in centrifuge tube.
5. The column is washed with two bed volumes of wash buffer followed by centrifuge at 700 g for 2 minutes. Additional and final wash was performed with 300 mM sodium chloride to remove any non-specific binding of proteins onto the Ni-NTA resin. During the washing step, all the unbound proteins will wash off the Ni^{2+} NTA column.
6. The bound 10X his tag CCR3 proteins was eluted with 300 mM imidazole elution buffer. One resin bed volume of elution buffer was added to resin and centrifuge at 700 g for 2 minutes. Three sequential elution of bound 10X polyhistidine tagged CCR3 were collected as separate fractions in centrifuge tubes, labeled as E1, E2 and E3. Final elution was carried out with 500 mM Imidazole with 500 uL and labeled E4. Imidazole in the elution buffer increases pH up to 9 -10. pH was adjusted to be at physiological 7.4 pH for further SDS PAGE analysis of the E1, E2, E3 and E4 elutions.
7. Finally, the IMAC Ni-NTA columns were regenerated with 20 mM MES regeneration and stored in 20 % methanol for further use.

Ni-NTA Purification of CCR3 under Denaturing Conditions: CCR3 10X Histag purification is also carried under eight molar Urea denaturing conditions. The overall purification steps are same as the non-denaturing method of purification and a molar strength of 8M Urea. Urea is a chaotropic agent that facilitates opening of the protein and accessibility of the 10X polyhistidine tag to the Ni-NTA resin.

Ni-NTA Column Regeneration: After elution of the protein, Ni-NTA column was regenerated with MES salt regeneration buffer, followed by ultrapure DI water wash and stored in 20 % ethanol at 4 C for further use.

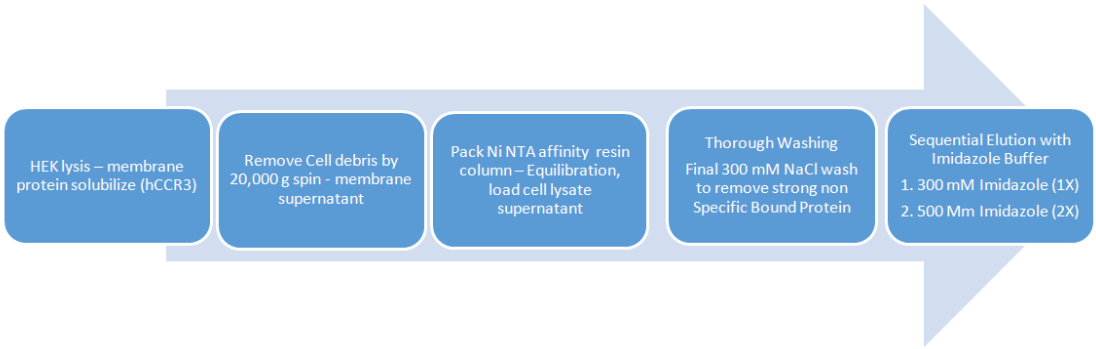


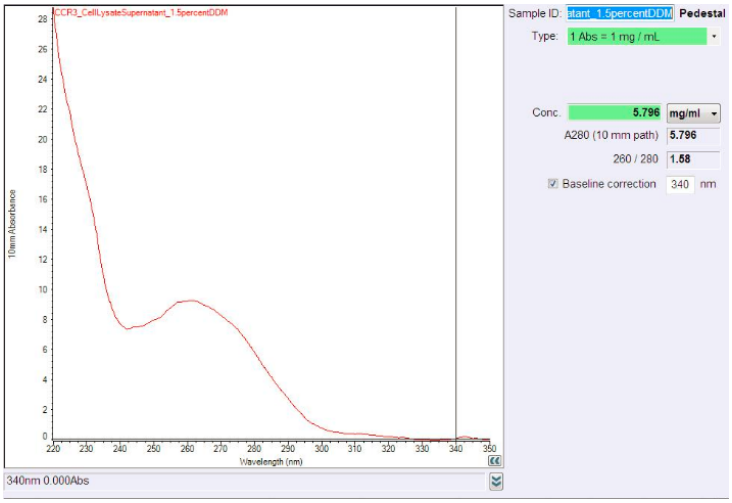
Figure 36. CCR3 Purification Method Optimization with Changing Buffer Composition.

5.10 Absorbance A280 Protein Concentration Determination

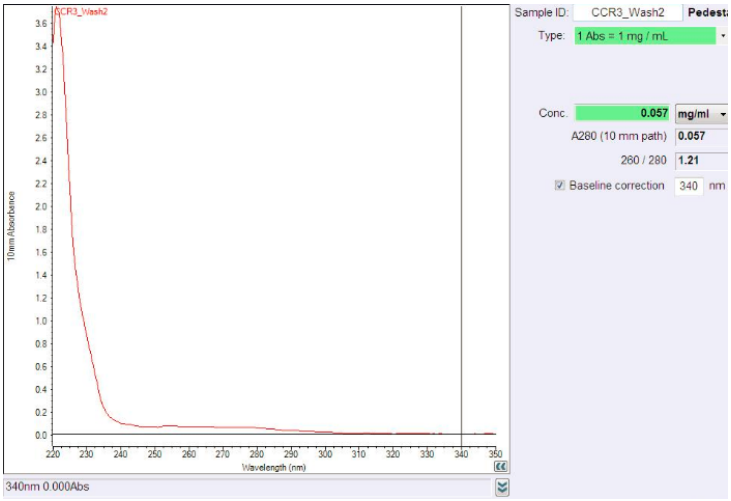
Absorbance A280 spectrophotometric method is a colorimetric method widely to estimate total protein concentration of soluble protein in solution [11, 12]. Absorbance maxima for aromatic amino acids such as tryptophan and tyrosine is at 280 nm without any interference absorbance from the cell lysate media. Each purification elution was diluted three-fold with 1X PBS and was estimated with Nanodrop UV 2000 spectrometer, UV measurement is carried out at A280 [13]. Blank reading is first taken with the

spectrometer before the sample reading were taken analysis. Approximately 0.9 ug/uL of CCR3 protein was estimated in the purified and pooled elution fractions.

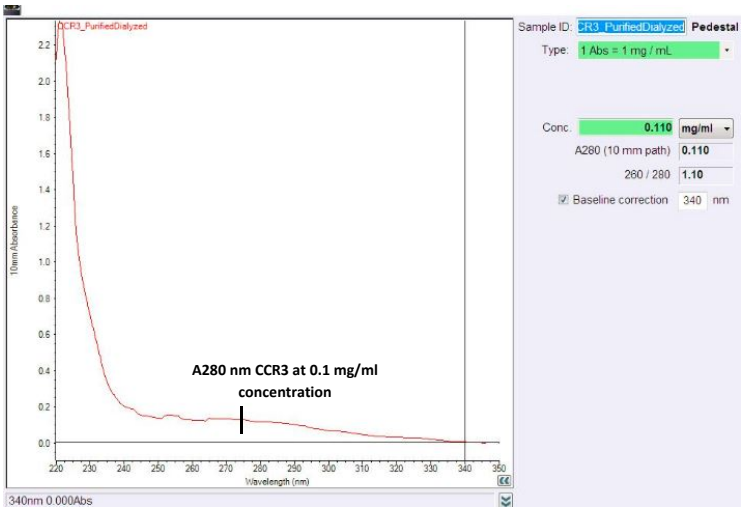
The Nanodrop UV spectrophotometer is blanked with three-fold diluted of 500 mM Imidazole elution buffer before protein concentration estimation was carried out with Nanodrop UV spectrometer.



A - Lysate Supernatant, A280 nm of 5.79 mg/ml.



B-Final Wash 2, A280 nm of 0.057 mg/ml.



C-Purified and Dialyzed CCR3, A280 at0.1 mg/ml Concentration.

Figure 37. A,B and C A280 Measurements of Purified Human CCR3 by IMAC Ni NTA Affinity Chromatography.

Note: The presence of Imidazole in the purification elution can lead to protein aggregation (Hefti et al. 2001) so buffer exchange and dialysis methods were carried out to remove excess imidazole as soon Ni NTA purification process is completed.

5.11 Purified CCR3 Protein Desalting - Buffer Exchange

Buffer exchange is a process of removing salts and low molecular weight species from protein and DNA samples. Here a 42 kDa protein, CCR3 in 300 mM Imidazole buffer is buffer exchanged to remove Imidazole [14]. Buffer exchange process was carried out with basebuffer having 0.05 % DDM mentioned earlier. 0.05 % DDM is incorporated in all the buffers to prevent the dissociation of CCR3 membrane protein micelle. Buffer exchange was carried out with Vivaspin 5000 kDa molecular weight cut-offfilter at 4 C by spinning at 12000 g for 15 minutes each, three successive exchanges to remove most of the imidazole and other salts from the sample. Finally, the concentrated CCR3 purified protein

was stored at -80 C for further mass spectrometry based sequencing efforts. The purified protein tends to precipitate out of buffer during the buffer exchange process at 4 C, visual observation have shown fine particles.

Acetone Precipitation of Purified CCR3 Receptor: Cold acetone precipitation is a comparatively easy process to precipitate protein out of solution and solubilize back in a protein denaturing or similar buffer [15,16]. -20 Celsius cold acetone precipitation of imidazole elution was carried out to remove salts and detergent from the purified receptor and to re-suspend the CCR3 protein precipitate in 8M urea denaturing buffer.

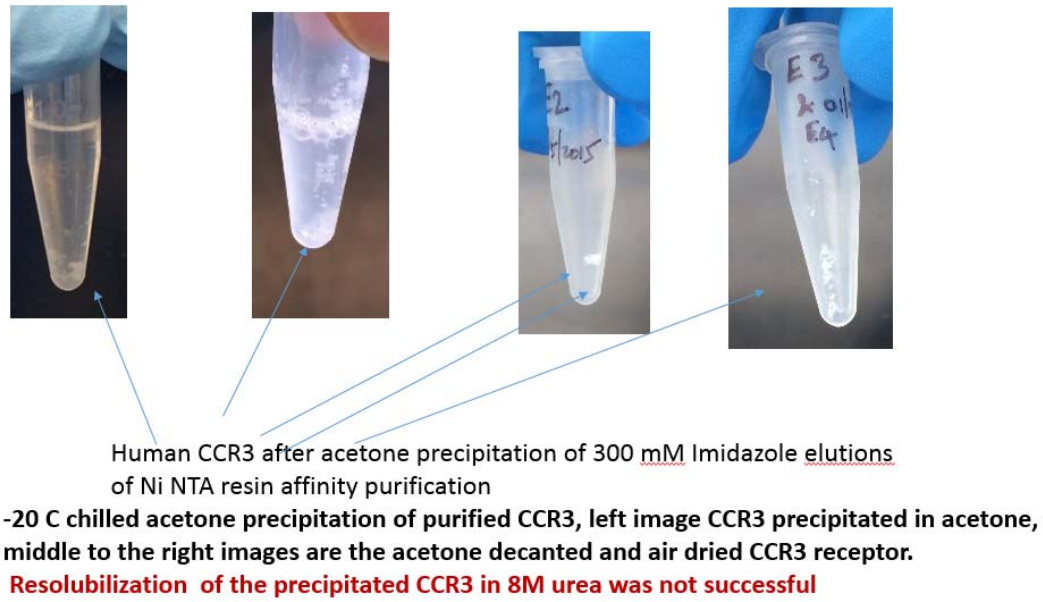


Figure 38. - 20 C Acetone Precipitated Purified CCR3 Receptor.

5.12 Dialysis of Purified CCR3 to Remove Imidazole

Dialysis is one of the milder methods to eliminate excess salts from purified protein samples, it is an osmotically driven process where molecule diffused based osmotic potential gradient across a semi-permeable membrane [17]. Several membrane proteins such as bacterial rhodopsin was cleaned by dialysis process [18]. One of the main disadvantages of dialysis based methods is, the protein may stick to the dialysis membrane and leads to low recovery of the protein sample after dialysis. Especially for low concentration protein samples, regular dialysis is not a strategic option. The loss mentioned above can be minimized by using a dialysis cartridge with a smaller dialysis membrane. A 500 uL volume 10 kDa molecular weight cut off (MWCO) Slide-A-Lyzer cartridge was used in dialyzing the protein (Thermo Scientific Slide-A-Lyzer catalog # 66383), Imidazole concentration of 300 to 500 mM and 300 mM NaCl is removed from sample elutions. Sequential two-step dialysis was carried out with the dialysis buffer in cold room for three hours each. CCR3 solubilizing buffer, mentioned in earlier sections with 0.05 % DDM concentration is used as the dialysis buffer.

After the dialysis procedure, the purified CCR3 and the dialyzed protein sample was taken for SDS PAGE analysis for protein recovery and further analytical methods such as A280 and enzymatic digestion for mass spectrometry determination.

5.13 CCR3 Receptor Characterization by SDS PAGE and Western Blotting

A small aliquot of the above purified CCR3 protein samples, the Ni NTA purified elutions, E1, E2 and E3 and the dialyzed sample were used in gel electrophoresis with a 4-12 % Bis-Tris Gradient gel NuPAGE electrophoresis. Initially, the gel was stained with

Thermo Scientific gel code blue, the protein band in the purified samples were very faint. Later the samples were analyzed and stained with Thermo Scientific mass spectrometry compatible silver staining. After imaging the gel with Biorad Quality One Imager. The SDS PAGE gel is saved for band excision for destaining followed by in-gel digestion methods for mass spectrometry based analysis.

SDS PAGE Protein Electrophoresis: CCR3 expressed HEK 293 cell lysate supernatant, all of the purified samples, wash 3, Imidazole elutions 1, 2, 3 and dialyzed samples are used for SDS PAGE analysis.

Approximately five micrograms of each protein were taken for SDS PAGE analysis. All the samples were reduced with 50 mM DTT at room temperature for 45 minutes through mixing in eppendorf thermomixer. Later the samples were mixed with 4X LDS buffer and deionized water to the respective proportions to a final concentration 1X LDS buffer.2 uL of Invitrogen Sea blue plus 2; the prestained ladder was used as the ladder.

Samples were analyzed with both 1X MOPS buffer and repeated with 1X MES running buffer. Power settings on the power are 200 volts, 30 milliamps for 40 minutes. After electrophoresis the gel was later washed three times with deionized water and fixed in fix solution, 30 % Ethanol, 10 % Acetic acid, 60 % water for 30 minutes, followed by three washed with ultrapure deionized water.

The gel is stained with gel code blue stain for 2 hours followed by destaining overnight with three changes of ultrapure deionized water. For mass spectrometry compatible silver staining procedure, the ethanol-fixed gel was stained with mass

spectrometry sample preparation compatible silver stain reagents and imaged with Bio-Rad Imager. Thermo Scientific Silver Staining Reagents, Catalog # 24612 is used as the silver stain.

Western blotting of purified CCR3: Both denatured and non-denatured sampleswere reduced with 50 mM DTT, these samples are used for Western blot procedure. Approximately twomicrograms of protein samples areutilized for SDS PAGE analysis.

Two Western blots were carried out, for the different epitope, anti-His-Tag primary antibody and other for the anti-CCR3 primary antibody.

Western blot procedure*:* Mildly reduced samples were analyzed on a 4 -12 % NuPAGE gel and described earlier in previous chapters.

Note. Dry transfer of the membrane was carried out with Invitrogen iBlot dry transfer apparatus.

Blocking buffer: The blot was blocked with 5 % BSA in PBST for one hour, followed by three washes three times with PBST for five minutes each. Primary mouse-human CCR3 antibody dilution in 10 % blocking buffer at 1.900 ratio. Incubation overnight at 4 C. The primary antibody solution was decanted, and the blot was washed three times for five minutes each with PBST. HRP conjugated anti-mouse antibody in the dilution range of 1:2500 was used, and incubated at room temperature for one hour and later decanted and finally washed three times with PBST for five minutes. The blot was incubated in ECL plus substrate (Advansta ECL substrate) for five minutes. Finally, the substrate is decanted, blot was dried and developed with X-Ray film.

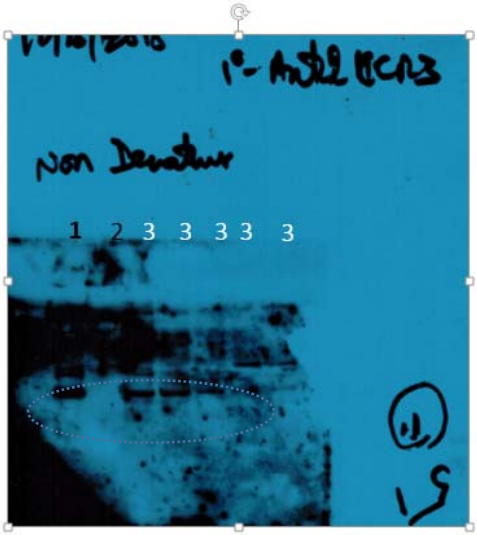


Figure 39. Western Blot of Ni NTA (IMAC) Purified CCR3. Mouse monoclonal anti CCR3 Primary Antibody and HRP secondary antibody with ECL plus development and final Blot imaging by Chemiluminescence.

