

## LECTURE-04

### SAMPLE PREPARATION FOR PROTEOMIC ANALYSIS

#### HANDOUT

#### PREAMBLE

Proteomics aims to study and characterize proteins by involving functional elucidation, their interaction with other biomolecules and their physiological significance in the system. Hence for proteomic analysis of any sample, the first and by far the foremost important factor is protein extraction and sample preparation. The amount of information that can be gathered from the proteomic data hugely depends on the quality and quantity of sample. The techniques involved in proteomics are extremely sensitive to interfering compounds and hence they need to be eliminated before any proteomic analysis can be done. The type of sample to be processed determines the protocol that should be used for preparing the sample. In this lecture various protein extraction protocols, and strategies for good sample preparation will be discussed.

#### OUTLINE OF LECTURE

1. Sample preparation in proteomics
2. Generalized protein preparation protocol
3. Work Flow of sample preparation
  1. Cell disruption/lysis
  2. Protection from proteolysis
  3. Sample fractionation

4. Protein extraction and Solubilization
5. Contaminant removal
6. Quantification
4. Challenges
5. Protein Sample Preparation
  1. Bacterial
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6. Conclusion
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## **BOX-1: TERMINOLOGY**

1. Gentle Cell Lysis: A method whereby the cells are lysed by chemical treatments like enzymes or chemicals.
2. Harsh Cell lysis: A method whereby the cells are lysed by harsh physical treatments like sonication, French press or grinding.
3. 2-DE: Two dimensional gel electrophoresis where proteins are separated first by their isoelectric points and then by their mass.
4. Biomarker: A biomolecule (usually a protein), which serves as a signature for a particular state of a disease.

## 1. SAMPLE PREPARATION IN PROTEOMICS

Sample preparation is by far the most important step in proteomics. The quality of protein sample is directly proportional to the quality of proteomic data that is obtained and hence the interpretation. The overall aim of sample preparation is to obtain a pure protein pool, devoid of any salt or other bio-molecule contamination. In any experiment, reproducibility of data reinforces the hypothesis proposed and hence for this purpose, the sample preparation is considered as one of the most important steps in proteomics. Proteomic samples range from cells (bacterial or yeast) to tissues (plant or animal) to organism as a whole to biofluids (cerebrospinal fluid or serum). With the ultimate aim of extracting proteins from the samples, all protocols need to be optimized accordingly. Sample preparation basically involves solubilizing the proteins followed by their denaturation, reduction and reconstitution in suitable buffer. The global protein extraction process ensures that all cytosolic and organellar proteins are extracted and the interfering compounds are removed. For example, in case of bacterial protein extraction, a gentle cell lysis results into the liberation of all cellular proteins into the medium, whereas in case of plant cells, a rigorous cell lysis method results in the liberation of only cytosolic proteins into the medium. Further, ultracentrifugation is required to separate out the organelles and their proteins. Hence, it is the sample preparation, which determines the fate of the proteins.

## **2. GENERALIZED PROTEIN PREPARATION PROTOCOL**

A typical protein isolation protocol involves the basic steps: Solubilize proteins, prevent protein aggregation, denature and reduce all the proteins, remove nucleic acid and other contaminations. A typical protein pool consists of various kinds of proteins varying in their physico-chemical properties. Therefore, a medium should be selected such that it is able to solubilize all the proteins and also prevents their aggregation. The proteomic techniques involved in the experiments are highly sensitive to contamination from salts or nucleic acids or any other bio-molecules. Also, repeated freeze thawing should be avoided and hence, the protein samples before processing are stored at  $-20^{\circ}\text{C}$  for long time. Usually buffer containing strong chaotropic agents like urea, thiourea and detergents like CHAPS are preferred for solubilizing the proteins. The quality of the sample is much more important than the quantity itself, as quantity can be compromised by pooling in samples together, but quality cannot be compromised as the errors are multiplicative in nature and the error made in the first step of protein isolation gets multiplied and pose a bigger threat to the downstream analysis of data.

