HANDOUT

LECTURE-09

SAMPLE PREPARATION FOR PROTEOMICS APPLICATIONS

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This lecture will discuss sample preparation for proteomics applications. A very good sample is essential to perform a good experiment, especially when one wishes to characterize thousands of proteins in that experiment. An ideal sample preparation procedure ensures that you have no contamination, good protein yield and no interfering substances in your extract. Often each type of biological sample poses its own challenges. For example, if you are working on bacterial cultures, plants, human samples, different body fluids such as serum, urine, saliva or cerebrospinal fluid, each sample with its own unique composition, presents unique challenges, requiring optimization of a generic protocol for each individual type of sample. An ideal sample preparation will ensure that you have all the protein present in your sample without any contamination from nucleic acids, salts and other interfering components.

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This lecture covers sample preparation for proteomics application, with the workflow for protein sample preparation being provided. The steps include

- Cell disruption/lysis, while maintaining protein integrity during this procedure
- Sample fractionation to simplify the complex proteome, to achieve adequate
- Protein extraction and solubilization.

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The workflow for proteomic sample preparation is now discussed.

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- Proteomics analysis can occur at various levels.
- The proteome can be examined at the whole organism level, or a tissue or fluid level, or at the level of a differentiated cell. Obviously, the higher the level of multicellularity of the sample, the more complex the analysis.

- Furthermore, proteomics can be global or it can be targeted or expression based.
- Highly reproducible samples are very important for performing comparative proteomic analysis, ie if you need to. know the difference in your sample and controls, sample preparation needs to be highly reproducible, in order to avoid sample artefacts

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Three different terminologies are commonly used during proteomic analysis; global proteome analysis, expression proteome analysis and targeted proteome analysis.

- Global proteome analysis involves characterization of all the proteins present in the given sample.
- Expression proteome analysis involves examination of the changes induced by to a chemical or treatment,, usually looking at the fold-change of proteins This kind of examination is the one most commonly used in clinical studies.
- Targeted proteomic analysis involves examination of say, a given cellular organelle for eg. mitochondrial proteome.
  So one needs to apply different sample preparation strategies tailored to fit each of the above types of studies. For example, targeted proteome analysis requires pre-fractionation of the sample in such a way that only that particular component is isolated and all the proteins from that organelle or cell are then extracted.

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Sample processing involves solubilization, denaturation, reduction and treatment of sample proteins.

But one needs to include additional steps depending upon the type of samples and objectives of the study, to improve quality of the protein extract.

However, some amount of protein or sample loss occurs at each step- a balance should be attained between improving sample quality and minimizing protein loss.

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Now protein extraction protocols need to ensure that majority if not all the proteins in a cell or organelle are extracted. The presence of interfering compounds should be minimized. Optimization of a good protein extraction protocol that ensures a very wide coverage of the proteome is crucial to the success of the experiment.

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So the steps involved in an ideal protein sample preparation include:

- Solubilising the proteins present in a mixture
- preventing protein aggregation
- denaturing and reducing all the proteins which are present in that mixture
- removal of nucleic acid and other contaminants
- removing salt and some other small interfering components.

Again depending upon sample type, different types of interfering components could be present. Plant samples contain phenolic contaminants. Serum contains a high concentration of salts that have to be removed etc.

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The guidelines for sample preparation are as follows:

- Start with a comprehensive literature search, to help modify the protocol based on the type of sample and experimental aim.
- Remove the nucleic acids, salts and different particulates.
- Samples should be prepared as fresh as possible, and then stored at -20°C in small aliquots. Avoid repetitive freezing and thawing of the sample.

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Why is a good sample preparation important?

- A good sample preparation includes all the proteins present in that mixture and it will provide high quality data with less interference from the artefacts.
- A good sample provides reproducible results with less variation amongst biological replicates and technical replicates of an experiment.
- Once a protein extraction protocol has been optimized, the same can be applied to in large scale studies.
- For example, if you are performing a clinical trial study on 200 patients. So once you have optimized the protocol with a small population then the same protocol can be applied for large number of samples. This will vastly improve the chances of success of that clinical study.
- The removal of contaminants also vastly improves the signal to noise ratio, allowing the detection of signal with a much higher sensitivity.

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The next sections continue with the step-by-step workflow of protein sample preparation.

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- Following extraction from the source material, the proteins should be solubilized before analysis. An ideal sample preparation disrupts all non-covalent bonds present within the proteins and also remove interfering components.

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- The workflow of the sample preparation could involve cell disruption or cell lysis, protection from proteolysis, sample fractionation, protein extraction and solubilization, removal of contaminants and quantification. Obviously this workflow can be modified depending on the sample type and a few steps can be moved about in this sequence. This workflow will be discussed in the context of some examples.

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Cell Lysis:

- First of all, the cells have to be broken open to release all the cell components. Cell disruption and lysis is very important and is often challenging because all the different sample types can't be lysed with the same method and this step has to be modified according to the sample.

