IS DESALTING IMPERATIVE IN ACCURATE ANALYSIS OF PROTEIN PROFILE UPON SUBCELLULAR FRACTIONATION

A Dissertation submitted in partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

By

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CERTIFICATE

This is to certify that the dissertation entitled "**IS DESALTING IMPERATIVE IN ACCURATE ANALYSIS OF PROTEIN PROFILE UPON SUBCELLULAR FRACTIONATION**" is a record of work done by Ms. AMINA AFNAN P T P, in partial fulfillment of the requirement for the award of the degree in MSc. Biotechnology.

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Date: 03/06/2020

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राजीव गाँधी जैव प्रौद्योगिकी केन्द्र, तिरुवनन्तपुरम 695 014, केरल, भारत.
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DECLARATION

I hereby declare that the dissertation entitled **"IS DESALTING IMPERATIVE IN ACCURATE ANALYSIS OF PROTEIN PROFILE UPONSUBCELLULAR FRACTIONATION"** submitted to Kannur University, in partial fulfilment for the award degree of Master of Science in Biotechnology is a record of work done under the supervision and guidance of **Dr. Rashmi Mishra,** Scientist EI, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

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Place: Kannur

Date: 03/06/2020

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ABSTRACT

Purified proteins or nucleic acids are required for the accurate analysis of target and there are different techniques to purify these compounds and desalting is one among them. Desalting is a process by which the salts and other unwanted molecules from sample is removed to make it purified and concentrated. Samples are usually taken directly after lysate preparation for analysis. Here we tried to study the electrophoresis ability of proteins with and without desalting, post lysate preparation. For this, a comparative study was conducted by obtaining two samples of lysates: one desalted and the other normal (not desalted).Both the protein samples were run on an SDS PAGE gel followed by western blotting. The study suggests an additional step of desalting to be done for accurately analyzing the protein sample concentration and to obtain better results in the consequent steps of experimentation. Through this study we found that there is a differential transfer of protein bands from the SDS-PAGE gel to blotting membrane during electro-transfer, in the presence of high salt concentration. High salt concentration is one of the major components in nuclear lysate preparation buffers, in the sub cellular fractionation protocol. The hypertonic solution used in nuclear lysis has to be desalted for the efficient transfer of protein from SDS-PAGE gel to blotting membrane. This study envisages light upon the importance of a commonly neglected step in the sample preparation for western blotting, that can have larger context dependent consequences like inaccurate results which can lead to misinterpretation of the data.

CONTENT

INTRODUCTION:

The molecules that plays a major role in supporting life was named as protein by Berzelius in 1838 to indicate its importance. The overall protein content present in a cell is represented as proteome. For understanding the cellular processes in a clear manner a direct comparison of protein distribution among nucleus, cytosol, and plasma membrane has to be done. The proteome of the cell can be studied through proteomics, which identifiesall the proteins present in a cell, tissue or organism. Mark Wilkins was first one to use the term proteomics in 1996. It helps in early diagnosis of disease, to monitor disease development, for drug development based on target molecules and in characterizing expression, function, and modification of any protein. Traditionally used technique for protein purification include chromatography based methods like ion exchange chromatography, size exclusion chromatography, and affinity chromatography andthe advanced techniques used for separation involves gel based like Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two dimensional gel electrophoresis(2-DE)and two dimensional differential gel electrophoresis(2D-DIGE) and protein microarray, mass spectrometry etc. Techniques like enzyme linked immunosorbent assay (ELISA) and western blotting can be used for analyzing the separated protein content. Large volume of proteomics data can be collected by high throughput techniques like X-ray crystallography, NMR spectroscopy etc. In this rapidly growing field of proteomics, methods like sub-cellular fractionation plays an important role.

Isolation of particular membrane or sub cellular organelle is required in several experiments to study the structure and function of different proteins having distinct localization. Disrupting cells helps in the isolation of subcellular compartment of interest in high yield. There are different approaches for the isolation of subcellular fraction involving traditional methods like density gradient centrifugation and innovative techniques like use of SPIONS or separation by specific buffers. Subcellular fractionation along with separating compartments of cell reduced the complexity of proteins by providing its identification easier. For unknown protein identification, the

ultimate goal is to simplify the protein pool, which is done by sequential centrifugation and more specific techniques like subcellular fractionation and chromatographic techniques are used in case the protein of interest is known.

Proteins are usually taken directly for quantitation and analysis after fractionation technique without considering the factors that can affect the concentration of proteins during fractionation technique taking in to wrong assumption. There are no reports on the effects of desalting after protein isolation but salts used in the buffers for the separation of cell compartments can interrupt the detection of actual protein concentration. So here, my aim was to determine the importance of desalting in accurate analysis of protein profile upon subcellular fractionation.

AIM AND OBJECTIVE:

AIM-

To determine whether desalting is imperative in accurate analysis of protein profile (sub-compartment distribution) upon subcellular fractionation.

OBJECTIVE-

- Growing single cell colonies from DH5α carrying HA tagged 55kD protein coding gene plasmid
- Isolating plasmid of HA tagged 55kD protein coding gene from single cell colonies of DH5α *via* midi plasmid DNA isolation method and quantitation of plasmid DNA yield
- To transfect a HA tagged 55kD protein coding gene plasmid in mammalian cell line (astrocytes)
- Perform immunohistochemistry mediated analysis for confirmation of plasmid transfection
- Perform subcellular fractionation to separate cytoplasmic from nuclear fraction using hypotonic-hypertonic (osmotic) lysis solutions
- Perform desalting or not of the separated fractions *via* spin column method
- Perform protein quantitation of desalted and non-desalted fractions *via* BCA method
- Estimate SDS page-Western mediated protein transfer efficiency in desalted *vs.* non-desalted fractions
- Analyze and report results.

REVIEW OF LITERATURE

SUB-CELLULAR FRACTIONATION-

Sub cellular fractionation, a process of separation of different parts of a cell from each other with the help of centrifugation is a procedure that depends on size and density of organelles. It is a process that helps in studying the specific intracellular protein of different compartment of a cell. This technique was applied for the first time in separating organelles derived from rat liver. Two steps are mainly involved in sub-cellular fractionation including, disruption of cellular organization (homogenization) and fractionation of homogenate to get organelles separated. This homogenate can be fractionated by differential centrifugation in to different population depending on size, charge density etc. which contains,

- Nuclei, heavy mitochondria, cytoskeletal networks, plasma membrane
- Light mitochondria, peroxisomes, lysosomes
- Endoplasmic reticulum, Golgi apparatus, Endosomes and microsomes
- Cytosol

The main aim of homogenization technique is to provide high degree of cell breakage using minimum disruptive force in order to release organelles of interest without any damage to avoid protein denaturation. It provides a sufficient stress to cells to disrupt the surface membrane and to release the cytosol and internal organelles present. Depending on different important factors like presence or absence of intracellular organelles, presence or absence of cell wall and the structural organization in case of organized tissue- the procedure for homogenization technique was chosen. In addition to plasma membrane, all eukaryotic cells except human erythrocyte contain number of intracellular organelles where the homogenization technique is terrible to break the surface membrane which may cause damage to releasing organelles where as in case of human erythrocyte and bacteria that does not contain any other internal membranes homogenization is concerned with breakage of surface membrane and its isolation. Most of the cells are comprised with different macromolecular structure peripheral to the plasma membrane involved in adhesion, movement, recognition and development

which has no significant role in this homogenization process where as this process is concerned in case of cells lining the lumen of mammalian digestive tract where the superficial layer is elaborated in to an extensive mucous layer. (Huber LA, et al.,2003)

For the isolation of all organelles other than nuclei from the mammalian cells, commonly used standard isotonic medium is 0.25M sucrose containing 1mM EDTA and buffering with suitable organic buffer like Tris, HEPES or Tricine (commonly used at pH 7.0 to 7.6). A high percent of cell lysis is documented specially for cell cultures when Triethanolamine used in conjunction with acetic acid and if homogenization is done for the recovery of nuclei, EDTA is replaced with low concentrations of divalent cations like MgCl_2 or $CaCl_2$ and KCl where $CaCl_2$ is a component of plant media and MgCl_2 is commonly used in animal media as calcium can activate phospholipases, proteases and inhibit RNA polymerase. There are media specific for the isolation of specific organelles. In case of microorganisms the media composition is entirely different which is comprised of 50mM Tris or phosphate buffer with low concentration of MgCl_2 and EDTA. To protect organelles from the damaging effects of proteases a cocktail of protease inhibitors are commonly included in the homogenization medium. For mammalian cells, mixture include phenylmethylsulfonylflouride (PMSF) stored in ethanol, leupeptin, antipain stored in 10% dimethyl sulfoxide and aprotinin stored in water. It is necessary to stress the cells osmotically by hypo-osmotic medium rather than homogenizing with standard iso-osmotic medium, where the commonly used medium is 1mM sodium bicarbonate of pH 8.When EDTA is added with it nuclei gets fragile and are more susceptible to leak DNA. (Graham John, David Rickwood,1997)

Most widely applied methods for homogenization include mechanical or liquid shear and the device applicable depends on the nature and severity of force required to disrupt the tissue. Stressing cells by suspending in a hypo-osmotic medium is sufficient to cause lysis as all cells are surrounded by osmotically active surface membrane or it can be used in combination with mechanical or liquid shear method. It is found necessary to stress the cells osmotically before disrupting by other methods. Chemical methods like the use of hydrolytic enzymes are usually used for the degradation of cell wall of plant tissue, bacteria, fungi etc. that produce protoplast or spheroplast whose surface

membrane can then be taken for fine disruptive forces. It is found that low concentration of Nonidet NP-40 may be used in few instances to weaken the surface of cultured cells especially for the internal organelle such as nucleus which is less susceptible to detergents than plasma membrane. Homogenizers are of two categories-Type 1 where the material subjected to disruptive force once and type 2 where the material exposed to continuous disruptive force in which these homogenizers disrupts cells or tissues in different means.

TYPE 1 HOMOGENIZER

Homogenizer rely on liquid shear to disrupt the cell, the suspension is propelled by a piston through a narrow orifice. Here, pressure is applied to the piston by compressed air repeatedly but it can also be electrically driven. For the homogenization of micro-organism with tough outer wall French pressure cell is commonly used in laboratory but it has been used for breaking membrane vesicles by Higgins which operates at high hydraulic pressure of about 15000-20000 psi. In addition to yeast and bacteria the Stansted cell disruptor that operates at low pressure has been used for homogenizing lymphocytes. The size of orifice and the pressure given will control the severity of shearing force.