## **3. WORKFLOW OF SAMPLE PREPARATION**

Usually sample preparation starts with disruption of cells or removal of contaminants. Ideally, all the protein extraction steps should be performed at 4<sup>0</sup>C to prevent proteolysis by addition of protease inhibitors. Centrifugation steps and precipitation steps should be carried out for adequate time points to prevent precipitation of unwanted substances. Ideally, the workflow of sample preparation in proteomics can be divided into the following steps, which are described in next section.

### **3.1. CELL DISRUPTION / LYSIS**

Cell disruption or lysis is the first step towards any sample preparation, be it for proteins or DNA or RNA. The level of strain that a cell can handle governs the cell lysis protocol. For example, a fragile eukaryotic animal cell can be effectively lysed enzymatically or by gentle sonication. However, a vigorous sonication is required for bacterial or yeast cells containing cell wall, while a liquid nitrogen crushing is required for algal or plant cell materials. The purpose of cell lysis is to make the proteins and all the bio-molecules available to the external media. Hence, according to the cell type, lysis steps are employed, which may be harsh or gentle or a mixture of both.

The gentle lysis of cells can be performed with the help of agents like osmotic stress (when placed in a hypotonic solution, the cell swells and bursts), detergents (solubilizing the membranes), enzymes (degrading cell walls mainly) and rapid freeze thawing (crystals generated pierces the cells). The cell types which are usually subjected to gentle lysis are mainly tissue culture cells, blood cells, bacterial cells etc. The harsh lysis involves procedures like sonication, French press, manual and mechanical

grinders. Usually, plant and algal cells having a tough cell wall are subjected to such treatments or combination of treatments. Sonication and liquid nitrogen crushing appear to be the best protocols for cell lysis in case of plant cells. Over-sonication leads to fragmentation of proteins as well as the DNA which when sheared improperly, leads to increase in the viscosity of the medium. Another problem which arises in liquid nitrogen crushing, is the space generated between the pestle and the cellular paste. To avoid this issue the acid treated sand or glass beads can be added, which further helps in grinding. In majority of the cases, especially when dealing with tough tissues or cells, a combination of more than one method is applied.

### **3.2. PROTECTION FROM PROTEOLYSIS**

Once the cells are lysed, all the ingredients come into the medium. Proteases are enzymes, which fragment the proteins into smaller pieces. At normal room temperature, proteases are extremely active and if cell lysis takes place at the room temperature it results into the enormous pool of proteases. To prevent this, either cell lysis is carried out at 4°C or protease inhibitor cocktails are added to the medium prior to cell lysis. Proteases fall under three broader categories – serine proteases, cysteine protease and metalloprotease. A protease inhibitor cocktail usually consists of chemicals that target these key residues of the protease. Apart from the proteases, phosphatases are another set of enzymes, which pose a threat to the proteins, especially, when studying post-translational modifications and signaling is a question. Hence, along with protease inhibitors, phosphatase inhibitors like sodium orthovanadate are also added.



## **3.3. SAMPLE FRACTIONATION**

A crude protein pool consists of proteins of varying concentrations. In fact the orders of concentration of proteins may vary upto 7- 8 orders of magnitude. Hence it becomes necessary to fractionate them so that a complete representation of the entire proteome pool is available in the sample. For example, in serum, abundant proteins such as albumin, immunoglobulin G and transferrin account for approximately 90% of the total proteins. The low molecular weight proteins either get masked under these abundant proteins or are extremely low in amount to be detected by any technique. Hence, there is a requirement of a suitable pre-fractionation step, which will remove all the unwanted proteins, either completely or partially, and thereby enriching the sample with other proteins. Majority of the pre-fractionation methods are based on selective removal of abundant proteins from complex mixtures and hence affinity chromatography or immunoprecipitation based methods are widely used.