5.14 Purification Optimization of CCR3 and SDS PAGE with Silver Staining

The purification method development was finally possible under mild reduction of HEK T-REx expressing CCR3 lysate supernatant with 20 mM beta-mercaptoethanol. The base buffer (composition described earlier), is supplemented with 10 % glycerol and 0.1 % DDM is used as the buffer in the preparation of all the Ni NTA buffers and the imidazole elutions concentration to 300 mM and 500 mM.

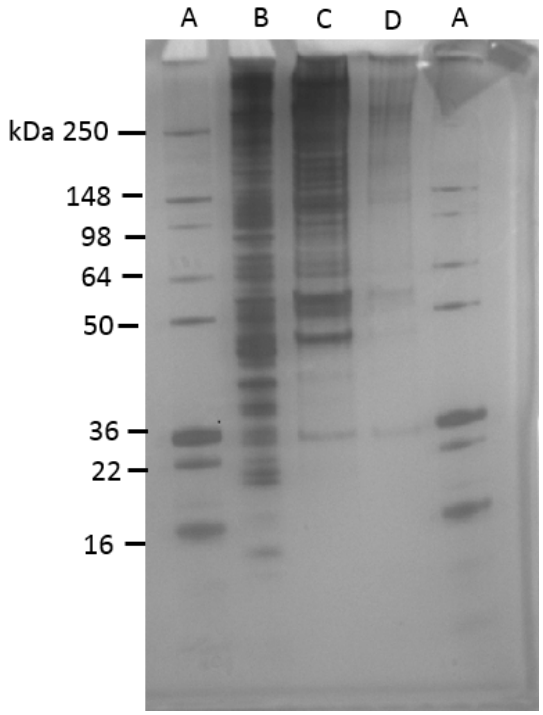


Figure 40. Ni^{2+} NTA Purified CCR3 for SDS PAGE Followed by Silver Stain Analysis; the First Experimental Trial of Ni NTA Affinity Purification of CCR3. A - protein molecular with ladder B - cell lysate supernatant, C - elution1 and elution 2 pooled 300 mM imidazole elution, D - elution 3, 500 mM imidazole elution.

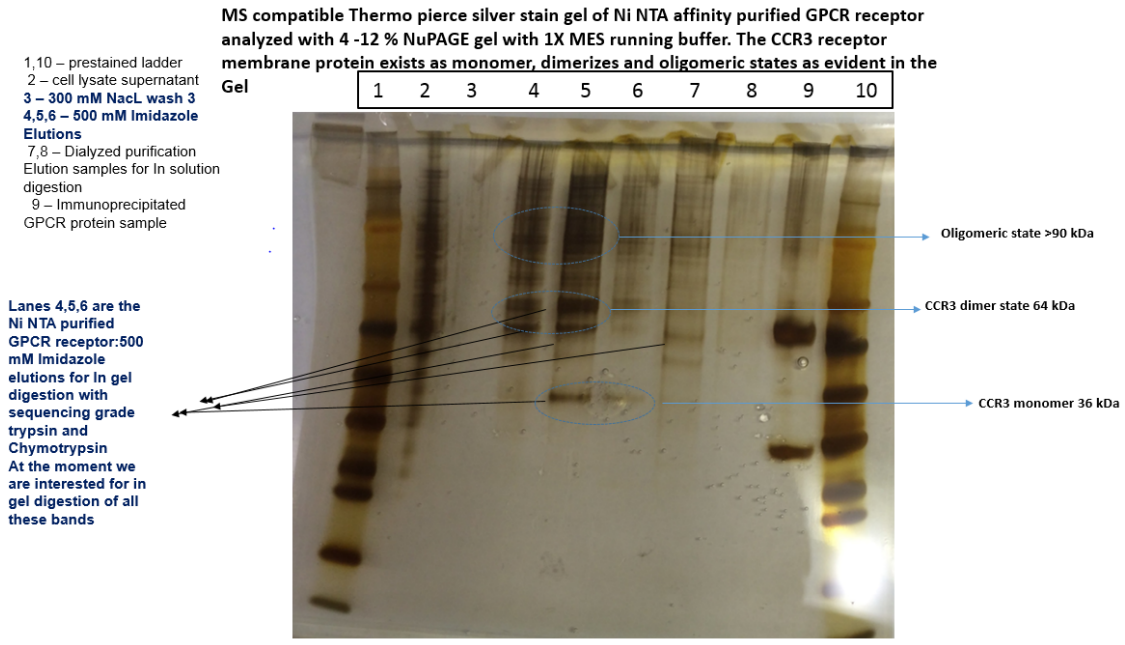
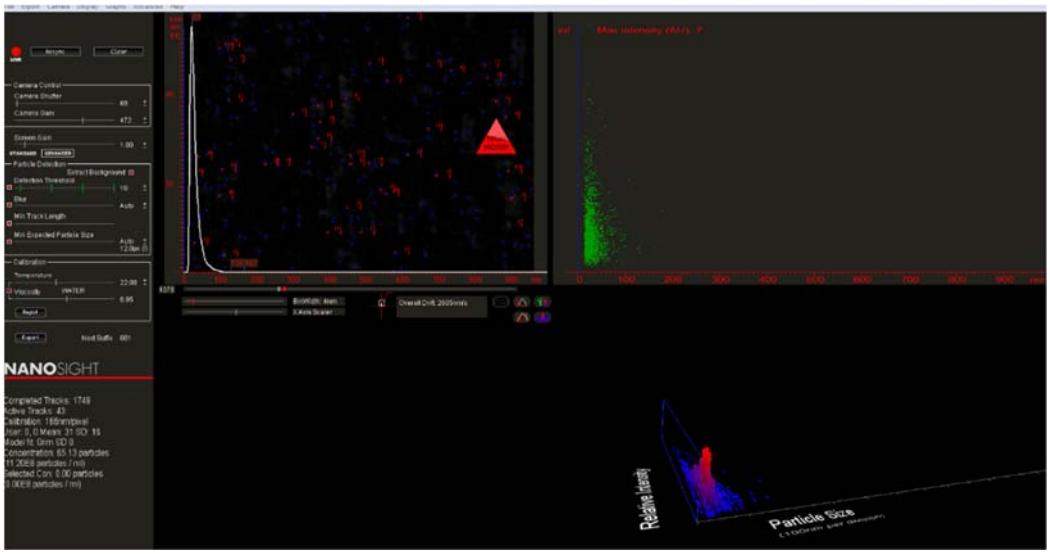


Figure 41. Optimized Ni-NTA Purified CCR3 SDS PAGE Followed by Silver Stain Analysis. Second experimental trial of Ni NTA Affinity Purification of CCR3.

5.15 Dynamic Light Scatter (DLS) Measurements of the Purified CCR3 Receptor

Dynamic light scattering (DLS) is a relative quantitation method to quantitate protein aggregation in solution. DLS may also be termed as photo correlation spectroscopy;this approach works on the principle of molecular translational diffusion coefficient (D_T) of macromolecules during Brownian motion in similar solutions [20, 21]. Nanosight dynamic light scattering instrument was used to assess the CCR3 receptor aggregation levels after IMAC purification with dynamic light scattering experimental protocol. 250 to 300 microliters of the purified sample wasused in the analysis. The blank analysis was carried out first with 300 mM imidazole buffer; later the IMAC eluted sample was used to acquire DLS measurements of particle size distribution. Protein aggregation are observed to an extent with an aggregate particle size of approximately of

80 - 100 nm diameter. Noise is also reported during data acquisition for uncertain reasons. Literature reports of imidazole and its tendency to protein aggregation during affinity purification of poly histidine recombinant proteins.



A. Dynamic Light Scattering, Blank Reading on the Right, and Relative Intensity vs. Particle Size.

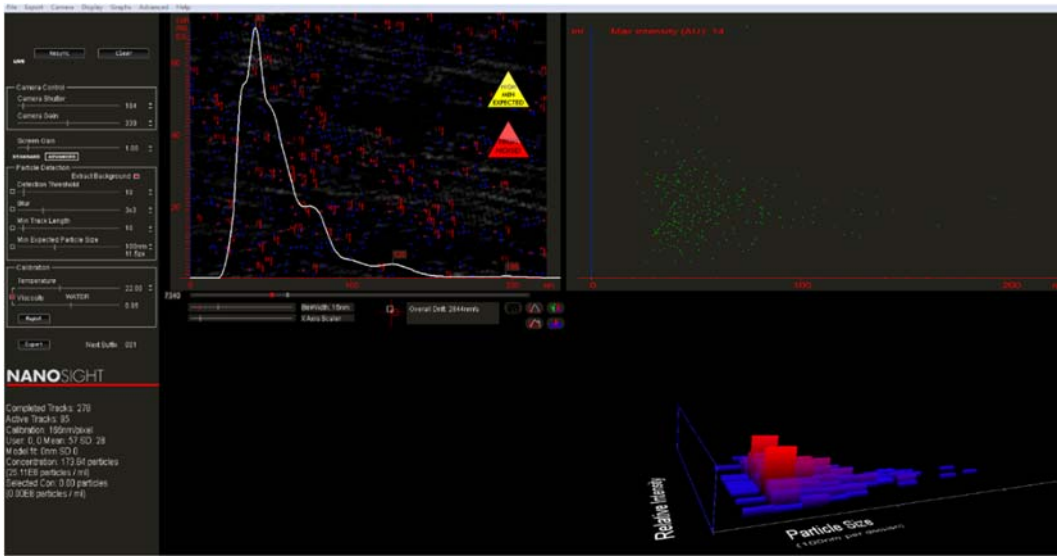


Figure 42. B. Dynamic Light Scattering of Purified CCR3. Relative Intensity vs Particle Size. Aggregations at 80 to 100-nanometer diameter range was observed for Human CCR3 Receptor in 300 mM Ni NTA elutions. There is some unavoidable noise observed during DLS Experiment.

5.16 Mass Spectrometry Based Characterization of CCR3: Electron Spray Ionization and Matrix Assisted Laser Desorption Ionization Methods

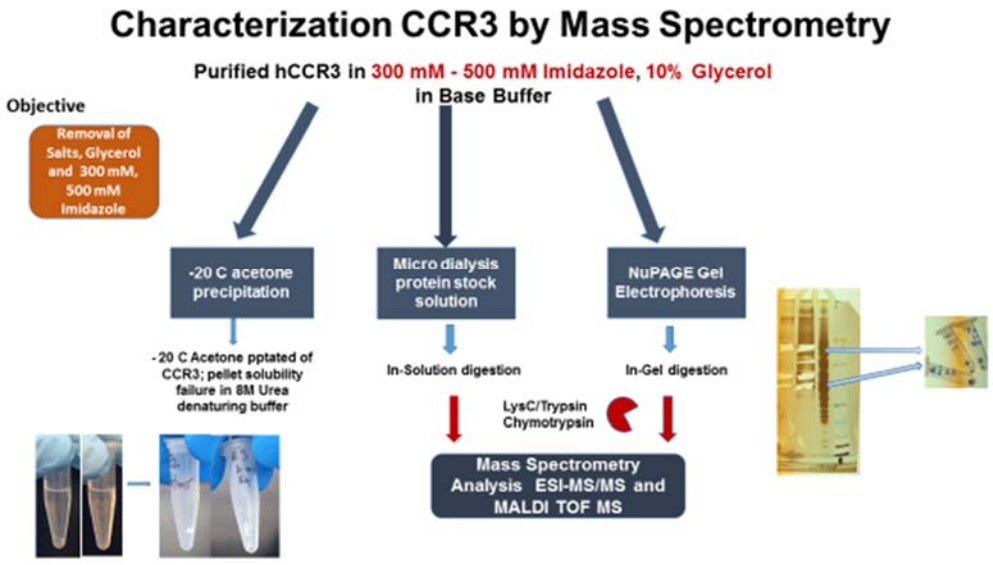


Figure 43. Sample Preparation Methods of Mass Spectrometry Based Characterization of the Purified CCR3 Receptor.

5.17 Purified CCR3 In-solution Digestion

In-solution digestion methods include efficient sequencing grade digestion and clean-up methods of peptides for mass spectrometry based characterization. The protein sample in solution is denatured followed by reduced-alkylated and finally sequencing grade enzyme digestion [22,23,24].The affinity purified CCR3 samples were dialyzed to remove excess Imidazole. After overnight dialysis in a SlideLyzer dialysis cassette, the sample is reduced with 20 mM TCEP a reducing agent and disulphide bond breaker. Immobilized enzymatic digestion with 0.1 % ProteaseMAX (Promega, Catalog # V2071)

solution is used for enzymatic digestion enhancement. ProteaseMAX enhances digestion efficiency of hard to digest proteins such as hydrophobic membrane protein.

Protein solubilizing enhancers such as ProteaseMAX and Rapigest SF (Waters Cat # 186001860) were used to facilitate protein solubilization of hydrophobic proteins such as membrane proteins. In low concentrations ranging in 0.1 to 1 % of V/V in 50 mM ammonium bicarbonate(NH_4HCO_3) promotes enzyme accessibility to the cleave sites on hydrophobic sites on proteins.

Freshly prepared stock of ProteaseMAX was prepared with 50 mM NH_4HCO_3 to have a 1 % solution and aliquoted and stored at - 80 C. Similarly Rapigest SF is suspended in 25 nM ammonium bicarbonate yielding a 1 % solution and aliquoted and stored in -20 C.

Recombinant Endoproteinase Lys-C (rLys-C)-Trypsin Digestion of CCR3: The purified CCR3 protein sample is pH adjusted to 8 with 50 mM ammonium bicarbonate (AMBIC), reduced with 20 mM TCEP for 45 minutes followed by recombinant Lys-C (Promega # rLys-C) digestion for 6 hours, later with Immobilized trypsin digestion was carried out. 30 uL of immobilized TPCK trypsin (Thermo Pierce # 20230) was spinned at 800 g for 2 minutes, and the supernatant buffer is removed. The Immobilized trypsin gel was washed three times with 500 uL wash buffer, 50 mM AMBIC pH 8.0. Finally, the gel was re-suspended with the rLys-C digested CCR3 sample. Digested overnight at 37 C, later the peptides were extracted from the TPCK trypsin slurry by spinning it at 1000 g for 5 minutes followed by pH adjusted to 2 with 0.1 % formic acid. The digested samples were C18 cleaned- lyophilized for MS analysis.

Chymotrypsin Digestion. Immobilized chymotrypsin is used (Proteochem # g4105), the resin was washed three times with 50 mM Tris buffer. The dialyzed CCR3 protein stock solution is added, reduced with 20 mM TCEP at room temperature for 45 minutes and digested for two hours with rapid shaking and another sample for overnight digestion.

5.18 In-gel Digestion Methods with rLys-C-Trypsin and Chymotrypsin

In-gel digestion is one of the most efficient ways to separate a protein by its molecular mass; then the protein is reduced-alkylated for sequencing grade digestion. Later the aqueous soluble peptides are extractedfrom the diced gel plugs for mass spectrometric analysis [25,26].

rLys-C and Trypsin Double Digestion: The purified 300 mM imidazole elution and 500 mM imidazole elutions were pH adjusted to 8 and separated on a 4 -12 % NuPAGE gel in 1X MES running buffer. The gel is later ethanol fixed and stained with mass spectrometry compatible silver stain as described earlier. The gel plug is carefully excised and dices in 1 mm pieces and de-stained with de-staining solution as recommended by the manufacturer.

Reduction-Alkylation: The gel plugs are shrunk with acetonitrile and air dried and rehydrated with 50 mM DTT in 50 mM AMBIC and reduced for 20 minutes at 45 C in the dark with shaking.

The excess 50 mM DTT was removed from the gel plugs and the gel plugs were shrunk back with acetonitrile and air dried in a speed vacuum apparatus. Rehydrated with 55 mM iodoacetamide in 50 mM AMBIC for 30 minutes at 40 C in the dark with

shaking. Later the excess iodoacetamide solution was removed, and gel plugs are made to shrink with acetonitrile and air dried in a speed vacuum apparatus

Freshly prepared rLys-C stocks of 12.5 nanograms/uLin 50 mM ammonium bicarbonate is used as the first step of digestion. The gel plugs are hydrated and adjusted to pH 8. After 6 hours, freshly prepared sequencing grade trypsin stock of 12.5 nanograms/uL concentration and further digestion to additional 12 hours.

Chymotrypsin digestion: In-gel digestion of chymotrypsin samples was carried out similar to digestion steps followed in rLys-C-Trypsin except the digestion enzyme used is chymotrypsin in concentration of 12.5 ng/uL.