In nitrogen cavitation, a pressure vessel is used which consist of a robust stainless steel containing an inlet port for the delivery of gas from a cylinder and an outlet tube with a needle valve. Under high pressure, nitrogen dissolves in the cytosol and suspension medium of cells, and the internal pressure forces the suspension through the outlet tube when the needle valve is opened and when it becomes exposed to atmospheric pressure a rapid decompression occurs . Nitrogen gas produce bubbles in the cytosol and in the medium and this sudden formation of bubbles results in cell disruption. Oxygen free nitrogen at pressures of about 800 psi for 10-30 minutes is given for equilibration of stirred cell suspension in the technique nitrogen cavitation which have many advantages as it is highly reproducible and is successful with any culture cells.

TYPE 2 HOMOGENIZER

It consist of methods like grinding, mimicking or blending and liquid shearing and the selection of method depends on the type of cell or tissue.

Liquid shear-

These homogenizer is used widely for applying disruptive force in soft tissues and in cells that lack tough outer coat. The homogenizer, Potter Elvehjem consist of a glass cylinder and a machined Teflon pestle attached to an electrical motor present overhead. The pestle is rotated normally at 500-1000 rpm. The pestle of Dounce homogenizer is normally a smooth glass ball which is operated by hand rather than Teflon cylinder. The magnitude of liquid shear force is controlled by thrust of pestle, speed of rotation of the pestle and the clearance between pestle and containing vessel. This clearance is smaller in Dounce homogenizer as compared to Potter Elvehjem where shearing force is grater greater which can lead to damage to released organelles like nuclei and cause difficulty in the early stage of the process in moving the glass vessel relative to pestle. Dounce homogenizer is used only when it is much important to use minimum shearing force. A machine called cell cracker is used by some workers in place of dounce homogenizer for the disruption of cultured cells.

Mechanical shear-

To undergo disruption of cells or tissue mechanical shear homogenizer uses rotating metal blades or teeth. It resemble modern hand held blenders where motor present overhead and most are the variation of traditional domestic liquidizer where the sample is placed over glass reservoir containing blade that are driven by motor present near to it. Ultra turrax and its successor, polytron homogenizer are widely used and can be used with small volume and for many years. Waring blender has been used for softening large number of plant and hard animal tissue. The rotation speed of blade varies and the

mostly acceptable speed for most application is about 2000-4000 rpm and the time of homogenization also varies according to the nature and amount of material.

Sonication-

This is the process that is widely used in the preparation of bacterial membranes and is effective in breaking open cells in suspension in which glass beads are sometimes added in order to increase the efficiency. It is applied in plant and animal cells in low amount as it is difficult to disrupt the plasma membrane by keeping the internal organelles un-affected and sonication are carried out in 5-10 second pulses with a gap for cooling (where heating in the sample is also a problem). For most of the work, sonicators with variety of work heads and maximum power of 150W are usually acceptable.

Osmotic lysis-

This method is used in little amount in case of eukaryotic cells except for the frequent rupture of tissue culture cells by liquid shear. In this case mammalian cells, protoplast or spheroplast are exposed to hypo-osmotic medium where swelling occur and bursting of substance happen depending on the fragility of structure.

Other methods-

Small pore metal or nylon screens are used for breaking some cells and tissue grinders are used for cells from lymphoid tissue where glass pestle present in it force cells through stainless steel screen. (Graham John, 1997)

CENTRIFUGAL METHODS FOR SEPARATION OF ORGANELLES:

DIFFERENTIAL CENTRIFUGATION-

Bensley and Hoerr introduced differential centrifugation for the first time in 1934 where they obtained large granule fraction containing nuclei and mitochondria. Claude recommended that the tissue should be passed initially through a metal screen for removing connective tissue thereby making homogenization easier, preventing damage

to intracellular organelles. He obtained three fractions, large granule (mitochondria), small granule (microsomes) and a non-sedimentable fraction.

Eukaryotic cells consist of different types of membrane bound intracellular organelles having specific function where this method called differential centrifugation is one of the widely used technique for the separation of these organelles which proceeds separation process by providing centripetal forces greater than that of gravity which is followed after homogenization process. The movement of particles in the centrifugal field depends upon the size, density and shape where the interaction between particles weight and resistance it acquire during movement through suspension medium and the exerted centrifugal force that act upon particle influences sedimentation. Larger particles move rapidly compared to smaller one in a given centrifugal force and differential pelleting is normally used for separation or for producing sub-cellular fractions from tissue homogenate. Depending upon different components of a cell the order of sedimentation in respect to density from higher to lower include nuclei, mitochondria, lysosomes, plasma membrane, endoplasmic reticulum and contractile vacuoles which may vary depending on the type of cell. Sucrose which are cheap, non-toxic, highly purified soluble material is introduced in the medium which provide biochemically active medium where 0.88M is normally used. (H Lodish,2000)

DENSITY GRADIENT CENTRIFUGATION-

This method provide cleaner separation of particles by applying a density matrix for the particles to move. The choice of matrix depends on the target molecule where the particles settle down in to clean particles by moving at different sedimentation rates through the matrix. Media used in this type of separation involves polyhydric alcohols, polysaccharides, inorganic salts and silica. Density gradient is usually formed by layering increasing concentration of sucrose in a centrifuge tube where other solutions like percoll and cesium chloride can also be used. It is of two types, isopycnic and rate zonal centrifugation. The problem of cross contamination of different particles of different sedimentation rates can be avoided by layering the sample on the top of the gradient as a narrow zone in rate zonal centrifugation where the speed of sedimentation

of particles will depend on the size and mass of the particle rather than density where the particles are denser than that of gradient resulting the in the formation of pellet of all particles when kept for centrifugation for long time. In isopycnic centrifugation, which is also known as buoyant or equilibrium separation, particles get separated depending on their density. Here the density of the gradient must be greater than the particles to be separated, where the particles will never moves to the bottom of the tube independent of the time of centrifugation and where the particles of specific density sediment when they reach to the point where the density is same to that of gradient media and the particles get separated according to the buoyancy. This isopycnic separation can vary according to the gradient medium as the particles are sensitive to osmotic pressure of gradient. (MK Brakke,1951)

SUBCELLULAR FRACTIONATION became the most useful technique to detect and analyze low abundant proteins specific to subcellular organelle. It also allows to detect the proteins that move between different subcellular compartments depending on stimuli. It is described for purification of endosomes based on continues and discontinues gradient of sucrose. (de Araujo ME, et al.,2008)

Subcellular fractionation of specific organelle followed by identification of proteins present in the target organelle using mass spectrometry is the traditionally used method for analyzing organelle protein profile where it is difficult to isolate and profile fresh organelles due to limited availability of fractionation methods. This technique provides an approach for profiling multiple organelle from same cell lysate which is required for determining the correct cellular localization of protein as there are many proteins that shuttle between different organelles where the method is based on subcellular fractionation of cell lysate by density gradient centrifugation, iTRAQ labelling, and MS analysis of protein content in selected cell fractions and its principal component analysis (PCA) (Yen W, et al., 2008)

They describe a modified method for the isolation of purified protein from three cellular fractions including cytoplasm, membrane bound organelles and nucleus by using gentle buffers allowing sequential lysis of cell membrane, organelle membrane and nuclear membrane where the method does not require expensive reagents or usage of

commercial kits and is applicable to tissue samples or cultured cells that will yield purified fractions of cytosolic, membrane bound and nuclear proteins. Sequential lysis of cell membranes by increasing the detergent strength of lysis buffer is the basis of this method where buffer A is used for the cytosolic proteins to get released and buffer B releases proteins from all membrane bound organelles except nucleus and buffer C helps in the release of nuclear proteins. HEPES is the buffer that stabilizes the pH of the solution where NaCl maintains ionic strength. (Sabhina Baghirova, et al., 2015)

Fractionation helps in studying the distribution of protein in different cellular compartments, tissues or cell types, allowing the analysis of protein of interest. It can also allow molecular information about the state of cell or tissue and the difference in distribution of proteins among different cell compartments. Western blot analysis is the process used in validating the purity of different fractions including cytoplasmic, mitochondrial and nuclear compartments of muscle cells with marker proteins specific for each compartment which allowed the isolation of these compartments with good purity without employing ultracentrifuge permitting the samples to be to frozen at -80℃ for future analysis. (Ivan Dimauro, et al.,2012)

Translocation of proteins between cytoplasm and nucleus is a common process that occurs in response to stress or other manipulations. To separate nuclei from cytoplasm, one of the widely used methods is the density gradient centrifugation where the cells are exposed to non-physiological hyperosmotic conditions for more time resulting in leakage among nucleus and cytoplasm. Agents such as leptomycin B have been used to maintain the ratio of nuclear and cytoplasmic proteins which helps in analyzing the nuclear cytoplasmic transport finely. For subcellular fractionation of primary and transformed human cells Rapid Efficient And Practical (REAP) was developed, which is a two minute non-ionic purification technique based on detergent that uses table top centrifuge, micropipette and micro-centrifuge tubes. This method however provide efficient separation of nuclear and cytoplasmic proteins without any cross contamination of nucleoporin and lamin A markers or pyruvate kinase and tubulin cytoplasmic markers. (Keiko Suzuki, et al., 2010)

The presence of huntingtin in cytoplasm and nucleus is confirmed by biochemical subcellular fractionation studies where Huntington's disease is a neuro-degenerative disorder caused by expansion of polymorphic repeats in the coding region of Huntington's disease gene. (Karien E De Rooij, et al.)