### **Illustration: Methodology for the removal of abundant protein from the serum**

*Complex biological samples like serum requires a multistep robust protocol to remove the interfering compounds and other highly abundant proteins. Such abundant proteins interfere in the separation and hinde the separation of low abundant proteins. The serum consists of high abundance proteins such as IgG, alpha-1 antitrypsin, IgA, transferrin, haptoglobin, which will interfere during protein separation. These proteins mask the movement of low abundant proteins. A representative protocol is demonstrated in following animation.*

- *Add binding buffer to the column and centrifuge. Buffer treatment is done to activate the bonding property of resins inside the column.*
- *Discard the liquid collected in the tube below, now the column is ready for depletion. Add the serum to the binding column and keep it in ice for 5 mins.*
- *High abundance protein binds to the affinity column, while other proteins do not bind and get eluted at faster rate. This step helps in depleting the sample.*
- *Centrifuge the column for 30 sec at 800 rpm. Centrifuge helps to collect the unbound protein sample at the bottom of the column.*
- *Transfer the serum to the new tube and store it till further usage.*



### **3.4. PROTEIN EXTRACTION AND SOLUBILIZATION**

Protein extraction is usually performed using organic solvents like acetone or trichloroacetic acid. The organic solvents are responsible for increasing the protein-protein interaction by removing the solvation spheres around the proteins. As more and more proteins interact, they aggregate and hence precipitate down. For solubilizing the proteins, chaotropic agents Urea, thiourea and detergents such as CHAPS are used. The chaotropic agents and surfactants are responsible for denaturing the proteins, which further break their intermolecular and intramolecular interactions. Once these interactions are broken, the water molecules are able to solubilize the proteins much easily. The proteins are first separated from nucleic acids and membranes by suitable treatments and then precipitated from the solution by use of organic solvents. In certain cases, especially where membrane proteins are involved, their greater hydrophobicity prevent them from easy dissolution. Thio-urea in this aspect becomes extremely helpful, in terms of stabilizing the membrane proteins. In many cases, ionic detergents like SDS pose a problem in downstream processes like electrophoresis. Zwitter-ionic detergents such as CHAPS (3-((3-cholamidopropyl) dimethylamino)-1-propane sulfonate) are extremely useful in solubilization and are also compatible with electrophoresis. Addition of DTT (dithiothreitol) enhances solubilization by reducing the disulfide linkages. Even though acetone and TCA are effective precipitants, care should be taken during removal of excess solvents from the precipitants. The sample should also not be extremely dry, or else, solubilization in appropriate buffer becomes an issue.

### **3.5. CONTAMINANT REMOVAL**

Contaminants in the form of salts and nucleic acids are the predominant species that need to be removed to obtain high quality protein samples. Usually in TCA Acetone mode of cell lysis and precipitation, the DNA associated with the proteins also precipitate and hence clogs the gel pores during electrophoresis. Hence, the lysis buffer should contain nucleases to digest the DNA and also reduce the sample viscosity. Salts are extremely notorious contaminants in protein solution. They mainly interfere with the isoelectric focusing by allowing the passage of excess current and hence generating substantial heat to destroy the gels and the samples. When the samples are body fluids like serum, plasma or urine, the concentration of salts are very high and hence additional steps for desalting needs to be performed. The small carbon chain length containing columns are commercially available, which by virtue of hydrophobic interactions; adsorb the proteins allowing the salts to pass through them. The proteins are then selectively eluted using formic acid and acetonitrile solution. This approach is usually done before mass spectrometry analysis of proteins because mass spectrometer is extremely sensitive to high concentrations of salt.

Salt removal techniques usually involve dialysis, precipitation and resolubilization, gel filtration etc. But these processes results into diluting proteins and loss of protein quantity; therefore, now a days, commercial zip tipping columns are available for desalting purposes. Polysaccharides and lipids fall under the category of lower level contaminants. Usually they don't pose much problem except when they are in higher amounts, when they tend to clog the gel pores in electrophoresis. Polysaccharides and

lipids are highly soluble in organic solvents and hence during the process of protein precipitation, they are easily removed. Pigments like carotenoids or chlorophylls need an additional step of removal by organic solvents such as methanol and chloroform.