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- Cell lysis achieves the effective disruption of cells or tissues, isolates the proteins from intact cells and tissues while avoiding the loss or modification of proteins, obtains all the proteins which are present in a given sample and helps maximize the sample recovery and to retention of structural integrity of the sample.

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There are different steps involved in cell lysis:

- Disruption of the cell membrane, while protecting samples from proteolysis
- Homogenization and solubilization of cellular contents.

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Cell lysis can be performed by gentle or harsh methods depending upon the type of cells to be disrupted.

- Cell types which can be easily lysed, such as RBCs or cultured cell lines, can be disrupted by gentle lysis. Choices for gentle disruption include a) osmotic lysis, achieved by suspension of the cell in a hypotonic medium b) detergent lysis, achieved by the addition of a detergent, c) enzymatic lysis: For example, cellulase for cellulose-containing plant samples, lysozyme that disrupts peptidoglycan for bacterial samples etc d) Freeze-thaw; this is one of the very commonly used methods for gentle disruption which involves rapid freezing and thawing cycles. The samples are frozen in liquid nitrogen, and then thawed at 37C.

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- Harsh disruption methods include as sonication, High pressure homogenization using a French press, or homogenization by, manual grinding. The different types of cell lysis methods and some of the principles involved will be discussed later, but the most commonly used methods include manual grinding (using a mortar pestle)_ or homogenization using a bead beater or polytron homogenizers. The latter methods are used if you have very low amounts of sample are available, or contamination has to be avoided. The sonication and French Press methods are used more for bacterial, yeast or other cells which are difficult to lyse.

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The above slide provides an overview of the lysis methods.

- One can use detergent to gently lyse tissue culture cells.
- Enzymatic lysis is also a gentle lysis method, which can be used for plant tissues, bacterial cells and fungal cells.

- Freeze-thawing can be used for bacterial cells or cultured cells and this is a gently lysis method.
- High pressure using a French Press is often applied on bacteria, algae and yeast samples and is a vigorous lysis method.
- Glass beads are used with cell suspensions or organisms with a cell wall and this is a rigorous lysis method.

Grinding of solid tissues and microorganisms is another vigorous lysis method.

- The mechanical homogenization of solid tissues is another vigorous lysis method.
- Osmotic lysis can be used for blood samples and tissue culture cells which is a gentle lysis method.
- Sonication can be used for cell suspensions and other bacterial samples and is another harsh lysis method.

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Proteolysis may occur during cell disruption, especially during mechanical disruption by grinding, etc. Prevention of protein degradation, from cellular proteases also released by cell disruption is very necessary

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Need for protease inhibition

- As mentioned, cell lysis will release various proteases which cause proteolytic breakdown of proteins. During the sample preparation one can use different types of protease inhibitors which can minimize this process.

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- Effective protease inhibition requires a mixture of different types of protease inhibitors, both irreversible and reversible, which inhibit serine & cysteine proteases, as well as various metalloproteases. This step is important when preparing samples for gel-based proteomic applications.

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A few specific examples of protease inhibitors include:

- Phenyl methyl sulphonyl fluoride (PMSF) which is effective against serine and cysteine proteases. It can be inactivated by DTT and it is unstable when you are preparing samples for gel-based applications.
- Ethylene diamine tetraacetic acid (EDTA) is effective against metalloproteases and also inhibits nucleases. EDTA can protectt from proteolysis and partially inhibit nuclease activity.
- Ethylene glycol tetraacetic acid (EGTA) is effective against metalloproteases. Note: When preparing a sample for proteomic applications, it is very important to work on ice to reduce proteolysis.

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Sample Fractionation:

- Reduction of unnecessary components and simplification of the proteome prior to starting the experiment vastly increases the chances of success. For example, for proteome analysis of a given particular organelle, the cell lysate should be fractionated to reduce the complexity of the sample.

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- Sample fractionation makes it possible to isolate the group of proteins or fractions from a given total proteome.

Why perform fractionation?

- It simplifies the analysis of complex protein mixtures.
- This method allows the improved resolution of proteomic data. The data obtained after the pooling of information from individual fractions is more informative. The samples applied on a 2-DE gel after fractionation of the original proteome will be produce less cluttered, busy gels, providing a better resolved protein map, making fractionation useful in in gel-based and gel-free proteomic applications.

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The different types of fractionation methods available include.

- Centrifugation or ultracentrifugation: This is useful for the separation of sub-cellular organelles such as mitochondria or chloroplast.