To accomplish recovery of target protein which are free from contamination, usage of partial subcellular fractionation with the help of exact buffers rather than undergoing whole cell lysis is mostly preferred as the usage of single lysis buffer can cause solubility issues and proteins can get contaminated from distinct cellular compartments in response to the target protein. A single lysis buffer like NP40 or RIPA is commonly used in most cell lysis procedures to obtain total cell lysate comprising a collection of proteins. Hence, subcellular fractionation is a established protocol for sequential extraction of proteins enriched in cytoplasm, membrane bound organelles etc. from cultured mammalian cells. (Paul Holden, William A Horton-2009)

Subcellular fractionation of cadmium containing tissues of pokeweed helps in the identification of a fact that the content of this element was mostly located in soluble fraction and cell walls in case of both roots and leaves. (Xiaoping et al., 2011). Subcellular fractionation have been conducted on cells transfected with PS1 or PS2 for identification of full length presenilin protein and their fragments. (Jimin Zhang, et al., 1998)

Subcellular fractionation have been used for localization of proteins in the cytoplasm and nucleus of C. elegans which is widely used in research purpose for the identification of processes related to health and disease where fluorescently labelled protein can be used for in vivo or immunochemistry can be conducted for the localization of specific protein in the subcellular level. (Mata Cabana, et al., 2018)

Subcellular fractionation is used in determining the localization of ubiquitinated (inactivation of protein by attaching a small molecule, ubiquitin) misfolded polytopic membrane proteins extracted to the cytosol during endoplasmic reticulum associated degradation (ERAD) when these proteins get ubiquitinated and retrotranslocated to the cytosol for proteasomal degradation. (Kunio Nakatsukasa, Takumi Kamura, 2016)

Endocytosed ligands, receptors and plasma membrane proteins are placed in the intracellular, acidic, membrane bound subcellular compartment known endosome where the isolation of elements present in this compartment is done by using the combination of processes like density gradient ultracentrifugation, electrophoresis, gel filtration and immunoaffinity techniques. (Barbara M.Mullock, et al., 1987)

DESALTING-

Desalting is a process that helps in removing salts from protein solutions, phenol or unincorporated nucleotides from nucleic acid and it also undergo separation of excess crosslinking, labelling or derivatization reagents from conjugated proteins. As there are many different ways to clean up a protein lysate, the selection of procedure would depend on the size and properties of the target proteins. Particular contaminants present in the protein sample can be removed by loading the sample on to ion-exchange medium, eluting the protein with a high salt buffer and desalting the elute. Desalting spin column packed with size exclusion resin can be used for removing buffer salts, urea etc. present in the protein sample. Use of centrifugal filters with molecular weight cut-off (MWCO) membranes are suitable for easiest desalting of small volume of protein solution. (Penn State Log in, 2015)

A simple procedure for coupling polymer microchip injector to mass spectrometry is based on adsorption of protein on polyvinylidene diflouride (PVDF) membrane which is directly eluted in spraying solution where this micro-chip based experiments are successfully applied on drug, peptides and proteins originally diluted in phosphate buffered saline (PBS) (Neils Lion, et al., 2002)

Successful and rapid desalting of protein - containing urea at 100 fmol/µL - up to a mass of nearly 70 KDa was observed using C18-membrane technology where the C18 membrane was incorporated in to a micro-spin column for successful identification of protein by concentrating the protein through desalting (Mike J Naldrett, et al., 2005).

To desalt and purify proteins and peptides from mixture of buffer, micropipette solid phase extraction (SPE) tips have been used where characterization of electro-spray mass

ionization spectrometry (ESI-MS) followed by removal of salts and buffers improves the limits of detection and sensitivity of protein analysis. (D Seth Fornea, et al., 2006)

IMMUNOPRECIPITATION (IP)-

This is a widely used method in cell biology for several purposes which includes identification of molecular mass of an antigen, characterizing specificity of antibodies, identification of molecules associated with antigens and quantifying amounts of antigen with radioimmunoassay, etc. The basic steps of this process include solubilization of antigen preparation, clearing preparation of insoluble materials and bonded molecules, primary antibody incubation, washing immunoprecipitate and resolving immunoprecipitate on electrophoretic gels. (Joann J Otto, Seung-won Lee-1993).

Erin R Greiner, *et. al.* used a strategy including proteolytic digestion followed by immunoprecipitation using amyloid conformational antibody LOC to detect amyloid for the diagnosis of amyloid diseases and for identification of functional amyloids. (Erin R Greiner, et. al., 2014)

Protein-protein interaction are now available to examine in both cells and native tissues by precipitation of the protein complex of interest. Immunoprecipitation of the protein bait, purification of complex and identification of interacting partners are the three essential components used for successful identification of neuronal proteins using mass spectroscopy based proteomics. Coupling of mass spectroscopy techniques with successful co-immunoprecipitation provide rapid identification of members of the protein complex (R Benjamin Free, et al., 2009)

Usually the quantitative analysis of protein biomarkers in plasma is done by ELISA but is limited by the availability of high quality antibodies and so the alternative approach developed involves immunoprecipitation combined with multiple reaction monitoring mass spectrometry (IP-MRM) where proteins are identified by multiplex immunoprecipitation from plasma with the ELISA capture antibodies further purified by SDS-PAGE, analyzed by stable isotope dilution MRM. (D Lin, et al.,2013)

CHROMATIN IMMUNOPRECIPITATION (ChIP)-

It is a technique used to determine whether the protein binds to a specific DNA sequence thereby studying interactions between specific proteins or modified forms of protein and a genomic DNA region. It can also be used to determine whether a transcription factor interacts with its target gene. UV light from trans-illuminator was used to cross link proteins to DNA irreversibly in earlier ChIP studies. Later some components are added to modify the ChIP procedure that include formaldehyde that is used as reversible protein-DNA and protein-protein cross linking agent and PCR to detect precipitated DNA fragments. (Michael F Carey, et al., 2009)

ChIP assays usually takes several days to complete involving several tube transfers and uses phenol chloroform or spin columns to purify DNA. The procedure is also developed based on chelax resin that reduces time of experiment and uses only a single tube to isolate PCR- ready DNA. (Joel D Nelson, et al., 2006)

The activity of ChIP is highly dependent on the using antibody where it must recognize formalin fixed epitopes. One way to check the antibody involves immune-fluorescent staining of cells fixed under same condition as that used for ChIP. (Thomas A Milne, et al., 2009)

This assay has been recognized as a powerful method for the identification of proteins interacting within native chromatin environment and is good enough for variety of purposes. This method has been applied on yeast, drosophila, tetrahymena, *Caenorhabditiselegans* and in various mammalian cell lines. It is a multistep process and each step has to be standardized for obtaining correct results. (P Gade and D V Kalvakolanu,2012)

MATERIALS AND METHODS

CELL CULTURE:

Cell culture is an essential technique to study the physiological and biochemical properties of cell. Growth of cells in a given media depends on different factors that include nutrients, temperature, pH, humidity etc. To obtain a good culture of cell, culturing has to be done in appropriate sterile condition which requires several laboratory techniques. Here, the astrocyte cell line was used for the experiments. Astrocytes are glial cells found in the brain and spinal cord which play a major role in maintenance, support and repair of nervous tissue. The immortalized mouse astrocyte, SV40T is a suitable cell line for studies in astrocyte functions.

Materials-

Astrocyte cell line, DMEM, PBS, trypsin, T25 flask, CO_2incubator

Cell culture maintenance-

Cell culture are maintained in a proper way by providing growth media and optimum growth conditions. Media used here is Dulbecco's modified eagle's medium (DMEM) which is a basal medium consisting of amino acids, vitamins, glucose, salts and a pH indicator and as it does not contain any proteins or growth factors, it is supplemented with 5-10% FBS (fetal bovine serum). To maintain the pH, 5% CO_2 is provided in the incubator.

Cell seeding-

- Cells were fed every 2-3 days and is sub cultured based on the confluence of cells and pH of the media. Here T25 flask containing healthy cells was taken in to a laminar air flow hood.
- The medium was discarded and the cells were washed using PBS
- Fresh medium was added and the flask was kept for incubation at 37℃ in a humidified CO_2 incubator.

Cell splitting-

- The healthy cells containing T25 flask were taken to hood and the entire medium was discarded.
- Cells were washed with PBS to remove the remaining medium and FBS.
- 500µL of 0.5% trypsin was added through the sides of flask and the flask was slowly swirled to cover the monolayer completely with trypsin.
- Flask was incubated in the CO_2 incubator for 5 minutes.
- Flask was then observed under microscope and cell detachment was confirmed. This cell suspension was transferred in to a 15ml falcon tube and was centrifuged at 2000 RPM for 5 minutes.
- Supernatant was discarded and the pellet was re-suspended in fresh medium and transferred to T25 flask which was then incubated in the CO_2incubator.

Cell counting-

Hemocytometer, invented by Louis Charles is a device used for counting cells. It contains a glass microscope slide with a rectangular chamber. It consist of two chambers that are divided in to 9 large squares with length and breadth of 1.0mm which are separated from one another by triple lines. At the center, an H shaped chamber with a laser etched grid of perpendicular lines are created. Hemocytometer is used by placing a coverslip over the chambers at a height of 0.1mm. The coverslip used here are specially made and are more thicker as compared to the cover slip used in conventional microscopy and it should be placed on hemocytometer by making sure that it does not slide off and the cells are counted by transferring the liquid containing cells mixed with stain Trypan blue by touching the tip to the edge of the coverslip allowing the chamber to fill by capillary action. Certain colored stains are used in order to differentiate viable and dead cells as these pass through the membrane of dead cells resulting in identification of dead cells. After transferring the suspension, hemocytometer was placed on the stage of microscope and cells were counted. Average number of viable cells is dependent on the large squares taken for counting. In some cases four corner squares are taken for counting and in other case center square was also taken for

counting. Cells like WBCs are counted by corner squares and smaller cells like RBCs by center square. In case cells are found in the center of boundary line, cells present in the adjacent two lines of a square is taken for counting. Live cells appear colorless and dead cells are stained blue when viewed under microscope which helps in keeping a separate count of live and dead cells. Cell viability was counted as,

- Percentage of cell viability=$\frac{total\ viable\ cells(unstained)}{total\ cell(viable+dead)}$

- Viable cell/ml = average viable cell count per square× dilution Factor × 10^4

- $average\ number\ of\ viable\ cell/square$=$\frac{Total\ number\ of\ viable\ cells\ in\ square}{number\ of\ squares\ selected\ for\ counting}$

- Dilution factor =total volume (sample+ diluting liquid) ÷ volume Of sample.

- Total viable cell/sample=viable cells/ml× original volume of fluid from which cell sample was removed

- Volume of media needed=$\frac{number\ of\ cells\ needed}{total\ number\ of\ viable\ cells\times 1000}$

Cryopreservation of cells-

Cryopreservation, low temperature preservation technique provides a long term storage of cells or tissues by reducing the biological and chemical reactions in living cells. It is used to preserve the exact structure of cells. Refrigeration is also used for storage but it provide only short term storage which is not good for cryopreservation. Cryopreservation protects the cells from un-protectant freezing. In this the protective substance known by cryoprotectant (small molecules) enters in to the cell, preventing dehydration and intracellular ice crystal formation that destruct and cause cell death.

- Here trypsin was added to the flask and cells were kept for 5 minutes incubation.
- After incubation fresh medium was added to neutralise trypsin and the cell suspension was centrifuged at 2000 RPM for 5 minutes
- Supernatant was discarded and the pellet was re-suspended in 700µL of freezing medium containing DMSO and FBS in the ratio 1:9 and the suspension was transferred in to sterile vial and was transferred to -80℃ $in\ a\ cryofreezor\ container$.