### **Illustration: Methodology for the cleaning-up of protein sample**

*The protocol focuses on removal of impurities like metal ions, salts, phenol and carbohydrates compounds that interrupt the separation of protein during IEF and SDS-PAGE. Proteins present in the sample are treated to get precipitated by combined action of precipitant and co-precipitant, while leaving behind most of the impurities into the solution as supernatant. A representative protocol is demonstrated in following animation.*

- *Place the wash buffer at -20C for at least 4 hours before use. Wash buffer allows rapid and complete resuspension of the proteins.*
- *The sample can be any raw sample extract or processed serum sample, bacterial tissue sample, which after extraction are stored in freezer. Thaw the frozen sample by rubbing between the palms to start cleanup process.*
- *After thawing the sample, it needs to be vortexed for a brief interval. To make a homogeneous mix of sample solution.*
- *Add 300  $\mu$ l of precipitant to the sample and keep it in ice for 15 mins. Precipitant helps in protein precipitation while interfering substances such as detergents, salts, lipids, phenols, and nucleic acids are left behind in solution.*
- *Vortex for few seconds, which helps in proper mixing.*
- *Add 300  $\mu$ l of co-precipitant to the sample and vortex for 30 sec. Co-Precipitant also helps in protein precipitation. Incubate it for 10 min followed by centrifugation.*
- *Centrifuge the content for 5 min at 4C for 12000 rpm.*
- *Remove the supernatant thoroughly without disturbing the pellet. In case if pellet gets disturbed, repeat centrifugation step again.*
- *Add 40  $\mu$ l of co-precipitant to the sample and vortex the tube. Care must be taken to remove supernatant without disturbing the pellet formed.*
- *Add 25  $\mu$ l of de-ionized water to the pellet and vortex for 10 sec. The pellet should disperse, but not dissolve in the water. De-ionized water is used to avoid contaminants.*
- *Add 1ml of wash buffer (pre-chilled for at least 3 hours at -20°C) and 5  $\mu$ l of wash additives to completely disperse the pellet. Wash buffer and wash additive contains organic additives that allows rapid and complete resuspension of the proteins. The proteins will not dissolve in the wash buffer.*
- *Incubate the tube at -20°C for at least 60 minutes with frequent vortex after every 10 mins. At this stage, the tubes can be stored at -20°C with minimal protein degradation or modification.*
- *Centrifuge the sample at 12000g for 5 mins.*
- *Remove the supernatant thoroughly without disturbing the pellet. In case if pellet gets disturbed, repeat centrifugation step again. Keep the tube containing the pellet for air-drying to remove the traces of co-precipitate.*
- *Once pellet completely goes into the rehydration solution, the sample can be stored at -80°C for further analysis.*

### **3.6. QUANTIFICATION**

Quantification is an important step because it governs how much protein is analyzed in a proteomics experiment. Varying amounts of protein loading gives misleading results, especially when the purpose is to study the expression levels of proteins. Various methods of protein quantification are available. The most common and traditional method involves measuring the absorbance at 280 nm, identifying the aromatic amino acids like tyrosine, tryptophan and phenylalanine. However, it is a highly error prone assay with a biasness for aromatic amino acids. Any protein having an under-representation of aromatic amino acid is thus lost during the process of quantification.

Dye based assays like Lowry, Bradford or Bicinchoninic acid are much preferred over the conventional UV based assay. Lowry assay involves the combination of Biuret Assay involving reduction of cupric ions to cuprous ions and the stabilization of the complex by alkaline tartarate. The reaction yields a purple colour and the absorbance is measured between 500-800 nm. However, the disadvantage of this assay is in its dependency for tyrosine residues. Also, it is highly sensitive to chemicals like TCA, Tris and EDTA. A much more sensitive assay involves the use of bicinchoninic acid after biuret reaction. The complex is even more stable than the same in Lowry assay and is highly sensitive for membrane proteomes. However, it is also affected by chemicals like EDTA, though at a higher concentration.