Gel bands excision for in-gel digestion. The excised bands were diced to 1 mm pieces.

Peptide Extraction from digested Gel Plugs*:* Finally, the peptides of sequencing grade enzymatic digestion samples of rLys-C-trypsin and chymotrypsin digestion samples were extracted by spinning the gel plugs at 1000 g and collecting the supernatant with 50 mM AMBIC and the final collection with 70 % ACN in DI water. The peptide extracts were acidified with 0.5 % formic acid to pH 3 and dried in speed vacuum apparatus for LC-MS and MALDI-MS analysis.

Note. Complementarity in sequential grade enzyme digestions is an efficient method to characterize protein sequences by liquid chromatography-mass spectrometry (ESI-MS and matrix-assisted laser desorption ionization (MALDI) mass spectrometry methods

Table 6. Sequencing Grade Enzymes Used in the Digestion of Purified CCR3 Sample and Their Respective Cleavage Sites.

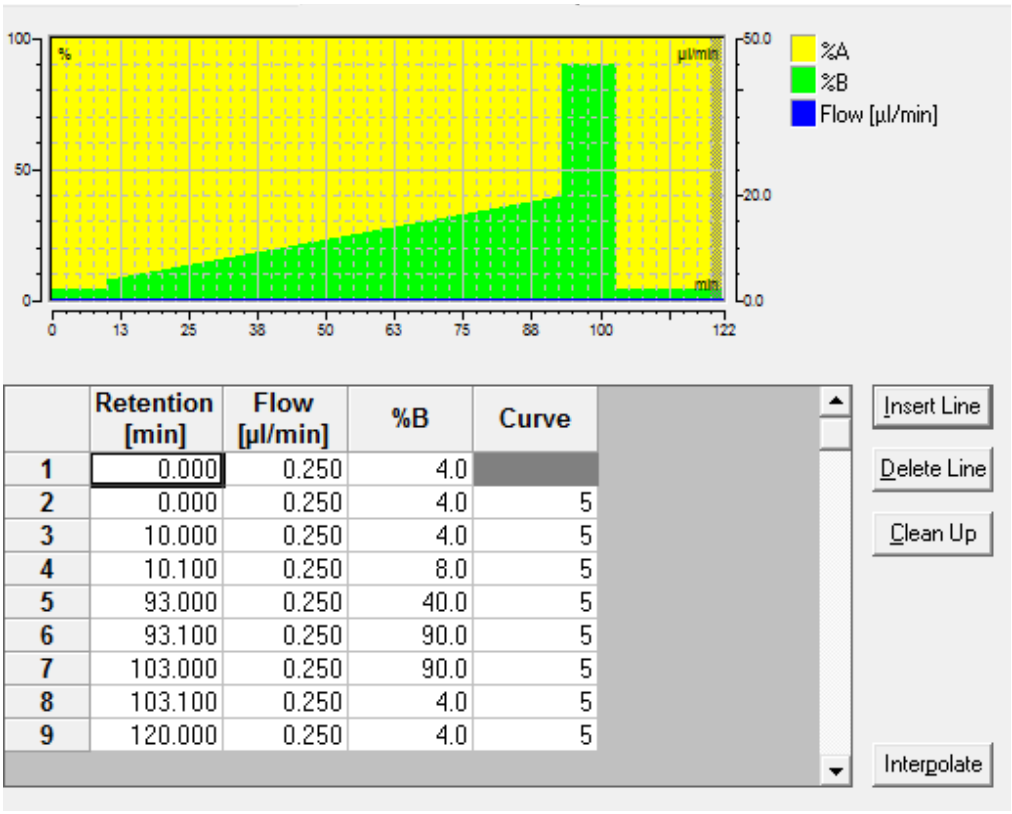
S. No	Enzyme	Cleave site	Comparative effectiveness	Additional comments
1	Trypsin	C (R, K)	Effective	Miss cleavages are possible
2	Chymotrypsin	Aromatic AA (FWY)	Highly effective	Miss cleavages happen under longer digestion conditions. Sequence-specific and optimal for cleaving hydrophobic residues
3	Lys-C	(K)	Highly effective	High sequence specific and can digest even under 8M urea protein denaturing conditions
4	Pepsin	non specific	Highly effective	
5	Proteinase K	non specific	Highly effective	

5.19 Electron Spray Ionization Mass Spectrometry (ESI-MS) Analysis

Mass spectrometry based proteomics and protein-peptide characterization involve several sequential steps from protein extraction to digestion with peptide clean-up and analysis by reverse phase chromatography coupled to a mass spectrometer [27, 28]. The mass spectrometer detects the mass over charge ratio and the charge state of the peptide ion in the first mass analyzer MS1, selective fragmentation of these precursor peptides

with collision activated dissociation products fragment ions in the second mass analyzer MS2 [29]. Thermo Scientific Q-Exactive HF (high frequency) high-resolution mass spectrometer with a mass accuracy under 5 ppm with front end ultimate 3000 nanoflow HPLC was used as the nanoflow LC-MS platform in analyzing the digested samples of purified CCR3.

The dried in-gel digested samples were re-suspended in 8 microliters of 0.1 % formic acid/5 % acetonitrile mobile phase A. C18 stationary phase 75 micron inner diameter (ID) 15 centimeters nanoflow LC column is used for binding and eluting the peptides by nanoflow reverse phase chromatography and sprayed in the Q-Exactive HF mass spectrometer with a nanospray source. Ultimate 3000 nanoflow HPLC is used with a flow rate of 250 nanoliters/ minute. The peptide tandem MS spectra areacquired.



	Retention [min]	Flow [µl/min]	%B	Curve
1	0.000	0.250	4.0	
2	0.000	0.250	4.0	5
3	10.000	0.250	4.0	5
4	10.100	0.250	8.0	5
5	93.000	0.250	40.0	5
6	93.100	0.250	90.0	5
7	103.000	0.250	90.0	5
8	103.100	0.250	4.0	5
9	120.000	0.250	4.0	5

Insert Line
Delete Line
Clean Up
Interpolate

Figure 43. Nanoflow Liquid Chromatography Gradient Table with a Flow Rate of 250 Nanoliters/Minute.

Top 15 MS method was setup in the Xcalibur acquisition interface to acquire tandem MS spectra of peptides. MS instrument method details are described below

1. Full scan MS Resolution of 120,000 at m/z 200

AGC target and maximum injection time. 3 e6, 50 ms

Scan range. 400 to 1950 m/z

2. MS/MS.

Resolution. 15,000 at m/z 200

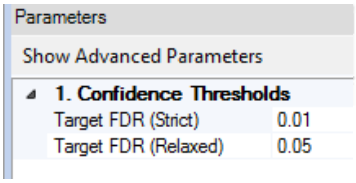
AGC (automatic gain control) target and maximum injection time. 1 e5, 100 ms

Top N loop. 15

1. Isolation window. 1.4 m/z
2. Normalized collision energy. 28
3. Charge exclusion. unassigned, 1, 7, 8, >8
4. Dynamic exclusion. 20 second
5. Profile mode

5.20 MS Data Processing with Proteome Discoverer 2.1 and MaxQuant Peptide Tandem MS Software

The tandem MS spectra acquired with the Q-Exactive HF, LC-MS platform was processed with Proteome Discoverer 1.4 and 2.1 for peptide spectral matches (PSMS) and protein ID hits [31]. The tolerance levels are 4 ppm precursor ion tolerance and 0.01 Da fragment ion tolerance. The raw MS/MS spectral data is searchedagainst SEQUEST HT search algorithm with a target false discovery rate of 0.01.



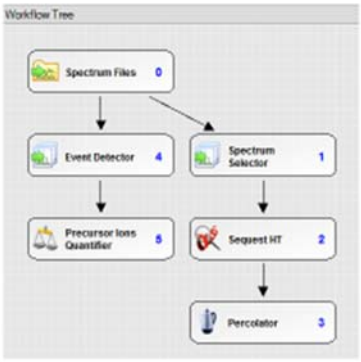


Figure 44. Proteome Discoverer 2.1 Analysis and Consensus Nodes Used in PSM Searches.

Table 7. Sequencing Grade Enzymes and Digestion Condition on the Purified CCR3 Digestion.

S.no	**Reduction conditions**	**Enzyme**	**AA Cleavage site**	**Digestion time**
1	20 mM TCEP at RT for 45 minutes	rLys-C only	K	16 hours
2	20 mM TCEP at RT for 45 minutes	rLys-C/Immobilized Trypsin	K,R	16 hours
3	20 mM TCEP at RT for 45 minutes	Immobilized Chymotrypsin	T,W,Y	2 hours and 16 hours

5.21 Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI) Mass Spectrometry

MALDI-TOF MS/MS methods are complementary to ESI-MS based characterization of peptides by mass spectrometry. MALDI methods have their applications in the analysis of small molecules, oligonucleotides, peptides and proteins in the mass range of 100 Da to 1,000,000 Da [30]. MALDI is a soft ionization method similar to ESI mode of ionization, the unique features of MALDI-MS methods is, MALDI-MS characterization can tolerate contaminants and salts in samples, unlike ESI based methods. When the protein is pure enough and sample quantity is enough MALDI MS method is a alternative option in mass spectrometry based characterization of proteins.Peptide sample analysis with MALDI-MS platform can be carried out in considerably less period over ESI-MS methods; in LC-ESI MS methods a front end separation of peptides by reverse phase chromatography with nanoflow HPLC is required before the peptide ions are transmitted into the mass spectrometer for characterization and sequencing by fragmentation.

Experimental Methods: The affinity purified CCR3 samples was analyzed by MALDI MS/MS to identify CCR3 derived peptides. In-solution digest and In-gel digested samples are prepared for MALDI TOF MS/MS analysis. SCIEX 5800 MALDI TOF-TOF mass spectrometer is used in acquiring peptide MS/MS spectra. Freshly preparedsaturated solution of alpha cyano-4-hydroxycinnamic acid was as the MALDI matrix with methanol. 0.4 uL of digested and C18 cleaned peptide sample and 0.4 uL of the above saturated solution of MALDI matrix solution are mixed in the ratio of 1.1 ratio

for spotting the MALDI plate with sandwich mode; first the peptide solution and later the matrix solution on top it. The MALDI plate is completely air driedanddata acquisition was carried out in Reflectron mode in the Sciex 5800 TOF/TOF MS. 5800 MALDI MS plate, the digested CCR3 peptides were spotted with MALDI matrix of 1.1 ratio. The mass spectrometer is calibrated first in MS mode and them in MS/MS mode before sample analysis was carried out.

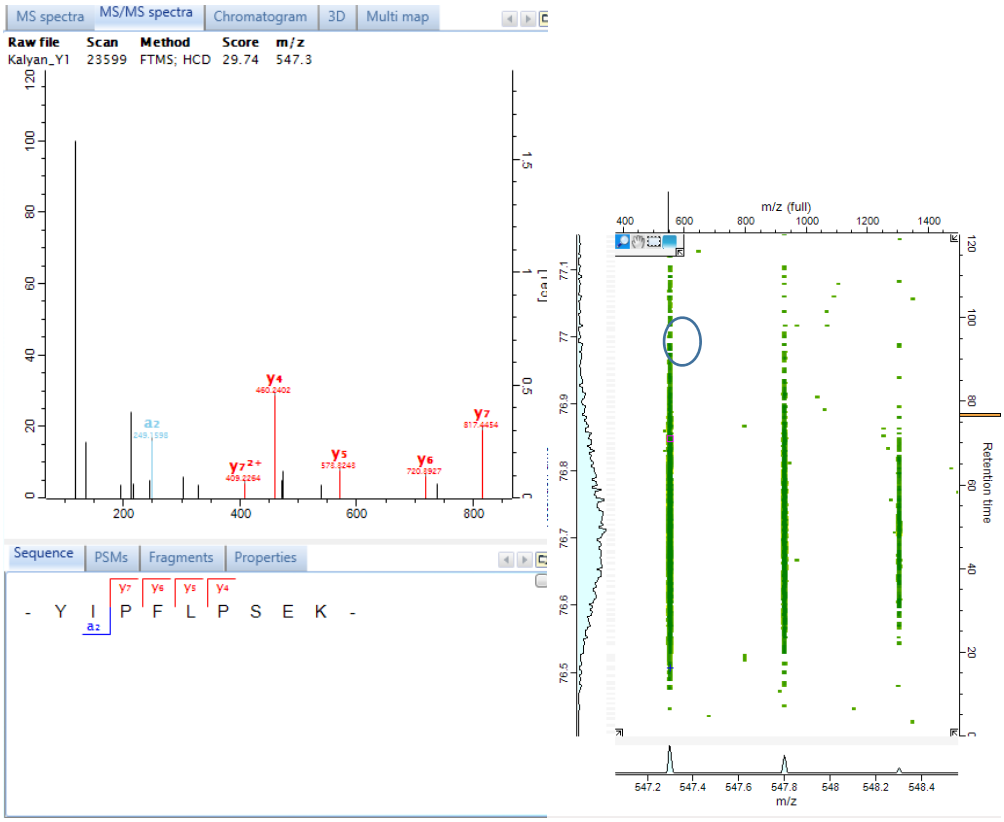
5.22 Mass Spectrometry Characterization of CCR3: Results and Discussions

Complementary sequences of the CCR3 digested and derived peptides were obtained by ESI MS and MALDI MSbased methods with sequencing grade rLys-C/Trypsin and chymotrypsin digestions of the purified CCR3. Fragmentation of peptide precursor ion for MS/MS was carried out in ESI MS method with the high-resolution Thermo Q-Exactive HF Orbitrap mass spectrometer. Two peptides are identified by MALDI MS methods with 11 % sequence coverage for CCR3. The experimentally identified mass of these two peptide ions by MALDI MS are 1598.6940 dalton with a 3 % sequence coverage and 3510.2561 dalton mass 8 % sequence coverage for the second peptide, two methionine oxidations and one carbamidomethylation is identified on this peptide.

With electron spray ionization (ESI) mass spectrometry-based characterization of CCR3 methods, one tryptic peptide with a sequence YIPFLPPSEK with m/z of 547.299, $M+H^+$ 1093.5245 charge state +2. By chymotrypsin digestion and ESI MS analysis, two peptides were identified AFVGERF (with $M+H^+$ of 824.41) and

HTLRMTIFCLVLPLLVMAICY ($M+H^+$ of 2563.35). The overall MS characterization data is represented in tabular form later in the chapter.

5.23 CCR3 Sequence Identification Results with ESI MS (Thermo Q Exactive HF Mass spectrometer) and MALDI TOF MS (Sciex 5800 MALDI TOF MS)



Figures 45. MaxQuant Tandem MS Search Data, Tryptic Peptide of CCR3 Digestion with rLys-C/Trypsin Digestion Characterized by LC-MS/MS analysis. Peptide sequence is YIPFLPPSEK with an m/z of 547.299, MH^+ 1093.5245 charge state +2. In HCD Fragmentation **a** and **y** series fragment ions of the precursor MH^+ in the Mass Spectrometer that matched with the theoretical fragment ions. Right image is the Peptide Natural Isotopic Distribution.

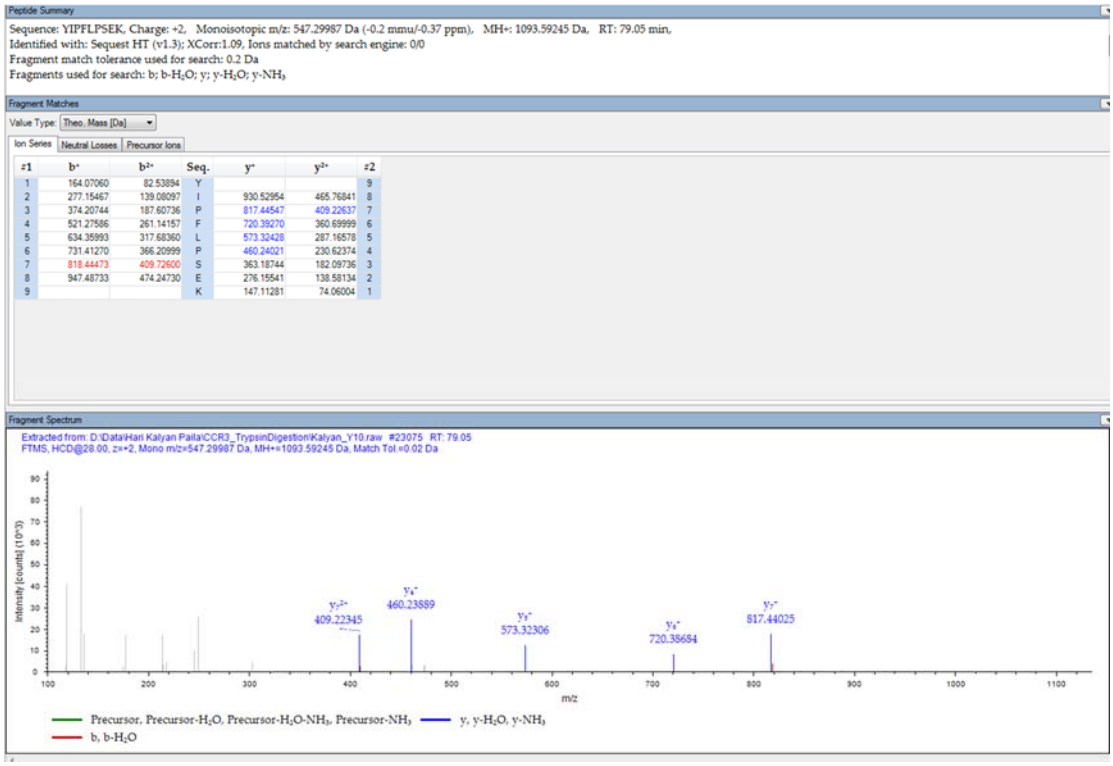


Figure 46. Peptide Tandem MS Search with Proteome Discoverer 1.4 of Trypsin Digested CCR3 Protein Sample and Fragment Ion Spectra One Peptide.