Revival of cells:

- The frozen cells taken from storage were first kept on 4℃ and was then transferred in to water bath at 37℃ for rapid thawing.
- Thawed cells were transferred to a centrifuge tube and pre-warmed growth medium was added drop wise and was centrifuged at 2000 RPM for 5 minute.
- Cells were re-suspended in fresh, complete growth medium slowly and was transferred to culture vessel which was then incubated in 37℃ and 5% CO_2 cell culture incubator.

TRANSFORMATION OF TARGETTED DNA IN TO E.Coli CELLS:

As they are easy to grow in large numbers, bacteria are usually used as host cells for studies. The process of intake of foreign genetic material from the environment by bacteria through horizontal gene transfer is known by transformation. A living donor cell is not required for this process but a persistent DNA. This process helps in making

multiple copies of DNA, large human proteins etc. Plasmid is a smaller circular DNA present in bacteria which is used as vectors to carry foreign gene into cell. Plasmid must contain sites likes origin of replication (where DNA replication begins), multiple cloning site and a resistant gene that allows particular type of cells to grow. The cells in which transformation is conducted must be in that condition that it have the capability to intake the foreign gene, for that the cells are to be made competent, which is done by treating with chemicals like calcium chloride that makes the cells swell by possessing the entry of water in to the cell to mildly disrupt the membrane in order to take the foreign gene inside. Such cells that are modified to intake foreign DNA are said to be competent cells. Some kind of receptors that are required for the initial binding of DNA gets activated or formed on the cell wall during the process of development of competence of cell. The complex thus formed will be resistant to DNases. On providing heat shock pores are created resulting in the uptake of DNA and the pores get closed on immediate chilling.

MATERIALS REQUIRED-

Plasmid DNA, modified E.coli as host, LB broth, LB agar, refrigerator, centrifuge, shaker incubator.

PROCEDURE-

1. Preparation of competent cell-

- Day 1: Revival of host bacteria
  - 0.1 ml of LB medium was added to the bacterial vial.
  - A loop-full of suspension was streaked on to LB plate and are incubated at 37℃ overnight.
- Day 2:
  - A single colony was isolated and inoculated in to 5 ml LB medium and incubated at 37℃ overnight.

- Day 3: preparation of competent cell

- 1 ml of overnight culture was inoculated in to 100 ml LB medium and incubated at 37℃ in a shaker.
- The culture was allowed to grow until the optical density reached 0.23-0.26 which takes about 2-3 hours.
- The culture was then transferred aseptically in to sterile eppendorf tube and centrifuged at 4℃ for 8 minutes at 6000RPM.
- The supernatant was discarded and 100µL 0.1M ice cold $CaCl_2$was added aseptically to the pellet and the pellet was re-suspended gently in the solution where the tubes are kept on ice during suspension.
- Tubes were placed on ice for 30 minutes.
- The supernatant was discarded and the pellet was again re-suspended with 20µL ice cold $CaCl_2$ solution.

- Transformation:
- 10µL of plasmid DNA (100ng) was taken and added in to the aliquot containing 100µL of competent cells and this was incubated on ice for 30 minutes.
- The cells after incubation were given heat shock by placing the vials in a 42℃ water bath for 90 second and was returned to ice to chill for 2 minutes.
- 300µL LB broth containing ampicillin was added to the vial aseptically and incubated on 37℃ shaker incubator at 200 RPM to allow the bacteria to recover and express antibiotic resistance and centrifuged at 4000 RPM for 4 minutes.
- Supernatant was removed by leaving some volume and pellet was re-suspended in it and spread over LB ampicillin plate (Ampicillin taken is 50µg/ml) and incubated at 37℃ overnight.

ISOLATION OF PLASMID DNA FROM TRANSFORMED CELLS:

Plasmids are double stranded, circular extrachromosomal DNA of bacterium, which are distinct from the chromosomal DNA of cell. The term 'Plasmid' was introduced by Joshua Lederberg in 1592. They are transferrable genetic elements or 'replicons'. Since plasmids are used as an important tool to study processes like replication, conjugation and as a vehicle for gene-expression; the isolation of plasmid DNA is an extremely important step in molecular biology. In molecular biology, the method alkaline lysis is used to isolate plasmid DNA. Bacteria with the desired plasmid of interest is first cultured in LB broth and then the bacteria are pelleted and re-suspended by using three alkaline lysis solution including alkaline lysis solution I or re-suspension buffer (Buffer P1) that contains basic pH, Tris buffer that helps to denature DNA, and EDTA (ethylenediaminetetraacetic acid) which binds to divalent cations and destabilizes the membrane and inhibits DNase activity. Buffer P1 also contain RNase which degrade the released RNA. Lyse-Blue was also added to the re-suspension buffer before use in the ratio 1:1000 so as to obtain the required working concentration. Lyse-Blue is a color indicator that provides visual identification of optimum buffer mixing, thereby prevents common errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA and cell debris. When Buffer P1 is mixed with Buffer P2 the Lyse-Blue precipitate is completely dissolved and the colour of suspension changes to blue. The bacteria are then lysed with alkaline lysis solution II, strong alkali (NaOH) and detergent SDS (Sodium Dodecyl Sulfate) [Buffer P2]. The SDS cleaves the phospholipid bilayer that lead to cell lysis and release of contents and the alkali denatures the genomic and plasmid DNA as well as the cellular proteins. Finally, the alkaline lysis solution III which is neutralization buffer of potassium acetate is added (Buffer P3). So that covalently closed plasmid DNA can re-nature and dissolve in solution while the single stranded genomic DNA, the SDS and other denatured proteins bind together by hydrophobic interactions to form a white precipitate i.e., the lyse-blue turns colorless. This confirms that SDS from the lysis buffer has been effectively precipitated. The precipitate and chromosomal DNA is removed by centrifugation. After which the soluble plasmid DNA is purified by binding the DNA to a solid support such as glass, silica or fibers. The plasmid will bind to QIAGEN resin under low-salt and pH

conditions. RNA, proteins and low-molecular – weight impurities were removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer wash, concentrated and desalted by isopropanol precipitation.

EQUIPMENTS AND REAGENTS-

- Standard microbiological equipment for growing and harvesting bacteria such as inoculating loop, culture tubes or flasks, 37^0C shaking incubator, centrifuge.
- Ice
- Isopropanol
- 70% ethanol
- TE Buffer
- QIAGEN Plasmid Midi Kit

COMPOSITION OF BUFFERS

a) Buffer P1 (Re-suspension Buffer)
   50mM Tris-HCl, pH 8.0;
   10mM EDTA;
   100µg/ml RNase A
   Storage - 2-8^0C, after addition of RNase A
b) Buffer P2 (Lysis Buffer)
   200mM NaOH, 1% SDS
   Storage – 15 – 20^0C
c) Buffer P3 (Neutralization Buffer)
   3.0M potassium acetate, pH 5.5
   Storage – 15 - 25^0C
d) Buffer QBT (Equilibration Buffer)
   750mM NaCl;
   50mM MOPS, pH 7.0;
   15% isopropanol;
   0.15% Triton X-100
   Storage – 15 – 25^0C

e) Buffer QC (Wash Buffer)

1.0M NaCl;

50mM MOPS, pH 7.0;

15% isopropanol

Storage – 15 – 25^0C

f) Buffer QF (Elution Buffer)

1.25M NaCl;

50mM MOPS, pH 8.5;

15% isopropanol

Storage – 15 - 25^0C

PROCEDURE-

- A single colony was isolated from a freshly streaked plate and inoculated in a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic. Cultures were incubated at 37℃ for about 8 hours with vigorous shaking.
- The starter culture was then diluted (1/500 to 1/1000) into selective LB medium. Cells were allowed to grow for 12-16 hours at 37℃ with vigorous shaking.
- Harvesting of the bacterial cells was performed by centrifugation at 6000x g at 4℃ for 15 minute.
- Re-suspension of the bacterial pellet was performed in 4 ml Buffer P1.
- 4 ml of Buffer P2 was then added, mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature for 5 min.
- 4 ml of chilled Buffer P3 was then added, mixed immediately by vigorously inverting 4-6 times, and incubated on ice for 20 min.
- Then Centrifugation was performed at 20000x g for 30min at 4^0C and supernatant was collected containing plasmid DNA.
- Equilibration of QIAGEN-tip was performed by applying 4 ml Buffer QBT, and allowing the column to empty by gravity flow. The supernatant was loaded onto the QIAGEN-tip.
- Washing of the QIAGEN-tip was performed with 2 x 10 ml Buffer QC.

- Elution of DNA was performed by washing with 5 ml Buffer QF.
- DNA was then precipitated by adding 3.5 ml room temperature isopropanol to the eluted DNA. Centrifugation was performed at 15000x g for 30 min at 4^0C. Supernatant was carefully decanted.
- DNA pellet was then washed with 2 ml of room temperature 70% ethanol, and centrifuged at 15000x g for 10 min. Supernatant was carefully decanted without disturbing the pellet.
- The pellet was Air-dried for 5-10 min, and re-dissolved in a suitable volume of buffer (TE buffer, pH 8.0, or 10mM Tris-HCl, pH 8.5).

NANODROP QUANTITATION OF ISOLATED PLASMID:

Nano-Drop is a laboratory instrument used to measure the absorbance and to calculate the concentration of nucleic acid and purified proteins. In this technique the sample is directly placed in to the pedestal, which is the space where sample can be kept rather than using cuvettes. This system provide quick and easy quantification to check the purity of samples like protein and nucleic acid which is done based on the absorption of ultraviolet light in specific pattern where absorbent ratio 260/280 of 1.8 is considered suitable for analysis.

MATERIALS:

Nanodrop spectrophotometer, TE buffer, plasmid DNA

PROCEDURE:

- Selected DNA from the option given by the nanodrop.
- Selected blank and placed 2μL of TE buffer on the pedestal and confirmed the concentration is 0 (blanking of control).
- Wiped out buffer and added 2μL of sample and measured the concentration.