Majority of protein quantification methods for proteomic analysis employ Bradford's method, whereby a complex is formed between Coomassie brilliant blue and the

proteins. Although the Coomassie dye prefers lysine and arginine residues, the overall assay is highly sensitive and is compatible with many chemicals, unlike lowry or BCA assay. The dye on binding with the protein shows absorption maxima at 595 nm. However, at higher concentrations of proteins, there is a deviation from the linearity, which affects protein estimation to some extent. Nonetheless, Bradford assay is most commonly used for protein estimation in proteomic studies.

### **Illustration: Methodology for the quantification of protein**

*Protein quantity varies based on the type of tissues, mode of extraction, handling techniques and the content of the cell. Proteins have to be quantified after extraction prior to starting any proteomics experiments. A representative protocol is demonstrated in following animation.*

- *Thaw the frozen sample by rubbing between the palms. Take the BSA standard and place it on the ice.*
- *To make a standard, use BSA stock (2mg/ml) and pipette out BSA in increasing volume for increasing concentration. Make standard graph so that concentration of unknown samples can be calculated.*
- *Make the volumes equal by using water.*
  
- *The Bradford reagent consists of Coomassie Brilliant Blue G250, ethanol, Phosphoric acid. Add the Bradford reagent to the protein sample and standard. Incubate for 15 minutes at room temperature.*
- *Red form of coomassie blue dye binds to the protein and the electron transfer reaction takes place, which results in protein denaturation and color changes from red to blue. The color intensity is directly proportional to the concentration of protein.*
- *Switch On the instrument and set wavelength at 595 nm.*
- *Keep the sample in the spectrophotometer and press 'absorbance' button to get the reading.*
- *Plot the graph between the Absorbance and the Concentration of BSA. The absorbance of the samples can be extrapolated and the concentration can be found.*

### **4. CHALLENGES**

Even though protein isolation is a routine work, it poses several challenges and these challenges arise depending on type of samples being handled. For example, algal and plant cells have a very strong cell wall, which is very difficult to disrupt by normal sonication method. Hence enzymatic pre-treatment or French press also accompanies sonication. Plant cells have high amount of pigments like chlorophyll and carotenoids and high amount of lipid. All these interfering compounds pose a challenge for preparation of good protein sample.

On the other hand, body fluids pose the problem of high salt contaminations and very dilute samples. Although both of these problems can be addressed simultaneously with the use of zip tipping (carbon columns), in certain cases like urine or cerebrospinal fluids, protein concentration is very low and hence isolation of proteins is extremely tricky. Also, the range of protein abundance varies in the body fluids and thus having a proteome profile of all the proteins becomes extremely difficult because many low abundant proteins get masked or are limited by the sensitivity of the technique used to identify them.

Bacterial cells are probably the easiest cells to harvest proteins, but due to their prokaryotic nature, the protein samples get highly contaminated with nucleic acids, which increase the viscosity of the medium and hence pose downstream problems. However, in all the samples, membrane proteins pose the greatest problem. They are



highly hydrophobic in nature and hence are not readily soluble in the buffer generally used. Also, they are extremely bound tightly with the membranes and disrupting the membranes at the level of individual building block is not possible. Hence, they are either under-represented or are totally lost from the proteome profile. Regardless of the sample to be analyzed, a combination of various methods is recommended for good protein sample preparation.

## **5. PROTEIN SAMPLE PREPARATION**

A workflow of protein extraction from few important sample types is described below:

### **5.1. BACTERIAL**

The bacterial cell is lysed by either gentle or harsh methods like lysozyme treatment and sonication, and the cell lysate is treated with TRIZol reagent (containing phenol and guanidium isothiocyanate) to denature the proteins. Chloroform and ethanol specifically separate out the RNA and the DNA. The protein is precipitated using acetone or a mixture of TCA and acetone. The advantage of TRIZol based method is that the protocol is devoid of any contamination from nucleic acid or lipids. There is also no need for desalting in this case. The proteins are highly soluble in the buffer and the yield is also very high.