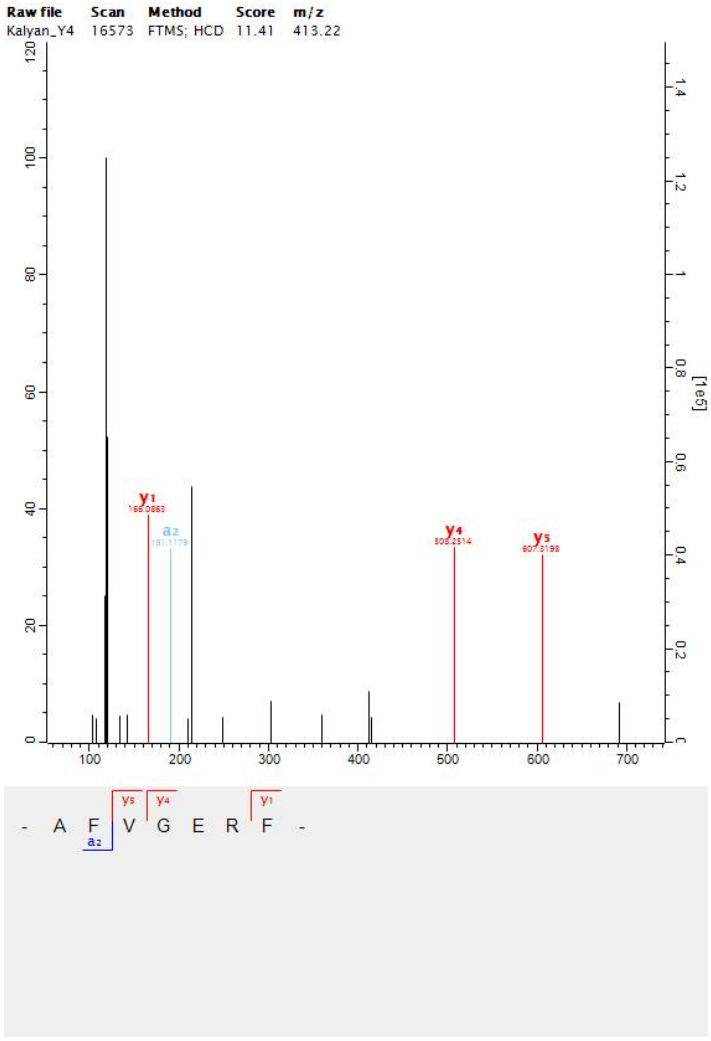


Figure 47. Chymotrypsin-Digested CCR3 Protein Tandem MS Fragment Ion Spectra.

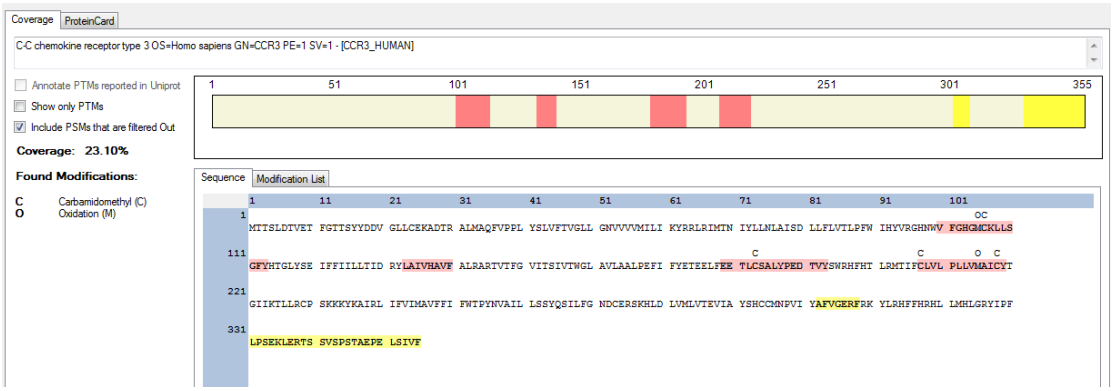


Figure 48. Sequence Coverage Map in Proteome Discoverer 1.4; Chymotrypsin-Digested CCR3 Sample.

Table 8. Total CCR3 Peptide Sequences Identified by Chymotrypsin and Trypsin Digestion on the Q Exactive HF Mass Spectrometer with Nanoflow Liquid Chromatography Electron Spray Ionization Mass Spectrometry Methods. Peptide Tandem MS (MS/MS) search of HCD spectra with MaxQuant and Proteome Discoverer 1.4.

S. No	Peptide sequence	$(M+H)^+$	m/z	Charge state
1	AFVGERF	824.41809	413.22	+2
2	HTLRMTIFCLVLPLLVMAICY	2563.3551	547.3	+2
3	LPSEKLERTSSVSPSTAEPELSIVF	2703.41233	901.80896	+2
4	YIPFLPPSEK (tryptic peptide)	1093.5245	547.299	+2

Table 9. CCR3 Digested Peptides Sequences Identified by 5800 MALDI TOF MS/MS Mass Spectrometer and Mascot Search Against a Non-Redundant Human Database.

Peptide	**M+H**	**Start and stop position**
LAIVHAVFALRAR	1598.6940	132-145
HLDLVMLVTEVIAYSHCCMNPVIYAFVGERF	1510.6808	278-307

1. 13 amino acids with the above sequence with 3 % percent sequence coverage. 1598.6940 Da is the experimental mass of the peptide
2. 29 amino acids match with the above sequence with 8 percent sequence coverage. Two methionine oxidations and one carbamidomethylation is observed, 3510.2561 Da is the experimental mass

5.24 Mascot Tandem MS Spectra Search Results of MALDI MS/MS Spectra [32]

Protein View

Match to: **sp|P51677|CCR3_HUMAN** Score: 4 Expect: 0.4
C-C chemokine receptor type 3 OS=Homo sapiens GN=CCR3 PE=1 SV=1

Nominal mass (M_r): 42387; Calculated pI value: 8.50
NCBI BLAST search of sp|P51677|CCR3_HUMAN against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 8%

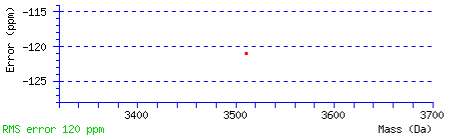
Matched peptides shown in Bold Red

```
    1 MTTSLDTVET FGTTSYYDDV GLLCEKADTR ALMAQFVPPL YSLVFTVGLL
   51 GNVVVVMILI KYRRLRIMTN IYLLNLAISD LLFLVTLPFW IHYVRGHNWV
  101 FGHGMCKLLS GFYHTGLYSE IFFIILLTID RYLAIVHAVF ALRARTVTFG
  151 VITSIVTWGL AVLAALPEFI FYETEELFEE TLCSALYPED TVYSWRHFHT
  201 LRMTIFCLVL PLLVMAICYT GIIKTLLRCP SKKKYKAIRL IFVIMAVFFI
  251 FWTPYNVAIL LSSYQSILFG NDCERSKHLD LVMLVTEVIA YSHCCMNPVI
  301 YAFVGERFRK YLRHFFHRHL LMHLGRYIPF LPSEKLERTS SVSPSTAEPE
  351 LSIVFHHHHH HHHHH
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
278 - 307	3511.2634	3510.2561	3510.6808	-121	0	K.HLDLVMLVTEVIAYSHCCMNPVIYAFVGER.F Carbamidomethyl (C); 2 Oxidation (M)



Protein View

Match to: **sp|P51677|CCR3_HUMAN** Score: 4 Expect: 0.4
C-C chemokine receptor type 3 OS=Homo sapiens GN=CCR3 PE=1 SV=1

Nominal mass (M_r): 42387; Calculated pI value: 8.50
NCBI BLAST search of sp|P51677|CCR3_HUMAN against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 3%

Matched peptides shown in Bold Red

```
    1 MTTSLDTVET FGTTSYYDDV GLLCEKADTR ALMAQFVPPL YSLVFTVGLL
   51 GNVVVVMILI KYRRLRIMTN IYLLNLAISD LLFLVTLPFW IHYVRGHNWV
  101 FGHGMCKLLS GFYHTGLYSE IFFIILLTID RYLAIVHAVF ALRARTVTFG
  151 VITSIVTWGL AVLAALPEFI FYETEELFEE TLCSALYPED TVYSWRHFHT
  201 LRMTIFCLVL PLLVMAICYT GIIKTLLRCP SKKKYKAIRL IFVIMAVFFI
  251 FWTPYNVAIL LSSYQSILFG NDCERSKHLD LVMLVTEVIA YSHCCMNPVI
  301 YAFVGERFRK YLRHFFHRHL LMHLGRYIPF LPSEKLERTS SVSPSTAEPE
  351 LSIVFHHHHH HHHHH
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
132 - 145	1599.7013	1598.6940	1598.9409	-154	1	R.YLAIVHAVFALRAR.T (No ma

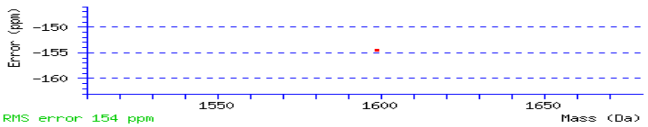


Figure 49. 13 Amino Acids with the Above Sequence with 3 % Percent Sequence Coverage. 1598.6940 Da is the Experimental Mass of the Peptide.

29 amino acids match with the above sequence with 8 percent sequence coverage. Two methionine oxidations and one carbamidomethylationis observed, 3510.2561 Da is the experimental mass.

Overall experimentally acquired CCR3 protein sequence characterized by electron spray ionization and matrix-assisted laser desorption ionization mass spectrometry.Sequence match with chymotrypsin and trypsin digestion with ESI-MS/MS.

>sp|P51677|1-355

MTTSLDTVETFGTTSYYDDVGLLCEKADTRALMAQFVPPLYSLVFTVGLLGNVV
VVMILIKYRRLRIMTNIYLLNLAISDLLFLVTLPFWIHYVRGHNWVFGHGMCKLL
SGFYHTGLYSEIFFIILLTIDRYLAIVHAVFALRARTVTFGVITSIVTWGLAVLAAL
PEFIFYETEELFEETLCSALYPEDTVYSWRHFHTLRMTIFCLVLPLLVMAICYTGII
KTLLRCPSKKKYKAIRLIFVIMAVFFIFWTPYNVAILLSSYQSILFGNDCERSKHLD
LVMLVTEVIAYSHCCMNPVIYAFVGERFRKYLRHFFHRHLLMHLGRYIPFLPSEK
LERTSSVSPSTAEPELSIVF

YIPFLPSEK

(Trypsin digested sample peptide sequence, a tryptic peptide)

Sequence match with trypsin digestion and MALDI TOF/TOF MS method:

MTTSLDTVETFGTTSYYDDVGLLCEKADTRALMAQFVPPLYSLVFTVGLLGNVV
VVMILIKYRRLRIMTNIYLLNLAISDLLFLVTLPFWIHYVRGHNWVFGHGMCKLL
SGFYHTGLYSEIFFIILLTIDRYLAIVHAVFALRARTVTFGVITSIVTWGLAVLAAL
PEFIFYETEELFEETLCSALYPEDTVYSWRHFHTLRMTIFCLVLPLLVMAICYTGII

KTLLRCPSKKKYKAIRLIFVIMAVFFIFWTPYNVAILLSSYQSILFGNDCERSKHLD

LVMLVTEVIAYSHCCMNPVIYAFVGERFRKYLRHFFHRHLLMHLGRYIPFLPSEK

LERTSSVSPSTAEPELSIVF

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CHAPTER VI

PHOSPHOPROTEOMICS STUDY OF HEK 293 CELLS: PHOSPHOPEPTIDE ENRICHMENT METHODS WITH TIO_2 RESIN

6.1 Cellular Proteome and Phosphoproteome

Proteomics involves a few selective analytical methods by which hundreds and thousands of proteins are detected detected simultaneous from complex protein samples. These Studies include protein identity and amino acid sequence level identification and validation with hyphenated techniques such as chromatographic, electrophoretic-based separation and following analysis with orthogonal mass spectrometry methods such as electron spray ionization and matrix assisted laser desorption ionization methods [1]. In the past few decades, several proteome analysis methods in complex biological systems such a cellular and organelle level led to several undiscovered proteins, and their splice variants and homologs. A few peptides derived from the corresponding protein are detected by liquid chromatography - mass spectrometry based methods [2]. The overall proteomic methods in protein identification methods provides the percentage of amino acid sequence coverage validated and the relative abundance level of the particular protein or a peptide in a complex high dynamic range samples such as cell lysates, biological body fluids, and tissue protein extracts [3].

The repertoire of the human genome has close to 20,000 genes that translate to proteins [4]. At any given point, the total diversity of proteins far exceeds the human genes that can actively translate to proteins [5,6]. RNA microarray validated the possible proteins expressed in cells [7]. Cellular ribosomes aid in protein synthesis-translation. This newly synthesized protein's higher order covalent modification also known as post translational modifications (PTM) happens on the side chain of the amino acids [8, 9]. Some of these PTMS are irreversible, such as glycosylation, ubiquitination, disulfide bond linkages between cysteine amino acids, whereas reversible modifications like methionine oxidation, and serine (S), threonine (T) and tyrosine (Y) undergo reversible phosphorylation[10,11]. Phosphoproteome constitute 2 -3 % of the total proteome in cells. The variability in protein expression changes from the same gene leads to the expression of protein isoforms, heterogeneity at the high order oligomeric assemblies, and post translational modifications [12, 13]. The above mentioned higher order and dynamic changesrender the global proteome a much greater analytical complexity over the corresponding human genome. The range of biophysical properties of proteins and peptides is attributed by the amino acid composition and theirrespective highly dynamic post translational modifications (PTMs), some of these PTMs are temporal in nature [14].The cytoplasmic space, especially the reticular endothelial system of the cell is the primaryloci of fixed PTMs in the cellular biochemical process to render the protein its higher order structure.

6.2 Phosphoproteomics: Phosphorylations in Threonine, Serine, and Tyrosine Residues

Phosphoproteome is a subset of the total cellular proteome; protein phosphorylation is one of the most common post-translation modification (PTM).Phosphorylation of proteins is the most studied PTM in the biomedical community, due to its immediate impact on cellular biochemistry and physiology and their functional relevance [15,16]. Some of the salient features of protein phosphorylation are the control of specific metabolic pathways, transient cellular signaling events, transcription and translation regulation, protein functionality and their associated life cycle in cells. Particularly in cancer cells, mostly cellular proliferation and differentiation processes [17,18]. Protein phosphorylation is a transient and reversible modification occurring on Serine (S), Threonine (T) and Tyrosine (Y). Phosphorylation can happen on histidine residues (H) as well, but it's a highly unstable process, and identifying histidine phosphorylation with confidence is low (19). Phosphorylation opens up several orders of functionality to the protein by affecting the protein conformational states. In any given state of cellular physiology, more than 50 % of proteins are phosphorylated in their respective lifespan [15]. More than 10^5 phosphorylation states can be observed in a normalhuman cellular proteome [20]. Only a small fraction of proteome undergo phosphorylation at a given time point, being its transient nature, detection of a specific protein phosphorylation is a challenging endeavor by standard phospho antibody probing methods.

Protein kinases catalyze phosphorylation, and two to three percent of the human genome encodes for protein kinases [21]. There are close to 100 phosphatases that dephosphorylated phosphoproteins [22]. Reversible phosphorylation events are catalyzed by kinase – phosphatase enzyme system.

Protein phosphorylation is a crucial biochemical process in which an inorganic phosphate ($PO4^{3-}$)is added to the tyrosine, threonine or serine amino acids in proteins. Phosphorylation is a reversible biochemical phenomenon, it plays a significant role in cellular physiological processes, and it may functionally activate a protein or inactivate it.