TRANSFECTION OF ISOLATED PLASMID DNA IN TO ASTROCYTE CELLS-

The process of introduction of nucleic acid (DNA, RNA or oligonucleotide) artificially in to the cytoplasm of eukaryotic cells is known as transfection. It helps in studying gene function and protein expression using various chemical and physical method. With cellular integration of plasmid DNA, mRNA or proteins this method can be used for gene silencing, editing and also for studying about over expression of a gene. This transfer can be done physically by electroporation or micro injection but are mostly toxic to the cells and so transfer with the help of chemical method is mostly preferred. This process include three steps involving complexation of nucleic acids, interaction with cellular membrane and entry in to the cell. In nucleic acid complexation, transfection reagents like polymers or lipids that are cationic, gets interacted with negatively charged nucleic acid form transfection complex which is protected from nuclease action. This positively charged transfection complexes enters in to the cell by interacting with negatively charged heparan sulfate proteoglycans (expressed by adherent cells on the external surface of cell membrane) and enters the cell through endocytosis and reside in endocytic vesicles. Transfection is difficult in case of suspension cells as they express very less heparan sulfate proteoglycans resulting in lower interaction with transfection complex. The transfection complex after entering the cell gets arranged in to intracellular vesicles and nucleic acid is released in to the cytoplasm with the help of more efficient transfection reagents through vesicle membrane rupture.

MATERIALS-

Transformed plasmid DNA, Astrocyte cell culture, DMEM (Dulbecco's Modified Eagle's Medium), buffer, lipofectamine reagent.

PROCEDURE:

- Astrocyte cells were cultured in 8 well chamber slides in 500µL DMEM one day before transfection, so as to enable 50-60% confluency, which is was preferred for transfection.
- 0.5µg DNA was then added to 30µL buffer taken in a vial and was mixed gently and then spinned down.
- 1.5µL lipofectamine reagent was then added to the buffer, re-suspended and kept for incubation for 10 minutes.
- 300µL media was then added to the mixture, re-suspension was performed and was then transferred to the culture plate.
- Plate was then kept for about 24 hour incubation in CO_2 incubator at 37℃

CONFIRMATION OF TRANSFECTION OF PLASMID VIA IMMUNOCYTOCHEMISTRY

The cells were fixed in 4% PFA for 20 minutes and 3 washes in 1X PBS were subsequently performed. For immunocytochemistry, cells were permeabilised in 0.25% saponin for 20 minutes and cells were subsequently washed with 1XPBS. The cells were then blocked in blocking solution containing 1X PBS, 5%BSA, 2% Donkey serum for 1 hour. 150µls of blocking solution was pipetted into each well. After blocking, 150µl of anti HA mouse primary antibody in 1:200 dilutions was added to each well and was kept for incubation for 16-18 hours at 4^0C. Next day, primary antibody was removed and cells were washed with PBS (quick wash for 2-3 times and 5 minutes incubation wash for 3 times). 150µl of secondary antibody (anti mouse alexa 488) in dilution of 1:250 was added and cells were kept for incubation at room temperature for 1.5 hours. 4 times quick washes along with 2 times of 5 minutes washes was given using 1XPBS. 150µl of 1X Hoechst staining solution was added to each well as a nuclear counter-stain and cells were kept for 5 minutes at room temperature. Again, quick PBS washes were given for 2 times. The cells were mounted on the with 70% glycerol as a mounting agent and were cover-sliped. The images were captured using 488 excitation laser in the confocal microscope.

CELL LYSATE PREPARATION OF TRANSFECTED CELLS FOR SUBCELLULAR FRACTIONATION-

Nuclear and cytoplasmic fractions are prepared by using two buffers, buffer A is a hypotonic buffer and helps in producing cytoplasmic fractions while buffer $A^{++}$is hypertonic buffer that fractionate nucleus using centrifugation where the complete centrifugation is done at 4℃ and the samples are kept on the ice through out the complete experiment.

MATERIALS-

- Buffer A (2 mL)-
  40µL 0.5M HEPES, 20µL 1M KCl, 20µL 200mM $MgCl_2$, 10µL 200mM EDTA, 20µL 20% NP40, 200µL 10x protease inhibitor.
- Buffer A^{++} (1 mL)-
  20µL 0.5M HEPES, 10µL 1M KCl, 10µL 200mM $MgCl_2$, 5µL 5M EDTA, 20µL 20% NP40, 250µL 100% glycerol, 100µL 10x protease inhibitor.
- Culture, PBS, Scrapper, syringe, needle.

PROCEDURE-

- Medium was removed from the plasmid transfected cultured cells (in 100mm dishes) and were given two washes with PBS (phosphate buffered saline). Cells were scrapped from the dishes and taken in a vial with PBS.
- Cells were then centrifuged at 2000 RPM for 5 minutes.
- Protease inhibitor was added to the buffer just before use.
- Supernatant was removed and buffer A was added to the cell pellet, pellet was resuspended and kept for incubation for 30 minutes.
- Stroke was given by passing the cell suspension using syringe through needle for about 20 times by intermediate checking of cell lysis by staining the sample with trypan blue.
- After all the cells got lysed, centrifugation was performed at 5000g for 5 minute.
- Supernatant was removed and the pellet was washed with the buffer A for two times at 5000g for 10 minutes.
- Protease inhibitor was added to buffer A^{++} just before use.

- After 2 washes of the pellet buffer A^{++} was added to the nuclear pellet, resuspended and kept for incubation for 30 minutes in the cold room.
- After incubation the sample was centrifuged at 18000g for 15 minutes.

DESALTING OF PREPARED CELL LYSATE-

Centrifugal filter devices like desalting columns provide fast ultrafiltration and recover concentration from dilute and complex sample. Processing time is usually 10-30 minutes. This column uses the principle of size exclusion chromatography. In desalting there is a large size difference between targeted protein molecule and unwanted salts. In fractionation, depending on size all the molecules need to be separated, are below the size exclusion limit of the column and so are retained by the column.

MATERIALS-

Microcentrifuge tubes, Amicon ultra 0.5 device, protein sample

PROCEDURE-

- Amicon ultra 0.5 device was inserted in to one of the microcentrifuge tubes.
- 500μL of sample was added in the device and was capped.
- Capped device was kept in the centrifuge rotor in such a way that strap of cap aligned towards the center of the rotor, balanced with similar device.
- Centrifugation was performed at 14000g for 30 minutes.
- Device was removed from the centrifuge tube and to recover the concentrated solute it was kept inverted in a clean microcentrifuge tube and centrifugtion was performed at 1000g for 2 minutes. Desalted and concentrated sample was collected in a tube.

BICINCHONINIC ACID ASSAY (BCA) FOR QUANTITATION OF PROTEIN:

BCA, also known as Smith Assay is a method used for determining the concentration of protein present in the sample. Bicinchoninic acid is a highly specific chromogenic reagent for copper. Here proteins get reduced in an alkaline solution from Cu^{2+} to Cu^{1+} with the presence of Bicinchoninic acid resulting in the formation of purple colored product which absorbs light at a wavelength of 562 nm. This process depends on temperature and the amount of Cu^{2+} reduced gives the amount of protein present in the solution, that is reduced amount of Cu^{2+} and protein concentration is proportional to each other. Measuring the absorption spectra and comparing with the known concentration of protein solution also gives the amount of protein present in the test sample.

MATERIALS:

Bovine serum albumin (BSA), Cell lysate, BCA solution, BCA working reagent- Reagent A is a 1000 mL solution containing bicinchoninic acid, sodium carbonate, sodium tartarate and sodium bicarbonate in 0.1N NaOH. Reagent B is a 25 mL solution containing 4% copper sulfate pentahydrate.

PROCEDURE:

- Bovine serum albumin standard were produced
- BCA working reagent is prepared by mixing 50 parts of reagent A with 1 part of reagent B.
- To the 96 well plates, standard BSA were aliquoted as 0,2,4,6,8,10,12 µL and 2µL of cell lysate was added to the well and all the wells wre make up in to 12µL by adding corresponding amount of distilled water.
- 190µL of BCA working solution was added in to each well.
- Plates were covered and incubated at 37℃ for 30 minutes and absorbance was measured at 562 nm.
-

WESTERN BLOTTING FOR ANALYSIS OF PROTEIN:

Western blotting, a process for detection of specific protein among a mixture of proteins is a widely used technique in the laboratory. This technique is proceeded by three steps that includes, separation of protein by size, transfer of it to solid support and marking of target protein using primary and secondary antibody. In this technique, the proteins are first separated based on molecular size through gel electrophoresis. Proteins used for separation can be collected from different kind of tissue or cells. Common form of sample used for this technique are cell lysate, a fluid containing the contents of lysed cells. Protein extraction is done by homogenization or sonication. The sample is mixed with loading buffer after checking the volume of sample, where the loading buffer contains glycerol which helps samples to sink easily in to the wells of gel. A dye called tracking dye, bromophenol blue is also added to the sample which helps in observing separation. In order to denature the higher order structure of protein without disturbing the sulfide bridges, the sample loaded with buffer is heated for about 10 minute where the negative charge of amino acid is retained enabling the protein to move with electric field toward positive electrode through gel. Two different types of gels are used by this technique including stacking gel and separating/resolving gel. Stacking gel, containing lower concentration of polyacrylamide and low pH (6.8) makes the gel more porous resulting in poor separation of proteins is placed on the top of resolving gel. It concentrates all the proteins in the sample in to a narrow band in such a way that all the samples enter the resolving gel at the same time. Resolving or separating gel at the same time is basic (8.8) containing high poly acrylamide that results in narrow pores on gel where smaller proteins can travel easily. Gels are made by pouring in between two glass and the sample along with dye is added to the wells made on gels using comb and the empty space is filled with sample buffer. Te assembly is connected to the power supply the proteins get separated based on their size to voltage applied. This separated protein is then transferred to the membrane, which is done by providing electric field where the membrane is sandwiched between gel and positive electrode. This sandwich includes sponge on each end and filter papers in order to protect the gel and sample resulting in the transfer of proteins from gel to the membrane. To obtain a clear image care should be taken while placing the membrane that the membrane and sample should be in close

contact so that the negatively charged protein gets transferred to membrane which is known as electrophoretic transfer. This process can be done in dry or wet condition, although wet condition is more suitable. There are two types of membranes, nitrocellulose and PVDF, where nitrocellulose show more affinity towards protein but it is fragile as it cannot be used for re-probing and at the same time PVDF provide mechanical support and can be used for re-probing. An important step in Western blotting is blocking that is done by 5% BSA or dried milk diluted in TBST, to prevent the binding of antibodies to the non-specific site of membrane, where the membrane can be detected by the signal produced by enzyme horseradish peroxidase (HRP) corresponding to the target protein when appropriate substrate is added which can be captured on a film that is done usually in a dark room.