#### **Illustration: Extraction of bacterial protein**

*Protein extraction from the sample requires an optimized protocol to increase the protein amount in the extract. The protein extraction from the cell requires suitable reagents and technique that can yield a better and efficient result. A representative protocol is demonstrated in this animation.*

- *Pick a bacterial colony using the toothpick from the master culture.*
- *Inoculate the bacterial colony in the sterile broth. Perform these steps in laminar hood and keep the burner on during inoculation to avoid contamination.*
- *Place the inoculated tube in the shaker incubator at 37°C for 6-8 hours.*
- *Transfer the bacterial culture into the clean centrifuge tube under the aseptic conditions.*
- *Centrifuge the culture for 10 min, 12000 rpm at 4°C to harvest the culture.*
- *Remove the supernatant completely without disturbing the pellet and take the pellet for further processing.*
- *Wash the pellet with phosphate buffer thoroughly to remove the excess broth. Once broth is removed completely, cell lysis need to be carried out.*
- *Keep the sample on ice and start sonication by providing 6 cycles of pulses for 5 sec, 20% amplitude with 5 sec gap.*
- *High frequency sound waves breaks open the cell wall and the contents are released into the buffer.*
- *Centrifuge the contents to remove the debris and collect the supernatant for further processing.*
- *Trizol reagent consists of guanidium thiocyanate, phenol and chloroform that separates DNA, RNA and proteins from each other.*

- *Add trizol to the supernatant and vortex it thoroughly.*
- *Add chloroform to the sample, mix thoroughly and incubate at room temperature for 5 min till you see the phase separation.*
- *Three layers are formed after centrifugation. The top layer contains RNA, Middle layer has protein and the bottom layer has DNA.*
- *Remove the aqueous layer containing RNA without disturbing the other two layers. Add the absolute alcohol to the remaining layers and mix gently till the middle layer dissolve and keep at room temperature for 3 min. Centrifuge the content for 5 min at 2000 rpm.*
- *DNA forms pellet and the supernatant containing protein is recovered. Add 4 volumes chilled acetone to the sample and vortex it to precipitate the protein.*
- *Place the sample at -20°C for at least an hour for complete precipitation and centrifuge the content.*
- *Remove the supernatant carefully and air dry the pellet. Wash the pellet with 0.3 M guanidium–HCl in 95% ethanol for 4 times to remove the color and for inactivation of RNAses. Each wash step needs to be followed by centrifugation to discard supernatant and carry out pellet washing.*
- *The rehydration buffer consists of CHAPS, which is used to solubilize the proteins including membrane proteins. Urea used to denature protein.*
- *Add 0.4 ml of rehydration buffer to the dried pellet and vortex till the pellet completely gets solubilized.*
- *The sample in rehydration buffer can be stored at -20°C.*

## **5.2. PLANT**

Plant and algal samples being extremely tough are first crushed using liquid nitrogen and then the lysate is treated with either TCA/Acetone or by TRIZol reagent as described above. Plant cells contain pigments and hence a depigmentation step involving chloroform or hexane is required prior to protein extraction. This step also removes majority of the lipids, which can pose problem later if not removed.

### **Illustration: Extraction of plant protein**

*A representative protocol for plant protein extraction is demonstrated in following animation.*