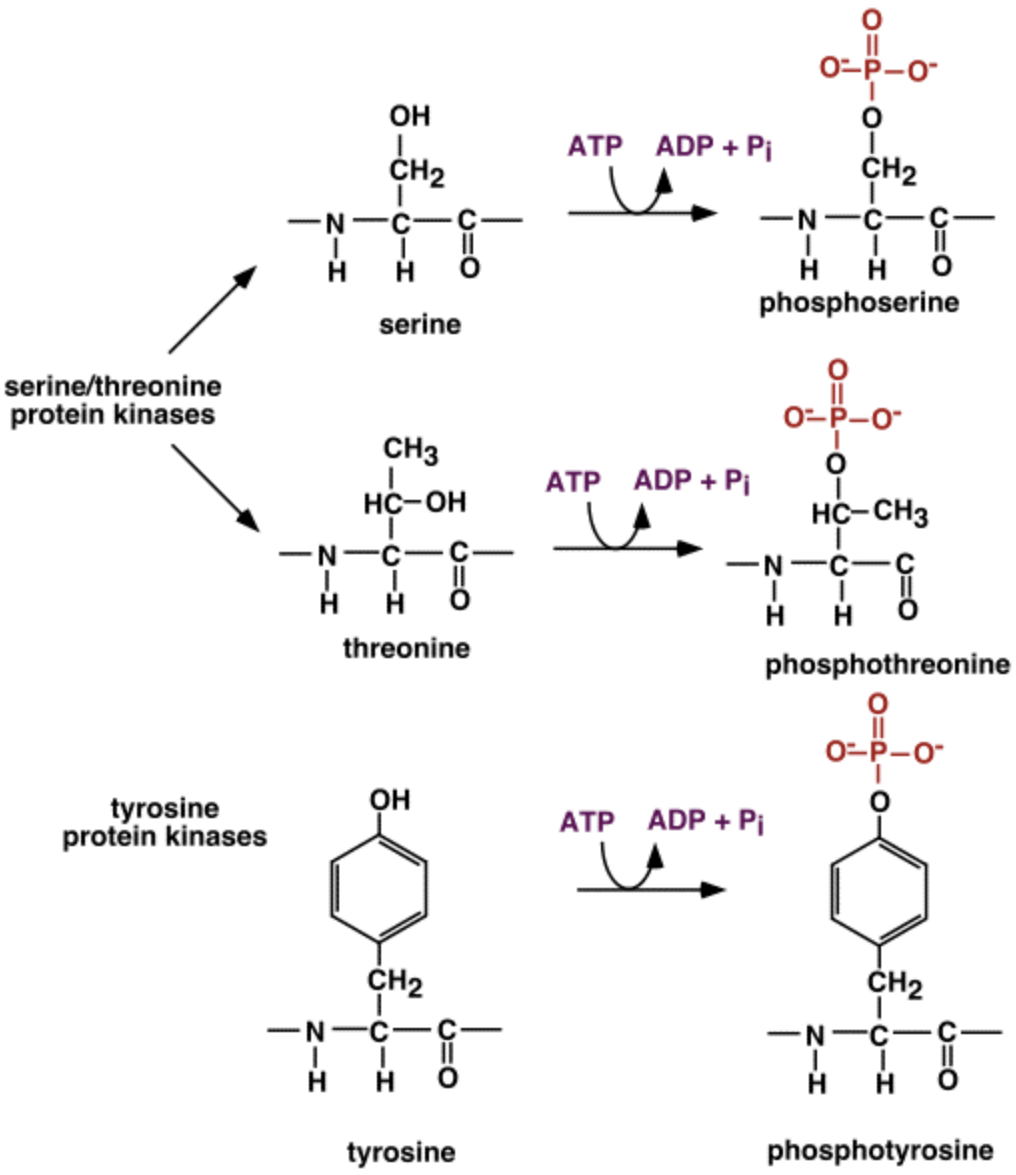


Figure 49. Mechanism of Phosphorylation in the Three Amino Acids, Serine, Threonine, and Tyrosine. Reference.www.bio.uci.edu.

A typical cellular proteome has a dynamic range close to ten orders of magnitude 10^{10} of protein concentration [23]. The most advanced mass spectrometry instrumentation used in proteomic applications in the discovery phase of global protein profiling to quantitative proteomics studies. The linear dynamic range for Ion Trap MS and Q-TOF MS systems of three to four orders (10^4), and five orders magnitude for triple quadrupole and quadrupole - linear ion trap MS platforms [24].There is a significant region in the proteome of complex samples such as a cell lysate or human biological fluids such as plasma, cerebrospinal fluid etc.,which cannot be analyzed-detected unless a given complex protein samples dynamic range is reduced to the range of less than 10^5. By reducing the dynamic range of a complex sample, the order of magnitude of the protein sample falling in the range of the MS instrumentation detector dynamic range for in-depth detection and quantitation of the target protein in a linear range. Several methods are available to fractionate (separate) proteins at a protein or peptide level to reduce the dynamic range of complex samples. These fractionation methods are on the molecular descriptors of proteins and peptides, such as molecular size based fraction by employing size exclusion chromatography (SEC), charge based separation with Ion exchange chromatography (IEX), hydrophobicity based fractionation method with reverse phase chromatography (RP-HPLC) at the complex protein sample or it enzymatic digest–peptide sample[25]. Protein sample complexity may be fractioned at the peptide level byHPLC-based separation, by harnessing the physical descriptors of the peptides. Charge of peptides or their hydrophobicity. Later the fractionated samples can be analyzed again

in a second dimension nanoflow HPLC-tandem mass spectrometer for peptide and the corresponding protein identification or relative quantitation methods [26, 27].

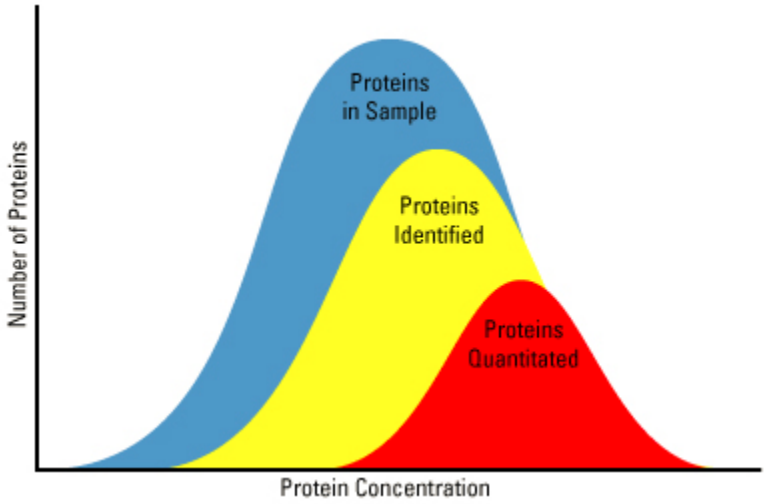


Figure 50. A Complex Protein Sample and the Relative Level That can be Identified and Quantified. Reference. Adapted from Thermo FisherScientific – Quantitative Proteomics Manual.

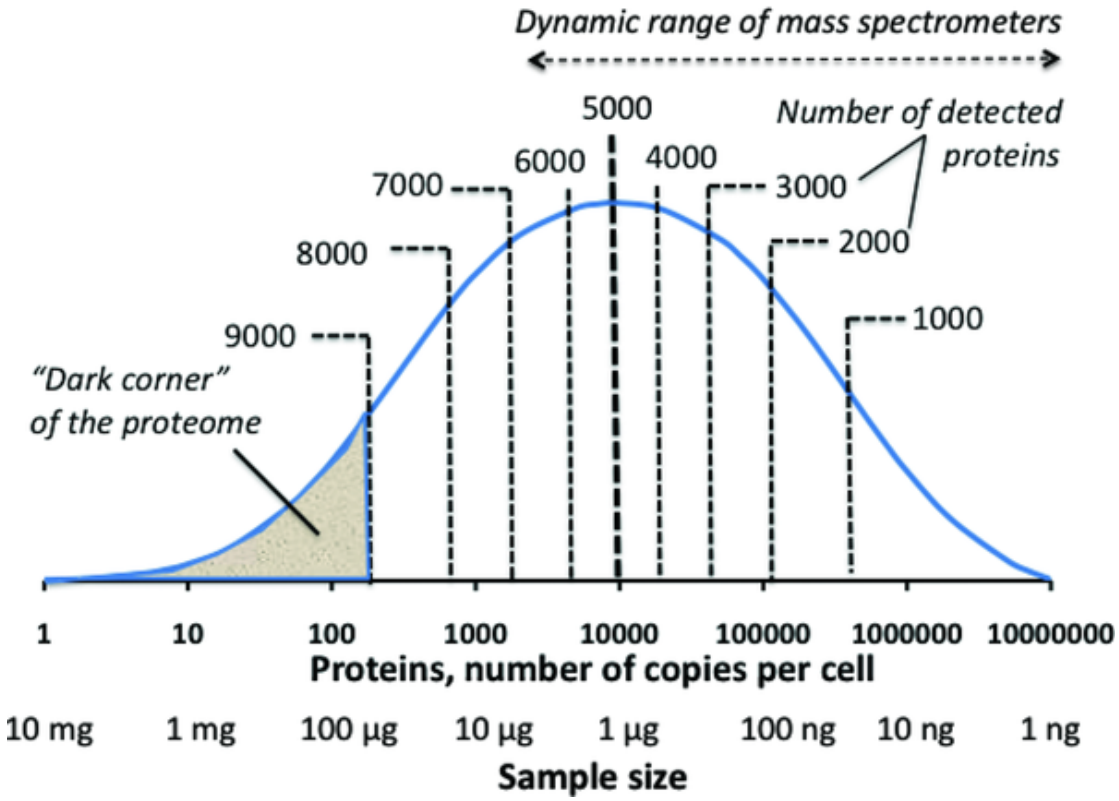


Figure 51. The Dynamic Range of a Protein Sample and the Detection Ranges of a Mass Spectrometers in Proteomic Studies [28].

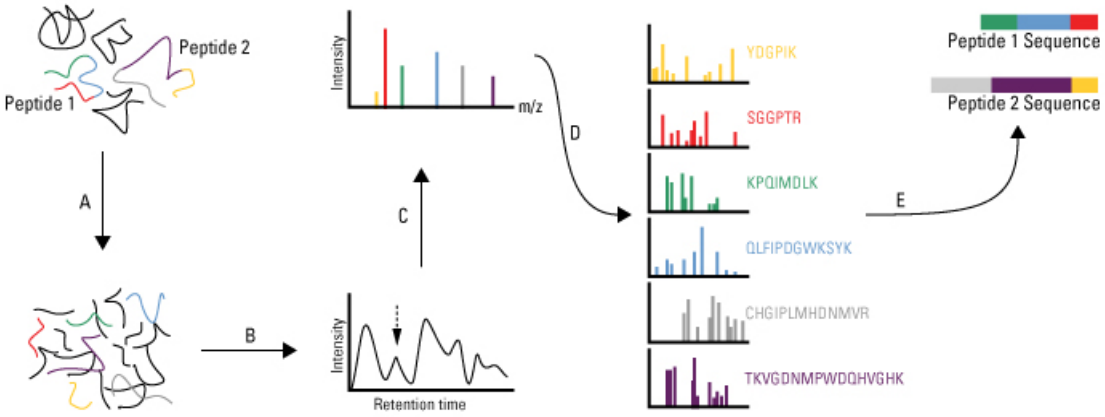


Figure 51. Overview of Mass Spectrometry-Based Protein Characterization in Complex Protein Samples such as Cell Lysates and Tissue Protein Extracts. Reference: Thermo Fisher Scientific Quantitative Proteomics Manual.

6.3 Signal Transduction from the Exterior to the Intracellular Environment via Membrane Proteins: Signal Transducers and Canonical Pathway

More than 50 % of the drugs act on membrane protein receptors, these are predominantly membrane ion channels like GABA receptors, ion channels, GPCRs, and membrane bound protein kinases such as endothelial growth factor receptors (EGFRs). The external signal is transmitted when physical stimuli or a ligand molecule bind to the membrane-bound drug receptor [29].The signaling is propagated into the intracellular environment by signal transducers or membrane receptors from the extracellular environment to cytosol bound signaling proteins in a canonical manner [30]. But recent evidence has shown a non-canonical models of biochemical signaling mechanisms as well. Duringdownstream signal propagation in the cell, few important signaling protein can be phosphorylation at any one or more of the three amino acids serine, threonine and tyrosine or in more than one of these. Protein kinase undergoes specific tyrosine

phosphorylation [31, 32]. Tyrosine phosphorylation of protein kinases is a critical PTM and comprises less than one percent of the phosphoproteome of the cell.

6.4 Analytical Strategies in Detecting Protein Phosphorylation Sites

Protein phosphorylation is sub-stoichiometric, it is a monumental task in identifying phosphorylation site. The role of specific motif phosphorylation in a given protein and its function features is validated by site-directed mutagenesis, kinase assay, and radiolabeled P32 Labelling techniques [33].

Phosphorylation is a ubiquitous phenomenon and is physiological state dependent, it is relatively a tedious task to specifically look for one phosphorylation even and annotate its relevance to protein functionality and its respective biological pathways [34]. Global phosphorylation studies are convenient methods to look forprotein that their temporal phosphorylation states.

While look for the entire cellular phosphoprotein in a particular cellular state, it is a daunting task, since phosphoproteome is a minute fraction of the entire global of the total cellular proteome. The occurrence of phosphoproteome in less than 1 % of the entire cellular proteome, and the stoichiometry of phosphorylation is also significantly less [33]. When detecting transient signaling proteins in total cellular phosphoproteome with a higher of magnitude of protein concentration, the detection of a biochemical signaling protein against high abundance proteins in the cells gets even challenging. The important strategy to identify and validate a specific phosphoproteome in a complex sample with a significant background such as entire proteome is an effective step to be undertaken in

phosphoproteomics studies. Enrichment methods of phosphopeptides derived by enzymatic digestion of phosphoproteins in the complex whole cell lysate sample is a practical means in phosphoproteomics studies.

The rate of phosphorylated in cellular protein is sub-stoichiometric. Phosphorylation may occur at S, T, Y residues as discussed earlier. The presence of these amino acids in the protein may not always guarantee phosphorylation with certainty. Phosphate group renders a negative charge when compared to its non-phosphorylated peptide analogs. These phosphopeptides moieties with the $P0_4^{3-}$has a negative charge, which can bind to positively chargesmetal ions. With this rationale, several metal ion based resins are employed in enriching phosphopeptides [35]. In chromatography methods,phosphopeptidesare selectively bound and eluted from an HPLC stationary phase due to their relative higher hydrophilic property over its non-phosphorylated peptides. The above stated methods are usedin the selective enrichment of phosphopeptides from the complex cell lysate and tissue protein extracts-digests.

Polyacrylamide gel electrophoresis methods is also a correspondingmethod in detecting phosphoproteins in cells and tissue samples. The rational in gel-based methods being the protein sample separation based on the size with SDS PAGE and consecutiveselective probing with phospho selective staining to identifying phosphopeptides on the SDS PAGE gel [36]. The differential phosphoprotein expression levels of the samples reported based on the degree of phosphor staining intensity of the gel. One of the drawbacksofgel-based methods is their lack the site localization of the

phosphorylated site at the amino acid sequence level. Furthergel-basedmethods identify a limited number of a phosphoprotein. Hyphenated analytical techniques such as chromatography - tandem mass spectrometry methods are advantageous in having the desired throughput in phosphoproteome of complex samples. Mostly due to the fact only a limited amount of protein concentration maybe separated by first dimension separation in gel-based methods.

Whole cell proteome is digestion with sequencing grade digestion enzymes such as endoproteinase Lys-C followed by trypsin, this sequential digestion increases efficiency and precludes any miss cleavages. With trypsin,alonethe protein digestion efficiency is not maximal.

6.5 Methods Overview: Temporal Phosphorylation Dynamics Analysis in Cellular Proteome

Experimental treatment and stimulation of cellular protein phosphorylation: In detecting phosphorylation states, two or more experimental conditions or physiological states are chosen to be studied. With one being the control experiment and the other as the treatment group that is perturbed the cellular to induce phosphorylation. Mammalian cell type are chosen as the experimental cells in which the intended cellular signaling mechanism affected by protein phosphorylation is the target of study. Another strategy is to look for one or a few specific proteins and how their phosphorylation is affected during excited and unstimulated states of the cells. Phosphorylation can be promoted or stimulated by several biochemical agents, such as growth factors, drug molecules, and

even serum is an excellent phosphorylation stimulant. In cancer cell lines phosphorylation is a major dynamic event predominantly with protein kinases as the key players.