MATERIALS:

- Resolving gel (12%):
  3.5ml autoclaved water, 2.5ml 1.5M Tris (8.8pH), 4ml 70% Bis-acrylamide, 100µl 10% SDS, 50µl 10% APS(ammonium per sulfate), 5µl TEMED (tetramethylethylenediamine).
- Stacking gel:
  6ml autoclaved water, 2.52ml 0.5M Tris (6.8 pH), 1.32ml 30% Bis-acrylamide, 100µl 10% SDS, 50µl 10% APS, 10µl TEMED.
- Loading buffer:
  4% SDS,10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCl
- Running buffer:
  25mM Tris, 190mM glycine, 0.1% SDS
- Transfer buffer:
  25mM Tris, 190Mm glycine, 20% methanol
- TBST(Tris-buffered saline with Tween-20) buffer:
  20mM Tris (pH 7.5), 150mM NaCl, 0.1% Tween 20
- Blocking buffer:
  3% BSA (Bovine serum albumin) in TBST

- Developer
- fixer

PROCEDURE:

- Electrophoresis:
  - Assembled the rack for gel casting solidification so that stacking and separating gel can be prepared.
  - After preparing separating/resolving gel mixture, it is added carefully to the rack until green bar. Water is added to the top of it and gel is left for 20 minutes to solidify.
  - After the solidification of separating gel the water is removed and it is overlayed with stacking gel and the comb is inserted carefully. Gel is left for solidification.
  - Comb is then removed the casted gel is inserted into the electrophoresis tank which is then filled with running buffer .
  - The electrophoresis tank is then connected to power supply (red to red and black to black)
  - Sample with marker is loaded in to each well.
  - The power supply is switched on and the gel is first run on 60V and then voltage is increased to 90V for when sample just enters the separating gel. The gel is allowed to run until the dye front reaches the bottom of the gel.

- Electro-transfer:
  - 4 filter sheets fitting the size of gel and one PVDF membrane cut to the size of the gel is taken.
  - Sponge and filter papers are wetted with transfer buffer and membrane with methanol.
  - Gel is taken out by separating the glass plates and western transfer sandwich is assembled as follows:,

    Sponge - 2 filter papers – gel - membrane - 2 filter papers - sponge
  - The sandwich was transferred to the transfer apparatus which was subsequently placed on ice (to maintain 4℃) and transfer buffer was added until the sandwich was covered and transfer is allowed to run for 90 minutes.

- Blocking and antibody incubation:
  - After the transfer of proteins to the membrane the membrane was blocked with BSA in TBST for I hour.
  - Primary antibody in 3% BSA was added and incubated overnight on a shaker in 4℃.
  - Membrane was washed with TBST for 3 minutes which was repeated for 3 times
  - HRP-conjugated secondary antibody in 5% BSA in TBST was added and incubated for 1 hour.
  - Membrane was then washed with TBS tween five times for 5 minutes.
- Detection:
  - Two chemiluminiscent reagents (peroxide and luminol) were taken in a vial in one to one ratio and mixed.
  - Membrane was incubated in it for 5 minutes without agitation
  - After incubation the membrane was taken out, excess reagent was wiped out and placed in a clear plastic wrap of screener.
  - X-ray sheet of size fitting to that of membrane (overlayed with the substrate) was cut out and placed above the plastic wrap. After exposure he x-ray sheet was taken out and kept for some time in developer and then in fixer where the complete process is done in dark room. The developed signal on X-ray is captured with the help of scanned and analysed.

RESULTS

RESULTS:

Plasmid DNA was transformed into freshly prepared competent cells by heat shock method.

Post transformation the cells were spread on antibiotic (kanamycin) containing LB plates and were incubated for 16 hours at 37°C in the bacterial incubator.

Bacterial cells which have taken the plasmid inside shows antibiotic resistance for kanamycin and grows into a single cell colony. Many single celled colonies were visible by the end of the incubation period.

Figure 1- Single cell colonies of HA tagged 55kDa protein coding gene in DH5α.

The colonies were obtained *via* transformation of the coding plasmid construct in DH5α.via standard heat shock method.

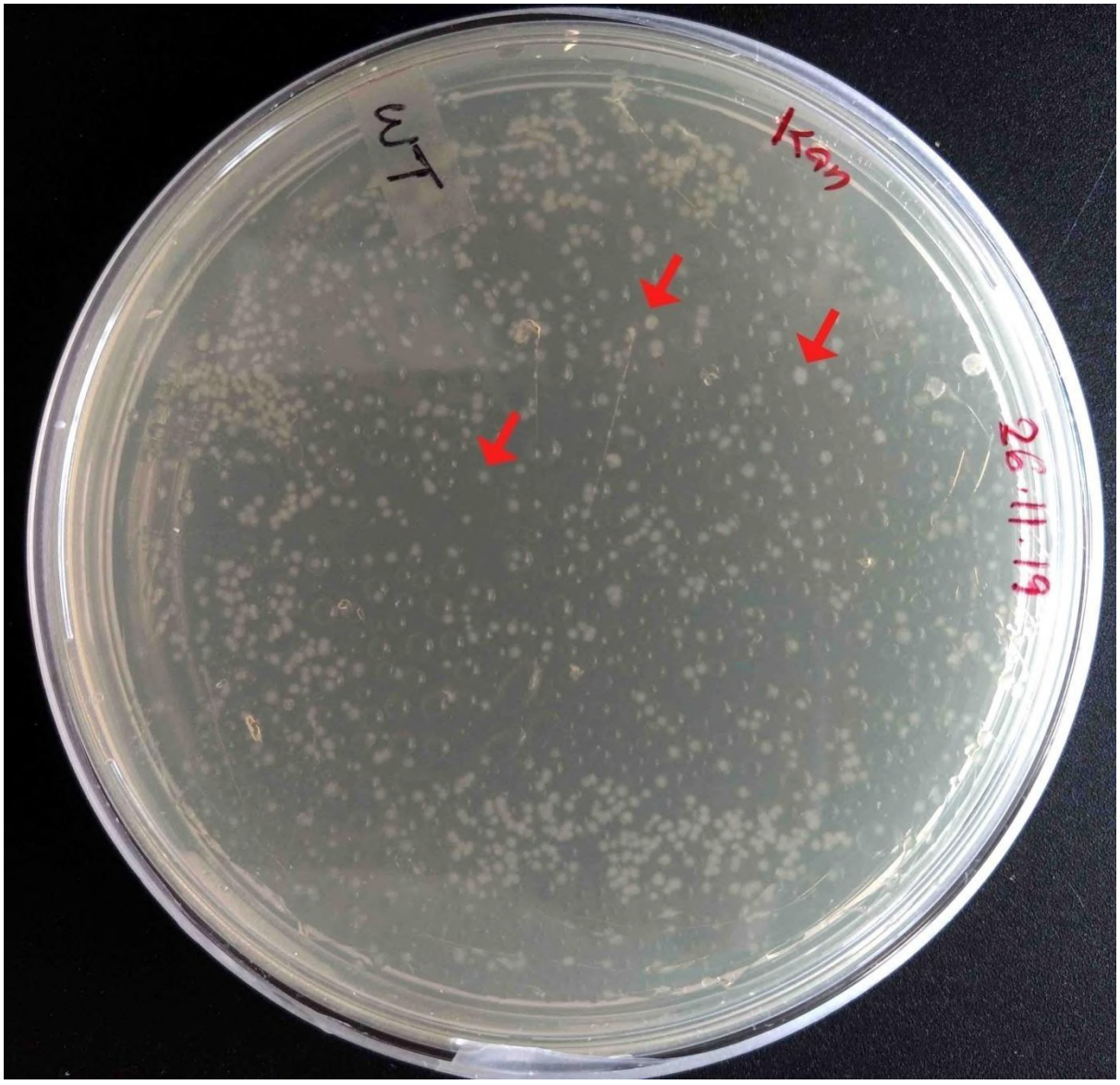


Figure 1

RESULTS:

Transformation

A single colony of E.Coli bacteria was inoculated into 5 ml Luria Bertani broth containing kanamycin (50μg/ml). The inoculated broth was kept overnight on a shaker (for proper aeration) at 37˚C and at 180 rpm. The culture was then transferred to a 60 ml LB Broth containing same concentration of kanamycin. The antibiotic provides a selection medium for cells containing the recombinant plasmids. The culture medium was then again incubated on a shaker for a period of 16 hours. After incubation the culture at logarithmic phase was then transferred to centrifugation tubes and centrifuged on a precooled centrifuge at 4˚C, 15000 rpm for 20 minutes. The medium was fully decanted and pelleted out.

Plasmid Isolation

The pelleted bacterial cells were lysed and the plasmid was isolated using Qiagen Midi plasmid Isolation kit by modified alkaline lysis method. Plasmid DNA binds to QIAGEN resin under appropriate low salt and pH conditions which is later eluted with appropriate buffers.

Plasmid Quantitation

Purity and concentration of the isolated plasmid is then quantitated using spectrophotometric method using Nano drop Instrument.

260/280 ratio of absorbance shows the purity of DNA over protein and phenol and 260/230 ratio shows the purity with respect to salt contaminants.

A260/A280- 1.757

A260/A230- 2.099

Concentration-3831ng/μl

Therefore the isolated plasmid showed a very good concentration and purity.

Figure 2:

A,B- Midi culture of HA tagged 55 kDa protein coding gene from single cell colonies of DH5α for isolation of plasmid DNA in higher quantities *via* Qiagen Midi plasmid isolation kit.