- *Clean the leaves with distilled water, and dry it on paper towel to remove the water. Weigh 300 mg of cleaned fresh leaves for the protein extraction.*
- *Add 10 ml of liquid nitrogen to the leaves. Add liquid nitrogen to mortar and pestle to prechill them.*
- *Transfer 300 mg of cleaned fresh leaves into the prechilled mortar and pestle. Grind the sample until it looks powdered form.*
- *To the powdered sample add the lysis buffer and perform grinding again.*
- *Transfer the paste to the clean, autoclaved eppendorf tubes.*
- *Incubate the samples at -20°C for 1 hour.*
- *Perform the centrifugation at 4°C, 14,000 rpm for 30 minutes.*
- *Remove supernatant without disturbing the pellet and discard it.*
- *Add 1 ml of ice cold wash buffer to the pellet and vortex it until it completely gets solubilized.*
- *Perform the centrifugation at 4°C, 14,000 rpm for 10 minutes.*
- *Air dry the colorless pellet to remove out excess acetone and add 400ul of rehydration buffer for storage.*
- *Incubate the sample at 4°C overnight..*
- *Perform the centrifugation at 4°C, 14,000 rpm for 15 minutes.*
- *Collect the supernatant in fresh tube and store it at -20°C.*

## **5.3. SERUM**

Serum is one of the most important body fluid, which serves as the network of many important reactions. Hence, a study of the serum proteomics is very important. Nonetheless, serum proteomic analysis poses the challenges of high levels of protein abundance and salt contamination. Removal of these abundant proteins and interference results in enrichment of low-abundant proteins that can serve as potential biomarker for disease diagnosis.

### **Illustration: Extraction of serum protein**

*A representative protocol for serum protein extraction is demonstrated in following animation.*

- *Collect the blood in the serum separation tube. Place the tube on ice for coagulation for an hour and perform the centrifugation at 4°C, 14,000 rpm for 10 minutes to separate the coagulants and blood cells serum.*
- *Store the samples in eppendorf tube at -80°C in aliquots until further use.*
- *Transfer the required amount of the serum to the fresh, clean eppendorf tube for further sample processing.*
- *Dilute the serum 5 times using phosphate buffer of pH 7.4 and vortex the sample to achieve uniform mixing.*
- *Keep the sample on ice and start sonication by providing 6 cycles of pulses for 5 sec, 20% amplitude with 59 sec gap.*
- *The serum consists of high abundance proteins such as IgG, alpha-1 antitrypsin, IgA, transferrin, haptoglobin. To enrich the low-abundant proteins, the sample needs to be depleted by removing high abundance proteins.*
- *Use the depletion column to remove the high abundance proteins.*
- *Centrifuge the column for 5 minutes at 800 rpm, this helps to pack the resin in column.*
- *Add binding buffer to the column, which helps to prepare the resin for the depletion step. Now carry out centrifugation step as explained earlier. Add the serum to the binding column and incubate it in ice for 5 mins followed by centrifugation.*
- *High abundant protein binds to the affinity column while the other proteins do not bind and get eluted at faster rate.*
- *Centrifuge the column for 30 sec at 800 rpm. Transfer the depleted serum to the new tube and store it till further usage.*
- *Centrifuge the column so that the depleted serum will be collected in the tube. Add 4X volume 10% (w/v) TCA-Acetone to the depleted serum and incubate at -20°C for at least 4 hours.*
- *Place the sample in the centrifuge, balance with equal number of tubes and centrifugation should be carried out at 4°C at 14,000 rpm for 30 minutes.*
- *Add 1ml of ice cold wash buffer to the pellet and vortex the pellet until it completely solubilizes.*
- *Place the sample in the centrifuge, balance with equal number of tubes and centrifugation should be carried out at 4°C at 14000 rpm for 10 minutes.*
- *Perform the centrifugation at 4°C, 14,000 rpm for 10 minutes.*
- *Air dry the colorless pellet and add 400 ul of rehydration.*

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- *Incubate the sample at 4°C overnight for the protein to solubilize in the rehydration buffer and vortex the pellet until it is completely solubilized in rehydration buffer.*
- *The sample in rehydration buffer can be stored at -20°C.*

## **6. CONCLUSION**

Protein isolation from various sources employ protocols for protein precipitation using organic solvents. Major difference lies in the cell lysis method. Yet there arises considerable difference in the protein quality. The physiological properties and the origin of the sample thus become extremely important in adopting a strategy for protein extraction.

## **7. REFERENCES**

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