6.6 Harvesting Cells and Lysis for Protein Extraction and Importance of Proteases and Phosphatases

Once the cell-basedexperiments are completed, cells are harvestedfor lysis and protein extraction. If the cells are cell suspension type in media, they are harvested by spinning at around 5000g at 4 C to collect the cell pellet. The cell pellet is washed with 1X chilled PBS and immediately frozen in liquid nitrogen and store at -80C before further processing. If the cell is theadherent type, they are washed with chilled PBS, harvested by scraping followed by 5000g at 4 C spinning and stored at -80 C.

The collected cell pellet is lysed for whole cell protein extraction and solubilization for the following steps of downstream analytical and phosphoproteome detection. Most protein denaturing lysis buffers constitute PBS, HEPES, and 50 Mm Tris-based buffer with six molars (6M) to 8M urea or guanidine and 0.5 to 1 % SDS. Urea and guanidine are chaotropic agents and aid in disrupt hydrogen bonding and hydrophobic interacts in protein, at these high concentrations they open up highly hydrophobic proteins to make them more accessible for aqueous solubility. SDS in low percentage aids in protein solubility, because SDS is a surfactant and amphiphilic in nature. To increase the denaturing and solubility of hydrophobic proteins 6M urea and

2M thiourea are used in lysis buffer. The total concentration of protein denaturants should not be more than eight molar concentration.

6.7 Cell Lysate Denaturation and Enzymatic Digestions

Denaturants and chaotropic agents such as urea, thiourea, and guanidine are helpful in solubilizing hard to suspend and related hydrophobic proteins in aqueous buffers. In most cases, urea is used at 8 Molar and guanidine at 7 Molar strength. These denaturants open up the tightly aggregated hydrophobic proteins, thereby creating access for protein reducing agents like dithiothreitol and digestion enzymes such as trypsin and endoproteinase Lys-C. High urea concentrations can denature trypsin, so after incubating the protein sample in 8M urea, the sample is reduced - alkylated and then diluted to less than 2M urea for trypsin to act. But the more rugged and efficient enzyme like Lys-C, which cleaves at C-terminus of lysine residues in the polypeptide chain can digest even at high Urea concentrations. Sequential digestion, first with Lys-C for 3 to 4 hours followed by trypsin overnight (12 -15 hours) is the most efficient method of protein sample digestion.

6.8 First Dimension Chromatography Based Fraction: Reverse Phase Chromatography under High pH Conditions (hPH)

The sequencing grade enzyme digested complex protein sample is cleaned with C18 stationary phase solid phase extraction (SPE) can be fractioned with several modes in chromatography. The principles of chromatography of peptides is predominantly based on the hydrophobicity-hydrophilicity and charge descriptor properties of the peptides.

These chromatography modes are by reverse phase, hydrophobic interaction chromatography, strong cation exchange and hydrophilic interaction chromatography are the most widely used modes in fractionating peptides [40]. High pH (pH 9) reverse phaseC18 method as the first dimension chromatography is predominantly for two-dimensional (2D) proteomics and phosphoproteomics methods. High pH chromatography is to reduce peptide sample complexity for second dimension low pH (pH 3) reverse phase nanoflow LC - MS/MS analysis. High pH (pH above 10) reverse phase chromatography is orthogonal to low pH (pH 3) reverse phase chromatography on complex protein digests such as cell lysates and tissue lysate samples [38]. Successfully method development studies were reported to compare orthogonal first dimension fractionation chromatography such as low pH reverse phase chromatography, strongcation exchange chromatography, and electrostatic repulsion chromatography (ERLIC).

First, reverse phase liquid chromatography offers better resolution over strong cation exchange chromatography [39]. Strong cation exchange (SCX) chromatography require longer chromatography run times due to SCX column equilibration – conditioning with test protein standards. Further in SCX method, after peptide fractionation, the fractions are to be desalted with solid phase extraction (SPE) methods before second dimension nanoflow HPLC - MS/MS analysis. In reverse phase HPLC-based methods, the mobile phases are aqueous and organic and the overall fractionation time is less. There is no necessity of fraction desalting by SPE method.

The prime rationale for hPH reverse phase liquid chromatography is, it renders a significant shift in charge distribution of peptides over low pH (pH 3) conditions. As mentioned earlier, the second dimension nanoflow HPLC is a low pH reverse phase chromatography. The high pH followed by low pH reverse phase is ideal for better orthogonality and increased peak capacity IN chromatography. This two-dimensional set up is a good alternative to SCX - Reverse Phase HPLC-basedtwo-dimensional chromatography [38].

6.9 Concatenation of First Dimension Chromatography Fractions for Phosphopeptide Enrichment

In chromatographic methods of peptide fractionation collected in consecutivetimeframes interval show linearity of the distribution in consecutive fractions. Concatenation of the first dimension fractions for second dimension nanoflow HPLC - MS/MS provides increased advantage of orthogonality of the separation – analysis methods [39]. The first dimension fractions are primarily divided into three distinct groups, early, middle and late fractions. And the individual fractions of these three different groups are pooled to one group. For example, if the total fractions collected in the chromatography run are 45, and the sequential ones are divided into 15 early, 15 middle and 15 late fractions. Each fraction from the three groups are combined to for one pooled fraction. Finally, 15 combined fractions are obtained. Each of the 15 fractions is analyzed as one distinct second dimension nanoflow LC - MS/MS run. In the case of

phosphopeptide enrichment, these 15 fractions are used for phosphopeptide enrichment for later second dimension nanoflow LC-MS/MS analysis.

6.10 Phosphopeptide Enrichment for Mass Spectrometry Based Analysis

Phosphorylated peptides have an additional negative charge moieties $PO4^{3-}$, this renders more hydrophilicity over its non-phosphorylated peptide. For selective enrichmentpurposes immobilized metal ion affinity chromatography is employed for selective enrichment of phosphopeptides. Metal oxide based affinity resin of Fe (III), TiO_2 (IV) are widely used in phosphopeptide enrichment process [40, 41, 42]. Caution is to be taken to prevent enrichment of acidic peptides; peptides having several acidic glutamate residues or aspartate residues.

TiO_2 nanoparticles (GL Sciences) are used in enrichment of phosphopeptides of HEK 293 lysate digest. The enrichment protocol is provided in later part of the chapter.

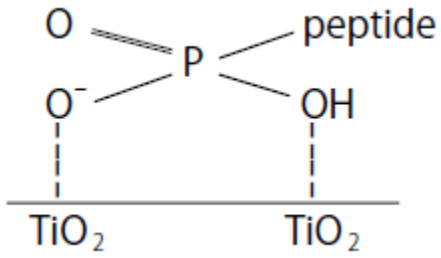


Figure 52. Selective Binding of Phosphopeptides on to TiO_2 Resin. Reference. GL Sciences TiO_2 Product Manual.

6.11 Carbon 13 (C13) and Nitrogen (N15) Labeled Lysine and Arginine Labelled Two Plex SILAC with Titanium Dioxide (TiO_2) Based Phosphopeptide Enrichment: Experimental Methods

HEK 293 TetR Stable cell line expressing hCCR3 for SILAC enrichment Zeocin antibiotic is used inselection of HEK 293 TetR transient transfected cell line, the clones expressing CCR3 was taken for phosphoproteomics study. This stably selected HEK 293 cell line is adapting to Stable Isotope Labeling in Cell Culture (SILAC) enriched methods by two plex SILAC methods; Lysine C13, N15 (+8 Da) and Arginine C13, N15 (+10 Da) is used in heavy labeling.

DMEM SILAC media is prepared with DMEM for SILAC, 10 % dialyzed FBS and incorporating with isotope labeled amino acids Lysine C13, N15 (+8 Dalton) and Arginine C13, N15 (+10 Dalton) and the antibiotics and antifungal agents to prevent cell culture contamination.

Media Preparation: Isotope labeled amino acids, Lysine +8 Da (C13, N15) and Arginine +10 Da (C13, N15 label) are incorporated in the Heavy DMEM media in the below concentrations, structures of lysine and arginine

DMEM : F12 for SILAC

10 % Dialyzed FBS

1 % Penicillin - Streptomycin

20 ug/ml Zeocin – selection antibiotic for pcDNA4/TO vector transfect

5ug/ml blasticidin – selection antibiotic for pcDNA4/TR vector transfect

1 % Fungizone - Antifungal agent

The isotope labeled amino acids, lysine (+8 Da) and arginine (+10 Da), antibiotics and antifungal were incorporated to DMEM: F12 for SILAC media (Thermo # 88215) and mixed thoroughly with gentle shaking and sterile filtered with a 0.2 micron disposable filter apparatus. To this filtered DMEM. F12 media, dialyzed FBS is added to a final concentration of 10 percent and mixed gently. The SILAC media is labeled as heavy media for cell culture enrichment.

Similar to the above media incorporation, unlabeled amino acids lysine and arginine were added to DMEM: F12 media, 10 % dialyzed FBS and all the antibiotics and 10 % Dialyzed FBS (Thermo # 88440) as the final concentration.

Table 10. Two Plex SILAC Media Composition for HEK 293 CCR3 Stable Cell Line.

DMEM. F12 Light Media Composition for 250 ml	**DMEM. F12 Heavy Media Composition for 250 ml**
DMEM F12 for SILAC	DMEM F12 for SILAC
10 % Dialyzed FBS	10 % Dialyzed FBS
1 % Penicillin-Streptomycin: antibiotic	1 % Penicillin-Streptomycin: antibiotic
20 ug/ml zeocin: selection antibiotic	20 ug/ml zeocin: selection antibiotic
5ug/ml blasticidin: selection antibiotic	5ug/ml blasticidin: selection antibiotic
1 % Fungizone: antifungal agent	1 % Fungizone: antifungal agent
12.5 mg – Lysine (L)	12.5 mg - Lysine (H) +8 Da (C13, N15)
6.25 mg – Arginine (L)	6.25 mg-Arginine (H)+10 Da (C13,N15)
25 ml Dialyzed FBS	25 ml Dialyzed FBS
Amino Acid Supplement	Amino Acid Supplement
L-Leucine 104 mg/liter	L-Leucine 104 mg/liter
L-Proline 10 mg/liter	L-Proline 10 mg/liter

Note: L-Leucine of 104 mg/liter, L-Proline of 10 mg/liter amino acid were supplement in the respective heavy and light SILAC growth media. Arginine to Proline interconversion can be inhibited by incorporation a little excess of L-proline in the culture media [43].

6.12 HEK 293 Cell Line Enrichment with SILAC Culture Media

Healthy HEK 293 cells grown in SILAC media were split in equal proportion and enriched with the above labeledheavy and light SILAC DMEM culture media. Cell growth was assessed for viability and split in 1.6 dilution for each generation of propagation. The enrichment process with split-dilution and passaging was carried out for six consecutive generations. This step is carried out to have isotope enrichment up to 95-98 % enrichment with the isotope labeled Lysine (+8 Da) and Arginine (+10 Da) in the entire proteome of the HEK 293 CCR3 stable cell line. Then the cell cultures were scaled up in three T 75 (225 cm^2) culture flasks for both heavy and light labeled HEK 293 cultures.

6.13 Serum Starving of SILAC HEK 293 and CCR3 Induction

After six generations of passaging in SILAC media heavy and light HEK 293 were induced to produce CCR3 receptor with1ug/ml of tetracycline as the final concentration in the DMEM media for 4 hours. This step is carried out for limited induction of CCR3 on the cell membrane, and not an overexpression of CCR3. Later the HEK 293 are let to starve for 15 hourswith starving media. DMEM media is changed with DMEM without FBS (cell starving media). The rationale for cell starvation is to promote cell cycle synchronization before stimulation the CCR3 receptor with eotaxin (CCL11).

Eotaxin Stimulation to Promote Downstream Signal Mechanism: Eotaxin (CCL11) is one of the potent natural agonists for theCCR3 receptor. Eotaxin (CCL11) stock solution was prepared with 0.1 % human serum albumin (HSA) in 1X sterile PBS. The final stock solution is prepared at 0.2 mg/ml or 0.2 ug/uL. Eotaxin stock solution is stored in -20celsius freezer in 10 uL aliquots for future use.

In the final step of SILAC methods to study temporal phosphorylation, eotaxin (CCL11) is used in stimulation HEK 293 for 5 minutes at 37 C. The *light* sample is the control untreated sample, no eotaxin stimulation, but exposed to 0.1 % HSA. The heavy isotopic amino acidlabeled HEK 293 were stimulated at 100 nanograms/milliliter of eotaxin for 5 minutes with gentle mixing with SILAC DMEM media. Later the respective HEK 293 were washed immediately with 4 C PBS, then the cells were harvested by scraped and pelleted by spinning at 8000 g for 20 minutes. The harvested cell pellet is snap frozen in liquid nitrogen and stored in - 80 C for further processing for protein extraction and digestion.

6.14 Cell Lysis and Protein Extraction, -20 C Acetone Precipitation and Solubilization

RIPA cell lysis buffer (Thermo # 89900) is used as the base buffer with 1X protease (Halt Protease inhibitor 100X tablets EDTA-freeThermo # 78437) and 1X phosphatase (Pierce phosphatase inhibition tablets EDTA free, catalog # 889669). The cell pellet was resuspended in this buffer with intermittent vortexing, once the cell pellet is re-suspended, the sample is sonicated for one minute intervals at 12 %, three times on

ice at 4 C. The soluble cellular proteinis precipitated with - 20C cold acetone (acetone chilled at - 20 C for 5 hours). Five volumes of chillacetone were added to the cell lysate, then vortexed well, and kept at - 20 C for 3 hours. Protein precipitation occurs upon addition of chilled acetone;the tube was spun at 5000g for 10 minutes at 4C.

The acetone-precipitated protein pellet is air dried and resuspended in protein denaturing buffer comprising of 6M urea 2M thiourea, 10mM HEPES, pH 8. The protein precipitated cell pellet was gently vortexed to resuspend the pellet until the pellet is completely soluble. The denatured protein lysate sample was reduced with TCEP (10 mM) at 45 C for 1 hour followed by alkylation with 50 mM Iodoacetamide at 40 C for 1 hour. A total of 3 ml of lysate was present. The sample was later diluted to less than 2 M urea with 50 mM Ammonium bicarbonate (AMBIC), before Lys-C-trypsin double digestion procedure.

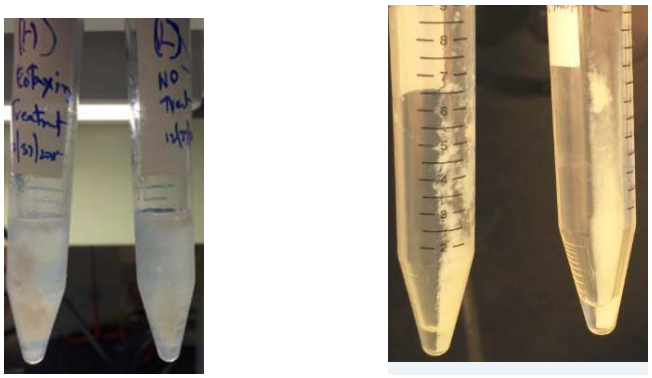


Figure 53. SILAC HEK 293 Cell Lysate and -20 C Protein Precipitated for Phosphoproteomics Study. Left image is the -20 C Acetone precipitated pellet, right image is the acetone decanted precipitated protein pellet.

6.15 BCA Colorimetric Assay: Protein Concentration Estimation

Thermo Scientific BCA assay reagents A and B (Catalog # 23225) are used with bovine serum albumin standards (BSA) for colorimetric estimation of the total cellular protein extract. BSA standards were prepared in increased concentrations of 2 mg/ml, 0.8 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.25 mg/ml and 0.125 mg/ml for creating linear calibration curve. The protein samples of heavy labeled and light samples were diluted by 3X and 4X and the protein concentration were determined.

BCA assay results: It was found the colorimetric assay failed to provide the concentration estimate. Later after checking the compatibility of BCA reagents with thiourea, incompatibility of thiourea is reported.

Note: The presence of 2M thiourea in the HEK 293 SILAC cell lysate supernatant interfere with BCA colorimetric assay (deep blue wells of columns D,E,F) rendering BCA assay incompatible with lysis buffers having thiourea.

A660 colorimetric protein concentration assay for thiourea containing cellular protein extracts: A660 colorimetric assay is an alternative protein concentration assay for cell lysate protein extracts in thiourea buffer. Fourfold dilution of the extracted protein samples of the *control* (Light) and *test eotaxin treatment* (Heavy) sampleis done before the assayis performed. For Heavy labeledsample, the protein concentration yield is estimated to be 2 mg/ml, and for Light sample it is 1.64 mg/ml.