C- Quantitation of the isolated DNA *via* Nano drop Method

Figure 2A

Figure 2B

A260/A280- 1.757

A260/A230- 2.099

Concentration- **3831ng/µl**

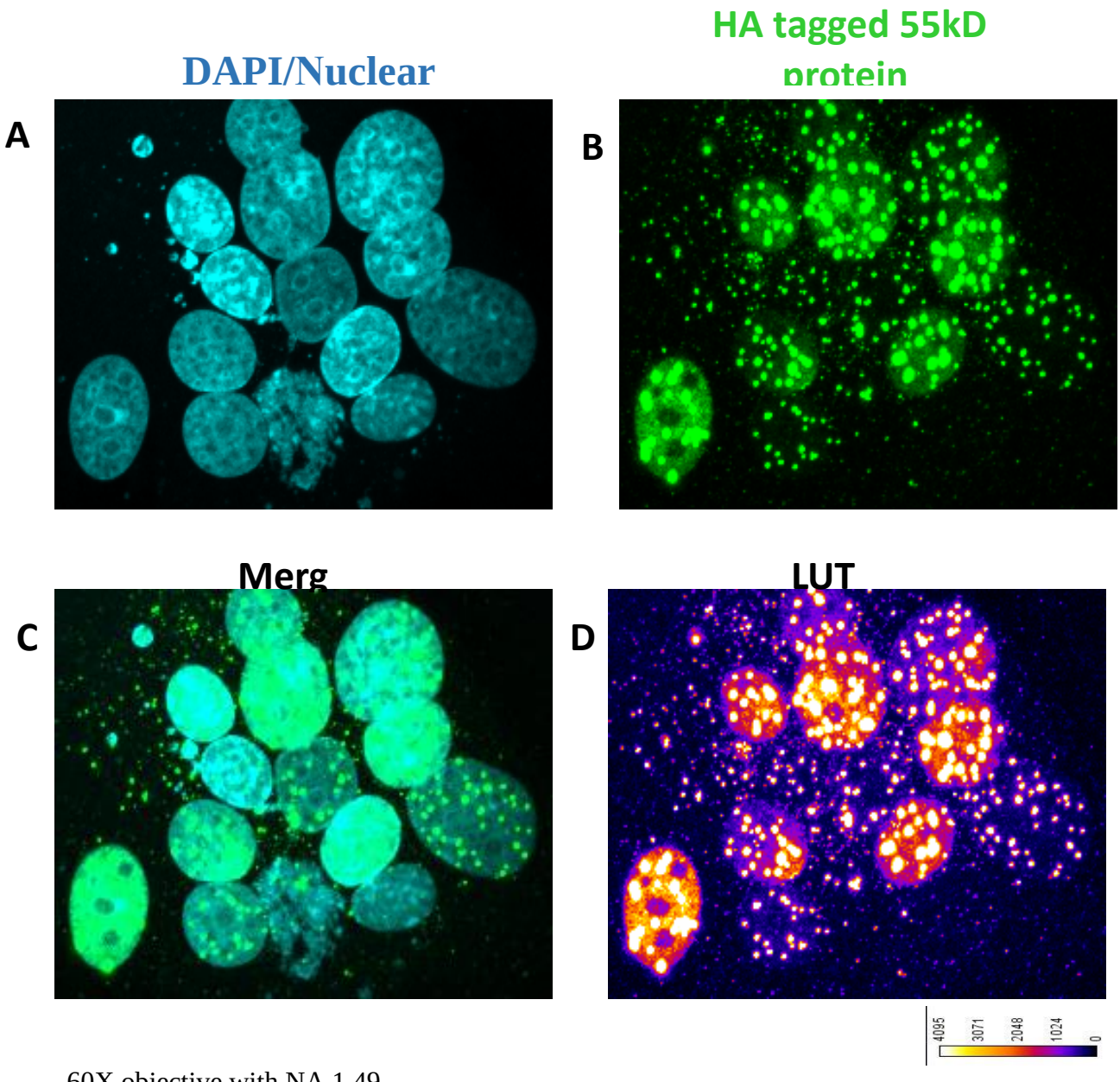
Figure 2C

RESULT

Cells plated on a 8 well chambered slide was transfected for the HA tagged 55 kDa protein coding gene using lipofectamine 2000. Lipofectamine is a cationic lipid-based chemical transfectant. They form liposomes taking up the plasmid (positive charges of the liposome *vs* negative charge of nucleic acids for DNA condensation). These transfection complexes (liposome) enter the cells through the endocytosis pathway. After disassembling of the late endosome, the nucleic acid is delivered into the cytosol for cellular action.

Transfected plasmid shows predominant nuclear localization in the mammalian cell line (astrocytes).

Figure 3-A) shows DAPI/nuclear staining of plasmid trasnfected astrocytes, 3-B) shows predominant nuclear localization of HA tagged 55 kDa protein, 3-C) shows merged image, 3-D) shows general polarised (GP) image of B where expression intensities can be assessed with a calibration bar showing differential intensities of the pixels in the image. The GP images were generated using Fiji Software. Images were acquired under



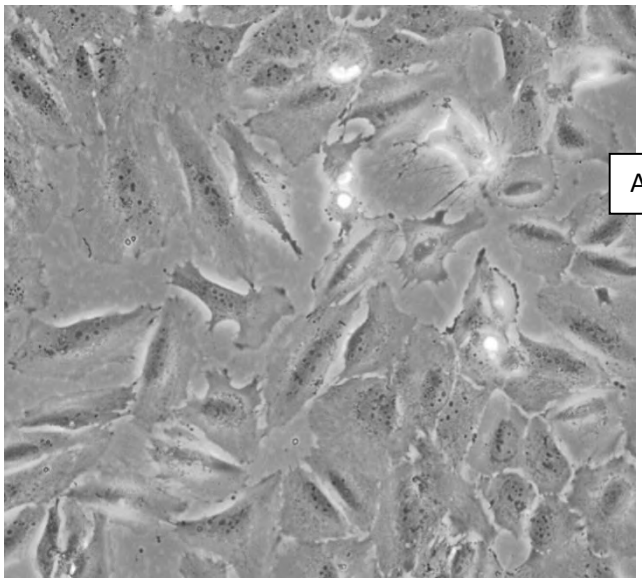
60X objective with NA 1.49

RESULT:

Two 100mm dishes where plated with cells and used for subcellular fractionation.

Successful subcellular fractionation of mammalian cells (astrocytes) yielded two fractions-cytosolic and nuclear. Since the protein was localized majorly in the nuclear compartment as evidenced from the confocal imaging post transfection, we took nuclear fractions for further studies and compared it with the cytoplasmic fraction.

Subcellular Fractionation

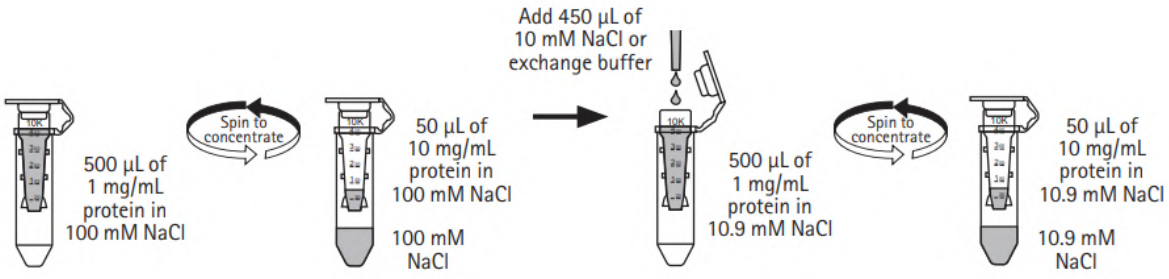


Cultured cells are scrapped in 1X PBS from the 100mm dish and collected in a 1.5 ml eppendorf vial.
Cells are then centrifuged at 2000 RPM for 5 minutes.
Supernatant is removed and buffer A is added
incubate for 30 minutes.
Cell suspension is passed through 23 gauge needle for about 20 times by intermediate checking of cell lysis by staining the sample with trypan blue.
Centrifuged at 5000g for 5 minute.
Supernatant is removed and the pellet is washed with the buffer A (hypotonic) for two times at 5000g for 10 minutes.
After 2 washes of the pellet buffer A^{++} (hypertonic) is added to the nuclear pellet, resuspended and kept for incubation for 30 minutes in the cold room.
After incubation the sample is centrifuged at 18000g for 15 minutes, collected and stored in deep freezer before further analysis.

RESULT

Nuclear and Cytoplasmic fractions isolated after subcellular fractionation were desalted using Amicon Ultra-0.5 mL centrifugal Filter from Millipore. The protein yield was then estimated using BCA assay.

Figure 4: Nuclear fraction and Cytoplasmic Protein lysates were desalted using Millipore desalting spin columns



RESULT

Protein yield of non desalted and desalted nuclear fractions is represented in the Figure. The protein concentration was estimated using the Bicinchoninc Acid Method.

Table shows a significant increase in the per microlitre protein yield post desalting procedure but equal concentration of protein was taken from non-desalted and desalted nuclear and cytoplasmic fractions for comparison purpose.

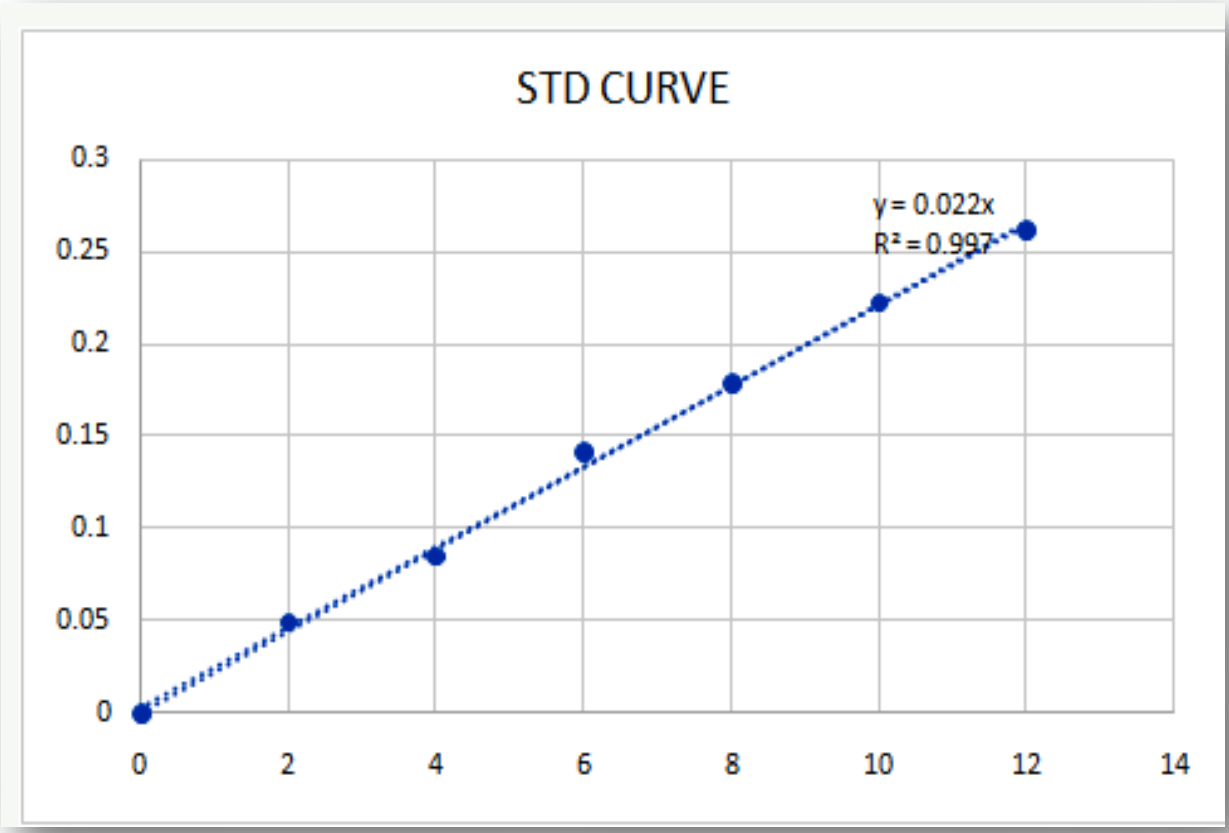


Figure 5: Protein Estimation Assay By Bicinchoninc Acid Method

Equation Y= 0.022x

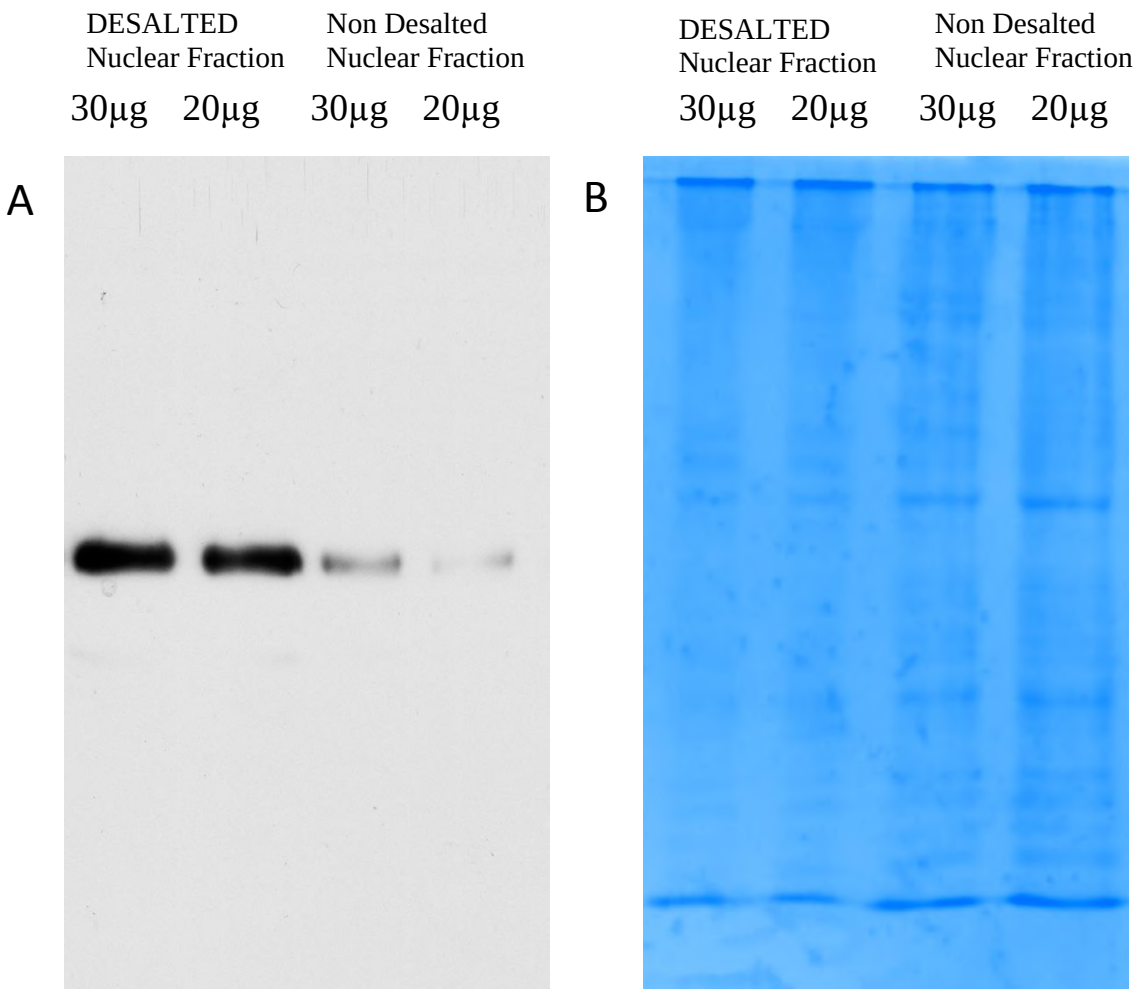
	Desalted Nuclear fraction	**Non Desalted Nuclear fraction**			Blank
Sample1	0.366	0.358		value1	0.335
Sample2	0.37	0.358		value2	0.347
Avrg	0.368	0.358		Avrg	0.341
Avrg-blnk	0.027	0.017			
x=y/m	1.216216216	0.765765766			
Conc. (µg/µl)	**0.608108108**	**0.382882883**			

RESULT

Result shows that upon desalting the transfer ability of the protein improved multifolds verses the non desalted nuclear fractions. Desalting of cell lysate prepared using hypertonic solution is better enough for efficient transfer and analysis of target as it is a single step based on gel filtration making the sample more concentrated.

Coomassie blue staining: This step is to visualize the protein bands formed during the electrophoretic run of the sample after SDS page. The stain used is coomassie brilliant blue G250 which binds non specifically to virtually all the proteins. The gel is stained post transfer to estimate the amount of proteins still on the gel after electroblotting at 100V for 90 minutes.

Figure 6: Expression level of HA tagged 55 kDa protein and its transfer efficiency in desalted vs. non-desalted fractions.

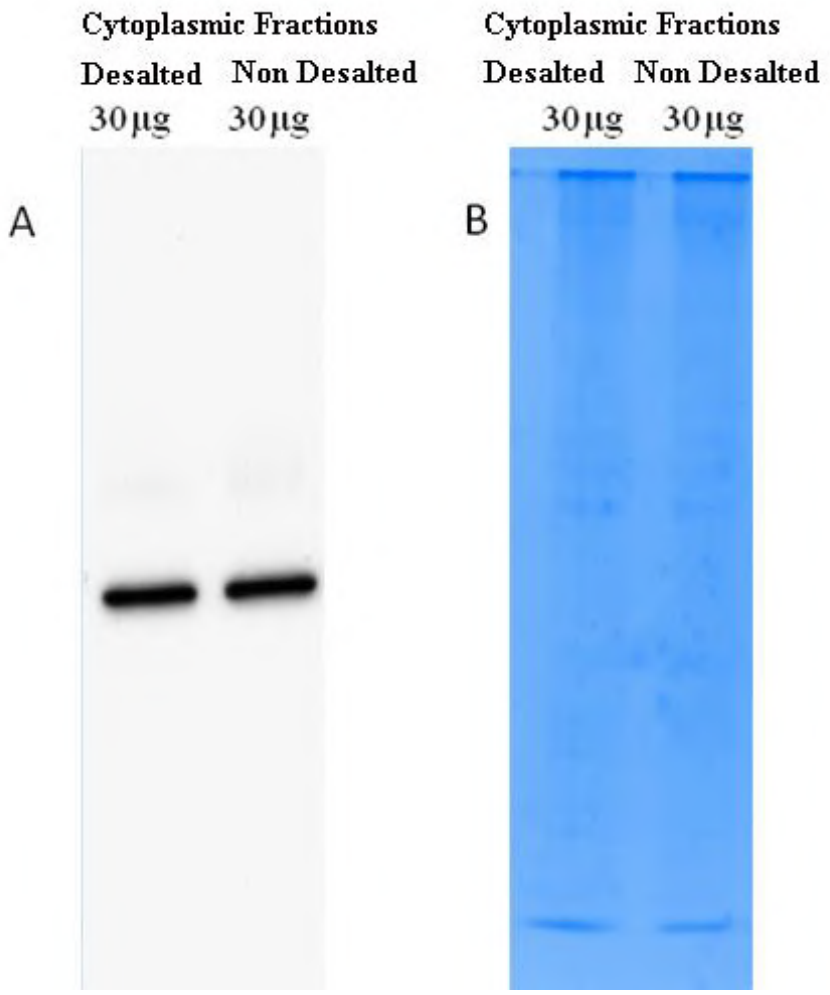


RESULT

Result shows that upon desalting the transfer ability of the protein did not change for proteins extracted in hypotonic buffer during subcellular fractionation verses the non desalted cytoplasmic fractions.

Coomassie blue staining: This step is to visualize the protein bands formed during the electrophoretic run of the sample after SDS page. The stain used is coomassie brilliant blue G250 which binds non specifically to virtually all the proteins. The gel is stained post transfer to estimate the amount of proteins still on the gel after electroblotting at 100V for 90 minutes.

Figure 7: Expression level of HA tagged 55 kDa protein in cytoplasmic fraction and its transfer efficiency in desalted vs. non-desalted fractions.



DISCUSSION

Protein immobilization on nitrocellulose membrane during Western transfer is thought to occur by hydrophobic interactions, and high salt and low methanol concentrations improve protein immobilization to the membrane during electrophoretic transfer, especially for proteins with higher molecular weights. However we find that the transfer process by itself is impeded by high salt in the protein lysateand this high salt will result in the transfer of proteins in unpredictable and unusual manner. So it is important to notice that the sample was free from excess concentration of salts or low molecular contaminants before applying the sample to electrophoretic gel.

Desalting of cell lysate prepared using hypertonic solution is better enough for efficient transfer and analysis of target as it is a single step based on gel filtration making the sample more concentrated which is required for acquiring good results. Concentration of proteins loading in each lane of gel during electrophoresis should also be equal in amount for accurate results as the increase in the protein expression of a lane will be masked if the comparative lane contains double amount of protein where the concentration of protein can be determined by measuring the intrinsic ultraviolet absorbance of protein or by methods based on protein dependent color change such as Bicinchoninic assay or Bradford dye assay.

CONCLUSION

Western blotting is a very sensitive method for visualizing specific proteins on the basis of molecular weight in SDS-gel electrophoresis where the use of membrane and electrophoretic mode of transfer provide complete and quantitative protein transfer and it is clear from the results that desalting of sub-cellularly fractionated proteins *via* hypertonic solution is required for the efficient electrophoresis of protein from SDS gel to nitrocellulose membrane. This is essentially because high salt impedes the electrophorectic mobility of proteins. Hence, for more accurate profiling of protein levels in different conditions, as the case may be, nuclear fractions which are isolated using high salt buffers should be de-salted.

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