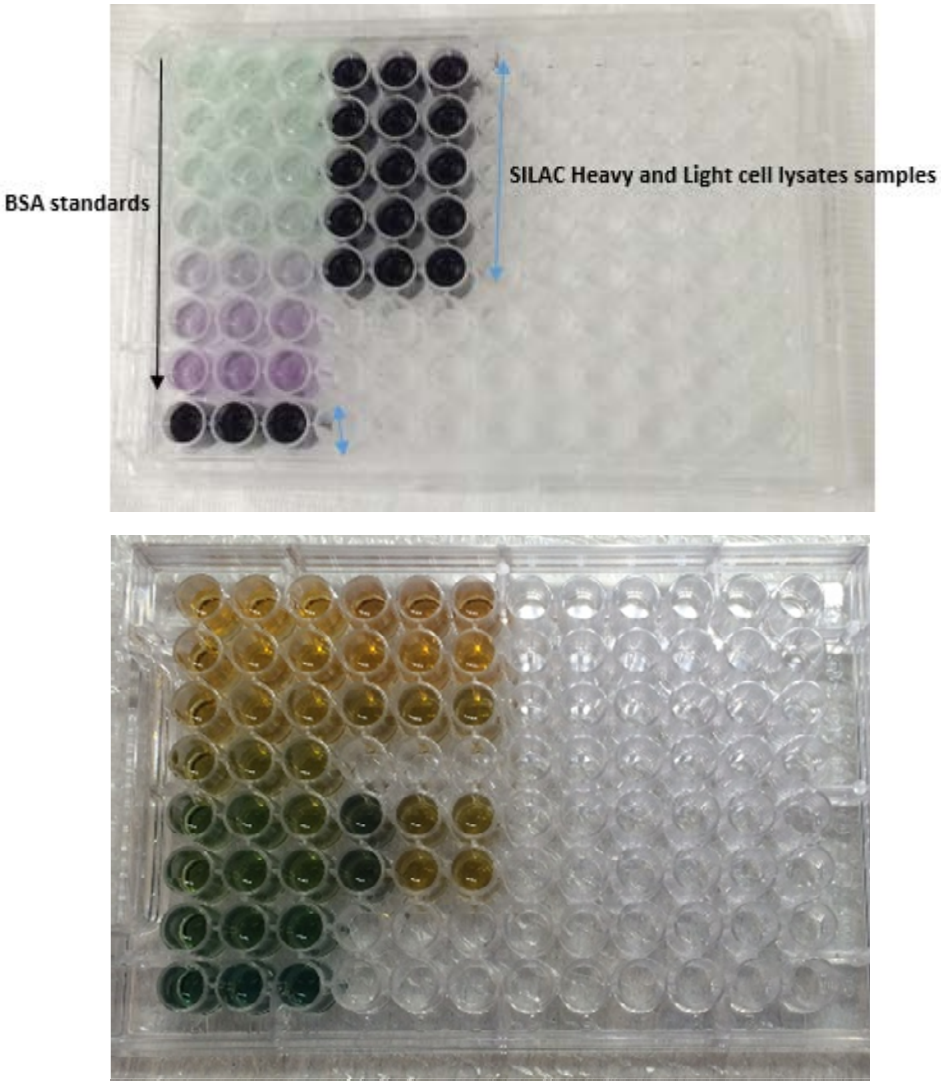


Figure 54. Top is the BCA Colorimetric Protein Concentration Assay Plate with Thiourea Incompatibility. Bottom Image is the A660 Colorimetric Protein Concentration Assay Plate.

6.16 SILAC HEK 293 Protein Lysate Mixing of Light and Heavy Labeled in 1:1 Ratio

Cell Lysate Protein Extract: After estimating the total protein concentration for light (control sample) and heavy (treatment sample), 1:1 ratio of Light and Heavy protein extracts of 2.7 mg light and 2.7 milligramsheavy lysate was mixed. Total of 5.4 mg is used for sequential digestion with sequencing grade recombinant endoproteinase Lys-C and later by sequencing grade trypsin

Sequencing Grade Enzymatic Digestion of SILAC Sample: Recombinant endoproteinase Lys-C (rLys-C) in the ratio of 1:150 was used to digest under denaturing conditions (6 M urea+ 2 M thiourea) at 37 C for 3 hours. Later the urea was diluted to less than 2 M and further digest with sequencing grade trypsin digestion at 1.50 ratio for 15 hours. Before the digestion steps is carried out, the sample is adjusted to pH 8 for trypsin activity. Final digestion process was quenched with 1 % formic acid and stored at - 80 C for further processing. A total of 12 ml of digested lysate is noted at the end of Lys-C/Trypsin digestion.

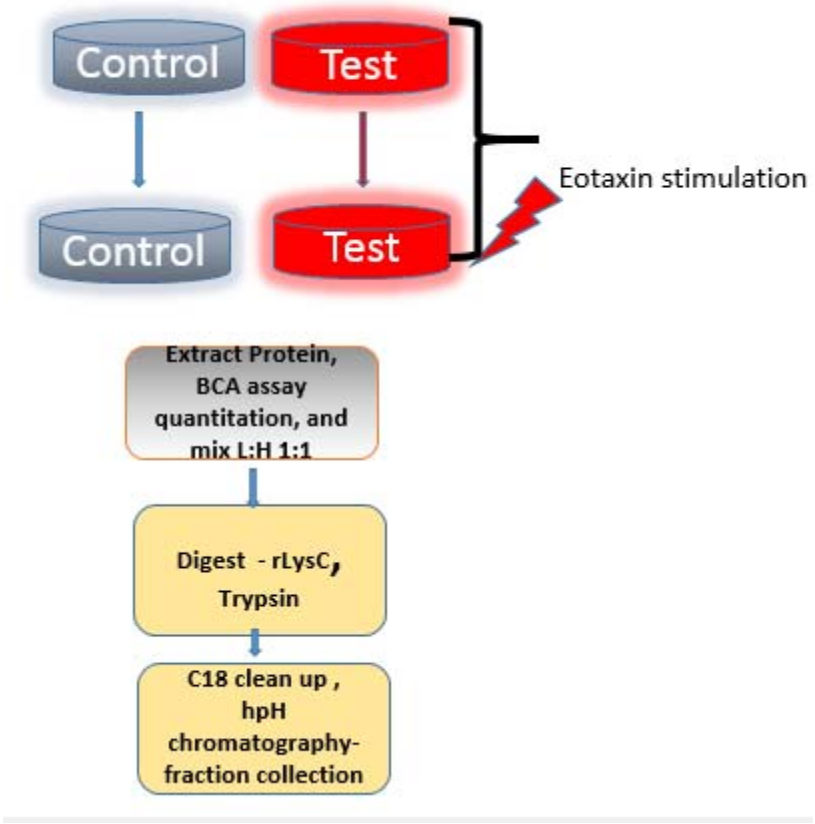


Figure 55. Two Plex SILAC Workflow for HEK 293. After hPH Fractionation, each fraction is enriched for Phosphopeptides for TiO_2 enrichment followed by LC-MS/MS Analysis.

Digestion Quenching and C18 Solid Phase Extraction-Peptide Clean-Up: The digested samples were quenched with 1 % formic acid and carried for C18 SPE clean-up. Three 1 CC (100 mg) C18 SPE cartridges (Sep-Pak, Waters Corporation).

Note: During C18 SPE clean-up process the vacuum applied on the manifold waslowat 5 mm Hg,and the C18 cartridge was never let to dry. Supelco vacuum manifold and Waters C18 SPE cartridge are used in peptide sample clean-up process.

SPE Protocol Steps: First step of wetting stationary phase is with 100 % methanol, followed by 100 % acetonitrile.

1. Equilibrating stationary phase with 3 ml of 0.1 % Trifluoroacetic acid
2. Peptide sample is acidified with 0.1 % TFA; pH confirmed to 2.3 - 3, later loaded slowly on to SPE C18 cartridge with vacuum for 10 minutes.
3. Washing the cartridge: after peptide binding to C18 stationary phase of the cartridge by hydrophobic interaction, the cartridge is washed with 0.1 % TFA in 95 % water, 5 % Methanol. 2 ml of wash solution was used in the washing process.
4. Trifluoroacetic acid removal: 0.1 % TFA from the cartridge was removed by washing with 300 uL 0.5 % Acetic acid in H_20.
5. Peptide elution: Two sequential elutions were carried, first with 40 % ACN, 0.5 % acetic acid, followed by 60 % ACN, 0. 5 % acetic acid.

Note: Low vacuum is applied on the Supelco extraction manifold, less than 5 mm Hg is used while operating the vacuum manifold in the C18 solid phase extraction (SPE) peptide clean-up process.

Total of 5.5 ml of cleaned peptide elutionsare pooled, and a small aliquot of the sampleis taken for Nanodrop UV 220 measurements for peptide concentration estimation.

Lyophilizing C18 Cleaned Peptide Samples: The C18 cleaned and pooled peptide sample was flash frozen in liquid nitrogen and then lyophilized for 9 hours to complete dryness in a Labconco Lyophilizer. Later stored at - 80 C for further fractionation process with Waters Alliance HPLC - DAD detector and fraction collector in tandem.

6.17 First Dimension Fractionation with High pH C18 Reverse Phase Chromatography: Method Development for HEK 293 Digests: Complex Peptide Samples

First dimension separation-fractionation is carried out to reduce the complexity of the sample for second dimension nanoflow LC-MS/MS analysis. Separation based upon hydrophobicity at basic pH or high pH (pH 10) is employed. Reverse phase chromatography under basic pH conditions are carried out. One of the primary advantages of reverse phase chromatography under basic pH over strong cation exchange chromatography (SCX) is the number of distinct peptides identified are significantly higher in RP HPLC at hPH conditions and also there is no necessity of having an additional C18 clean-up of SCX fractions before downstream phosphopeptide enrichment [44].

HPLC Mobile Phases used in hPH Reverse Phase Chromatography

Mobile Phase A - 10 mM ammonium formate (buffer A).

Mobile Phase B - 10 mM ammonium formate in 90 % Acetonitrile (ACN) (Buffer B).

Buffers A and B with 10 with ammonium hydroxide are pH adjusted to 10. HPLC method under hPH conditions is carried out with Waters Xbridge C18 reverse phase HPLC column (column dimensions; 4.6 × 150 mm, Waters product number. 186003034). Initially, 2 to 4 milligrams of HEK 293 digested samples were analyzed. The digested peptide sample are separated in a shallow gradient of 90 minutes.

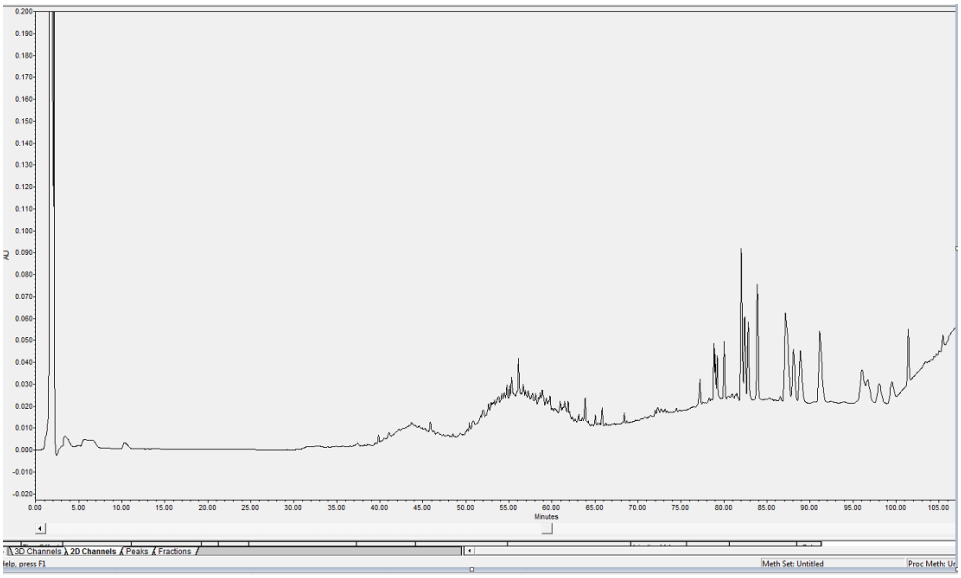


Figure 56. Reverse Phase Chromatography at High pH (hPH) of 1 Milligram HEK 293 Protein Digest. 220 nm UV Absorbance Chromatogram in Empower 3. Waters Xbridge C18 Reverse Phase Column (4.6 mm × 150 mm).

The C18 cleaned and totally dried peptide sample was re-suspended in Mobile Phase A (10 mM ammonium formate) is used as sample injection. The sample is loaded at 1 ml per minute with auto-sampler.

Rationale for a very shallow initial gradient is for selective elution of hydrophilic phosphopeptides over a longer time frame; The gradient was swallowed over a longer period 1 - 35 % B over 60 minutes.

Peptide separation was carried out with a gradient flow rate of 1 ml/minute from 1 % A to 35 % B in 60 minutes (0.58 % per minute gradient). From 35 % to 70 percent in 15 minutes and ramped up to 90 in 5 minutes and held for 5 minutes and brought back to 1 % B in 15 minutes and the column was re-equilibrated for 30 minutes at 100 % of buffer A.

A total of 65 fractions were collected at1-minute intervals and frozen in -80 C freezer for the following steps of lyophilization and phosphopeptide enrichment with TiO_2 enrichment and liquid chromatography mass spectrometry-based analysis for phosphopeptide characterization. Future experiments were stopped here for future analytical workflow.

6.18 Selective Enrichment of Phosphopeptides of HEK 293 Cell Line with Titanium dioxide (TiO2) Resin

To develop TiO_2 based phosphopeptide enrichment workflow, healthy HEK 293 were grown in three T 75 culture flask followed by serum starving for 18 hours with brief serum stimulated for 5 minutes. Later these cells are harvested, this cell pellet is lysed

mildly with a mechanicalsonicator. Then the lysate was spinned at 15000 g for 20 minutes at 4 degree Celsius to sediment cell debris. The cell lysate supernatant was denaturing, reduction with 20 mM TCEP at 40 C in the dark for 20 minutes and alkylation with 50 mM iodoacetamide at 40 C in the dark for 45 minutes. This reduced-alkylated sample is digested with sequencing grade trypsin enzyme; Methods described earlier for two plex SILAC HEK 298 protocol. This HEK 293 cell lysate supernatant digest-peptide sample is C18 cleaned with a 200 mg Waters C18 Sep-pack cartridge and lyophilized overnight. This peptide sample is used for phosphopeptide enrichment with TiO_2 enrichment protocol and consecutive LC-MS/MS for the number of phosphopeptide-phosphorylated protein identification and the extent of phosphopeptide enrichment for the overall experimental protocol.

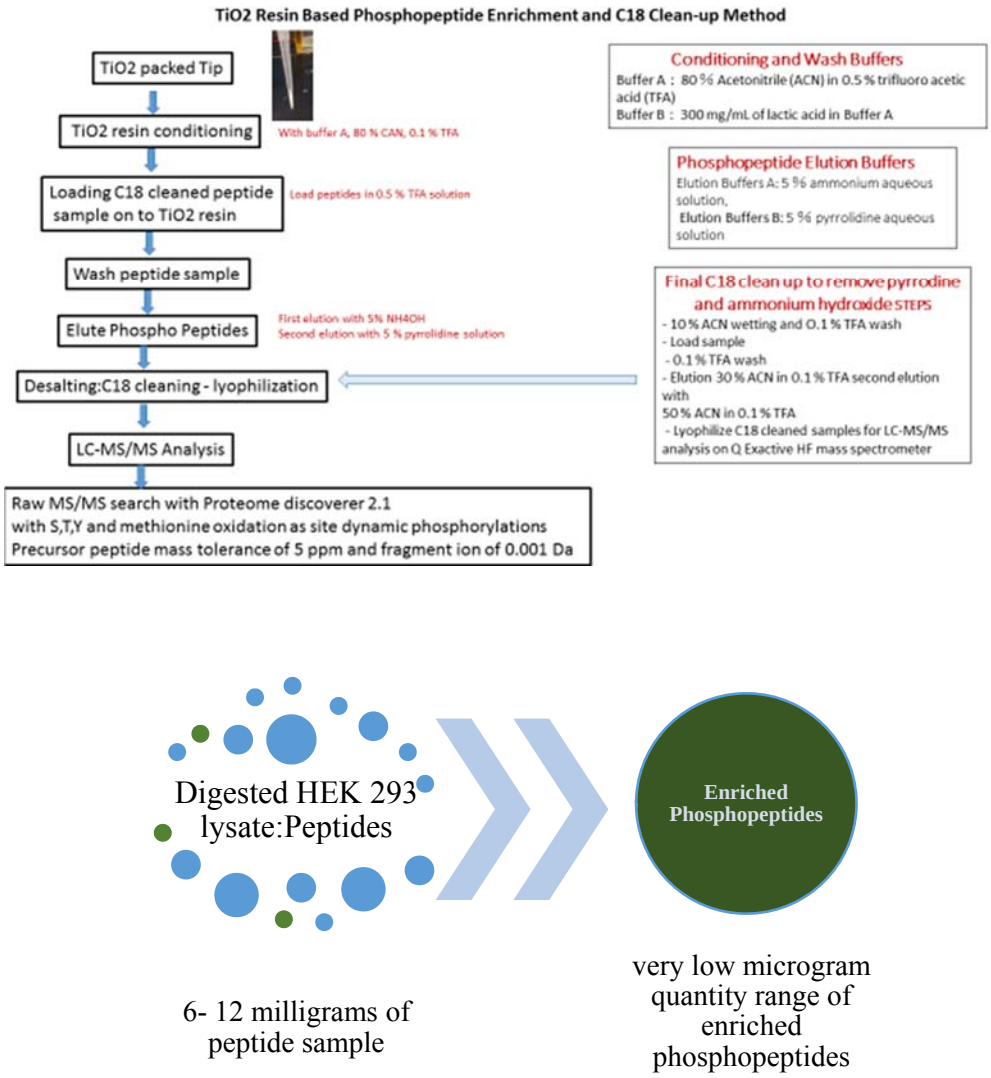


Figure 57. Top: Protocol Flow Chart: Phosphopeptide Enrichment with Titanium dioxide (TiO_2) Nanoparticle-Resin. Bottom: Phosphopeptides as a Subset of Peptides Enriched by Selective Enrichment and in Low Concentration.

6.19 LC-MS Analysis of Phosphopeptide Enriched Samples

The enriched phosphopeptide and C18 solid phase extraction cleaned are analyzed with an LC-MS platform, with front end Ultimate 3000 nanoflow LC coupled to an Orbitrap mass spectrometer, Thermo Q Exactive HF mass spectrometer. Phosphopeptides

were separated with a 75 μm i.d C18 reverse phase LC column. Details of the nanoflow LC gradient was discussed earlier in Chapter IV. Mobile phase A constituting of DI H_2O with 0.1 % formic acid is used to load the peptide samples on to the C18 nanoflow column. Nanoflow LC gradient is set at 250 nanoliters per minute. Phosphopeptidesare eluted with mobile phase B (98 % ACN in 0.1 % formic acid). Phosphopeptides are comparatively more hydrophilic in nature over their non-phosphorylated analogs. Hydrophilicity renders them to elute in the earlier phase in a reverse phase LC gradient: with lesser organic phase (mobile phase B under 30 %).Peptide sample mass spectra is acquired on a Q-Exactive HF mass spectrometer is operated in positive polarity with full survey scan range at 400 - 1950 m/z, survey scan resolution set at 120,000 at 200 m/z. Automatic gain control is setting at 100,000 and maximum injection time of 100 milliseconds. Data dependent top 15 methodsis used for HCD fragmentation of the precursor ions, isolation with of 1.4 m/z, normalized collision energy of 28, charge state exclusion of unsigned ones of 1, 7, 8, > 8. Dynamic exclusion is set to 20 seconds, and the MS spectra are in profile mode.

6.20 MS/MS Spectra Data Analysis with Proteome Discoverer 2.1 and MaxQuant

The raw tandem MS data is acquired with the Q-Exactive HF mass spectrometer. Raw MS/MS spectra are processed in MaxQuant, and Proteome Discoverer 2.1 for peptide spectral matches (PSMS) and protein ID hits with Sequest HT spectra search algorithm with a false discovery rate (FDR) of 0.01 under 5 ppm precursor ion mass tolerance and 0.01 Da fragment ion tolerance. Methionine oxidation (+16 Da) Serine, Threonine, and Tyrosine (+79 Da) is set as dynamic modification and

carbamidomethylation (+57 Da) was set as fixed modification in the Sequest HT search analysis nodes. The final peptide spectral matches (PSMs) identified were reported with their respective proteins and their functional annotations.

For two plex SILAC enriched samples the analysis workflow is similar to the above with an addition dynamic modification of C13, N15 lysine (+8 Da) and C13, N14 arginine (+10 Da).

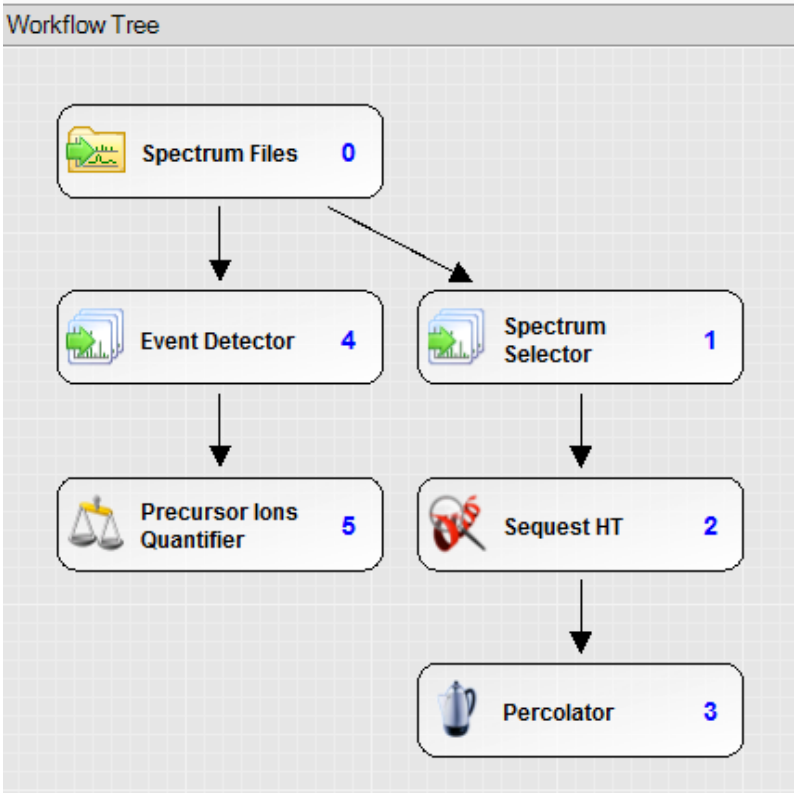


Figure 58. Analysis Nodes in Proteome Discoverer 2.1 for Phosphopeptide ID Search in 2 Plex SILAC Method Development Workflow for HEK 293.

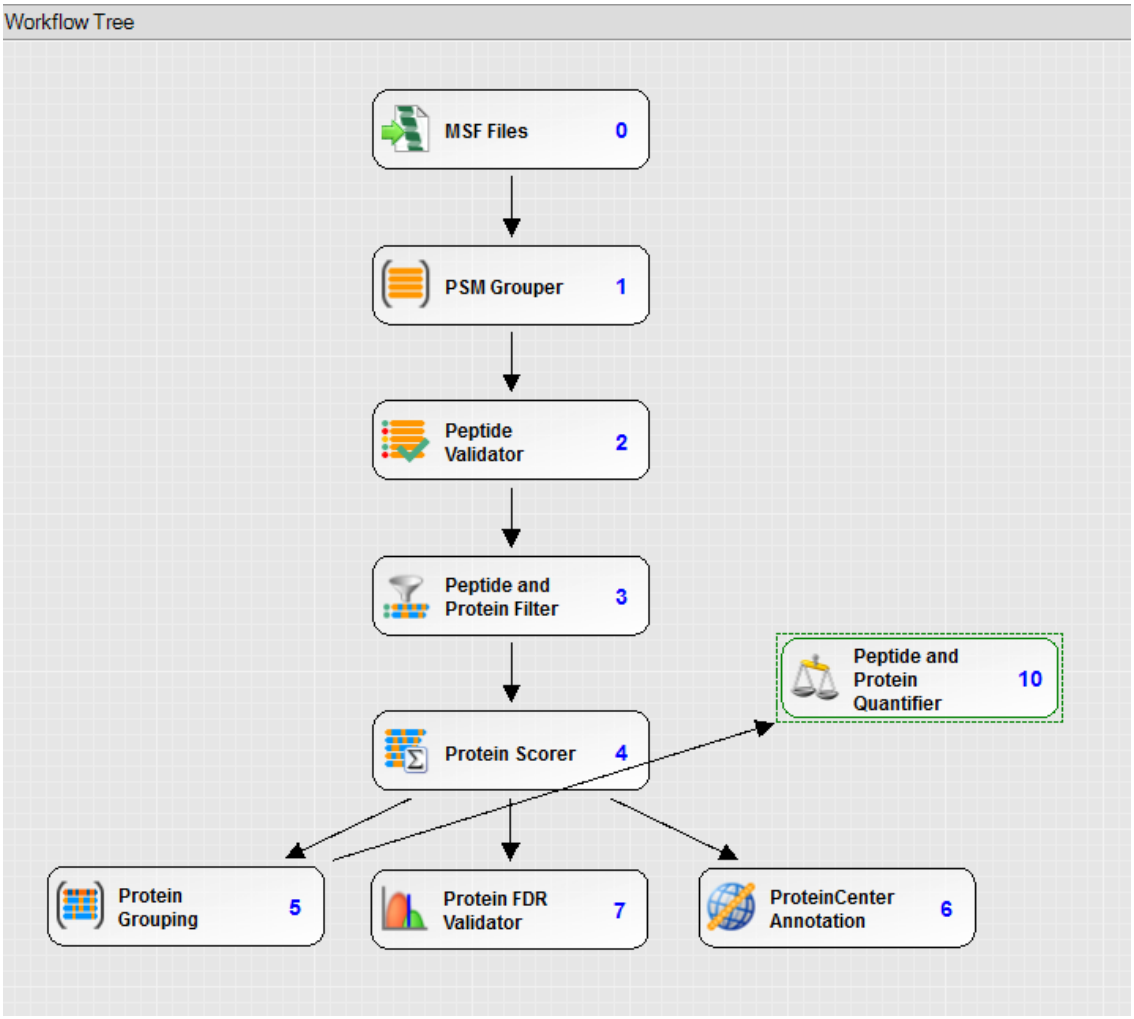
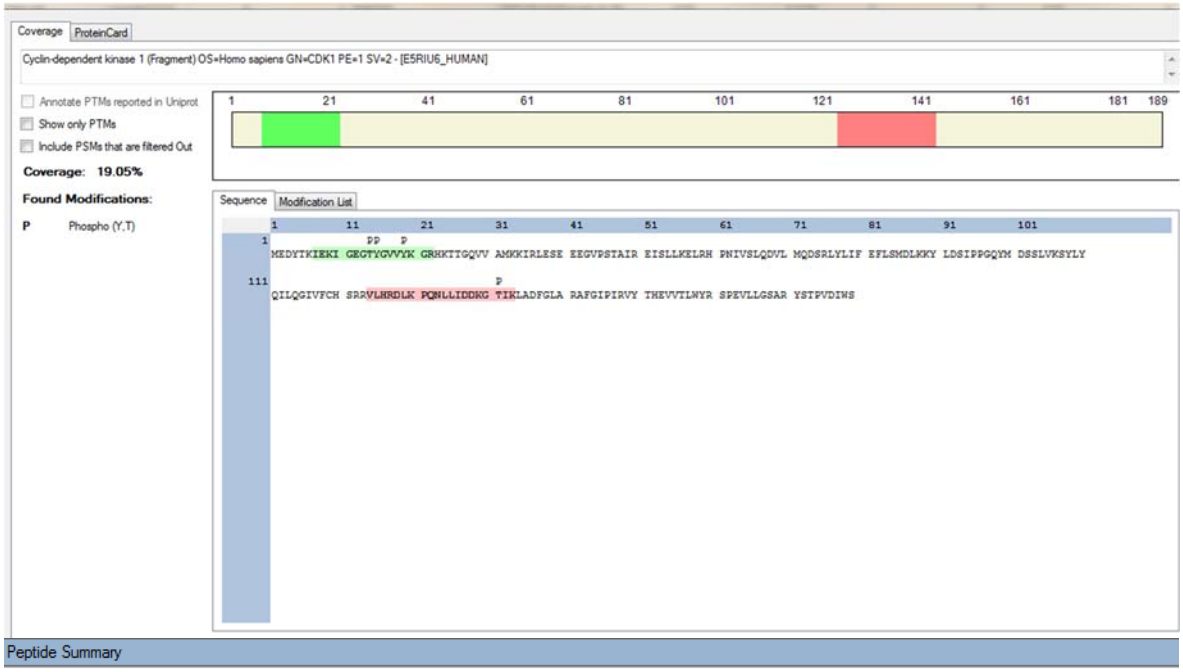


Figure 59. Proteome Discoverer 2.1 Consensus Nodes for Phosphopeptide ID Search.



Sequence: IGEGTYGVVYKGR, Y6-Phospho (79.96633 Da)
Charge: +2, Monoisotopic m/z: 739.85535 Da (-0.25 mmu/-0.34 ppm), MH+: 1478.70342 Da, RT: 49.7373 min,
Identified with: Sequest HT (v1.17); XCorr:3.62, Percolator q-Value:0, Percolator PEP:7.31e-09, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

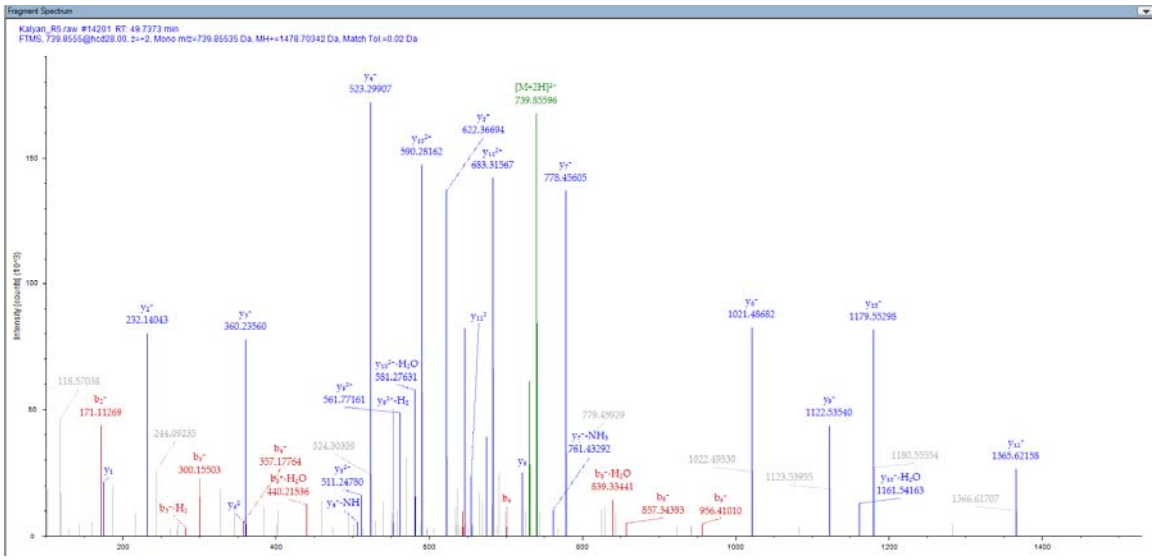


Figure 60. Tyrosine Phosphorylated Peptide of Cyclin-Dependent Protein Kinase with Phosphorylation Site Localization.

6.21 HEK 293 Cell Line Phosphopeptide Analysis: Method Development, Results and Conclusions

We Successful development of a two plex **S**table **I**sotope **L**abeling in **C**ell **C**ulture successfully (SILAC) enrichment workflow of the HEK 293 cell line with C13 and N15 labeled lysine and arginine. The HEK 293 CCR3 expressing stable cell line developed in our laboratory. This HEK 293 CCR3 cell line was used to induce phosphorylation with the nature ligand eotaxin (CCL11) on the CCR3 receptor; this cell line can be used to study temporal phosphorylation dynamics in two physiological states; *control* and *treatment* groups. Further to development TiO_2 based phosphopeptide enrichment. Serum starving followed by transient serum stimulation was carried out to induce phosphorylation on this HEK 293 cell line. Analytical workflow for selective enrichment of phosphorylated peptides over non – phosphorylation peptides was successful established. The overall methods of phosphoproteome analysis of HEK 293 cell line with first dimension high pH reverse phase (C18) chromatography followed by TiO_2 IMAC phosphopeptide enrichment - purification for mass spectrometry is successfully developed. We were able to observe greater than 80 % phosphopeptide enrichment with TiO_2nanoparticle-based phosphopeptide enrichment when a small aliquot of phosphopeptides enriched sample was analyzed with nanoflow LC Q-Exactive HF mass spectrometer. This overall analytical workflow to study temporal phosphoproteome of cellular states can be expanded to three-plex SILAC in HEK 293 cell line or similar cell line. Two or three-plex SILAC experiments provide a relative quantitative method between two or three biological states of the isotope labeled cell line after isotopic

arginine and lysine enrichment and their perturbation to alter their cellular physiology and in-turn their proteome states.

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