- Affinity chromatography: This method is useful for the removal of abundant proteins, such as serum albumin or IgGs Now affinity chromatography methods can be used for fractionation in different contexts.
- Sequential extraction: This method, based on solubility, utilizes different chemicals added sequentially to solubilize and extract certain proteins at each step. It uses the property of differential solubility-. Few proteins may more soluble in one composition of mixture and other proteins are more soluble in other solubilization buffer. Electrophoresis: This method can be also used for fractionation in say, a gel-free method wherein one wishes to directly extract the protein and analyse that using MS. Rather than fractionating the entire proteome with liquid chromatography, the proteome can first be simplified by isoelectric focussing in the liquid phase. This concept will be discussed in greater detail in the following lectures, during the discussion of OFFGEL fractionation

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Fractionation achieves the following:

It increases proteome coverage: You would like to analyse as many proteins as possible from that given sample.

- An effective fractionation separates highly abundant proteins from the proteins of interest that may be present at much lower levels and brings them into the dynamic detection range. This process thus increases the chances of identifying low abundance proteins or diagnostic or therapeutic interest.

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Following the optional step of fractionation, the next step in the workflow is protein extraction and solubilization.

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- As mentioned, this step can be preceded by sub cellular fractionation and succeeded by protein analysis.
- Protein extraction is carried out in an aqueousbuffer by various methods.
    - Tris-HCl method followed by desalting
    - protein precipitation by trichloro acetic acid (TCA),
    - acetone alone or TCA and acetone.
- The protein extracts should be soluble and free from contaminating proteases, DNA, RNA and metabolites. As reducing and denaturing agents are adding during this step, protein-protein interactions will also be disrupted

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Sample solubilization is important because proteins naturally form complexes with membranes, nucleic acids as well as other proteins. Protein can also form various

types of non-specific aggregates. Proteins can precipitate when they are removed from their normal environment.

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The effectiveness of the sample solubilization protocol depends upon the choice the method of protein disruption, concentration and dissolution, the choice of detergents and the sample composition itself. Hence, the solubilization methods differ from sample to sample. One can't provide a generic recipe for an effective sample solubilization.

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There are different components used in sample solubilization. They include:

- Chaotrops, which is a mixture of urea and thiourea. LUrea is used as a denaturant which can solubilise and unfold most of the proteins into fully random conformations. This exposes all the ionisable groups in the protein to the components of the solublization solution. Thiourea improves solubilization of membrane proteins more specifically.

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- Detergents: SDS is extremely effective at solubilizing hydrophobic proteins But its anionic nature limits its effectiveness during conventional proteomic analyses. SDS is not compatible with isoelectric focusing and hence should be avoided during sample solublization during the 2DE procedure. When you want to do 2-DE, DIGE or other advanced gel-based proteomic applications, zwitterionic and non-ionic detergents are the detergents of choice.

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- CHAPS, a zwitterionic detergent is one of the most commonly used detergents in protein solubilization in the 2-DE process. It prevents non-specific aggregation through hydrophobic interactions with proteins and helps in sample solubilization. Depending upon on your sample type, different types of detergents could be useful, in few cases ESB14 and sulfo-OB18 detergents can be employed.
- Additionally, Neutral detergents such as NP-40 are also available. A list of most effective solubilization agents cannot be provided;. no single zwitterionic or non-ionic detergent can completely solubilise all the proteins.
- Multiple detergents can be tested for any given sample, to determine the more effective solublization method during the optimization process

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Reductants:

- In the solubilization, reducing agents cleave the disulphide bonds, which are present within and in-between the protein chains.

- The most commonly used reductants are dithiotreitol (DTT) and ß-mercaptoethanol. A commonly used non-ionic reducing agent used to increase protein solubility is Tributylphosphine (TBP)

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Solublization bufferes used prior to iso-electric focusing should include the carrier ampholytes or IPG buffers. The Th charge-to-charge interactions of the ampholyte molecules minimize aggregation while enhancing protein solubility. Different buffers and bases are added which sometimes minimize the proteolysis and helps in the complete solubilization of the proteins.

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To sum up, the solubilization solution for a 2-DE experiment includes chaotropic denaturing agents such as 8 M Urea and 2 M Thiourea, detergents such as 2-4 % CHAPS, reductants such as 2-100 mM DTT or ß-mercaptoethanol and carrier ampholytes such has 0.5 % Bio-Lyte.

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The sample solution components ensure that the protein solubility is good during the extraction and protein separation. A typical sample solution for the gel-based 2-DE application includes 8 M Urea, 2 M Thiourea, 4% CHAPS, 2% IPG buffer (pH 4-7; Linear), 40 mM DTT as well as few other components depending upon your sample type.

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In providing the guidelines on good sample preparation, examples of different challenges being imposed by different sample types are provided.

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- Tissue Culture cells: These are grown in media rich in serum proteins and salts. These should be eliminated during sample preparation.
- Plant cells: The original sample material for this are very hard issues with many interfering substances such as phenolics and salts that need to be eliminated
- Fungal cells (such as yeast) and bacteria: These also have tough cell walls that be challenging to lyse. Proteolysis is a common problem encountered during sample preparation.

- Body fluids: Fluids such as cerebro spinal fluid (CSF) are dilute and require concentration during sample preparation. On the other hand, serum needs to be depleted of salts and abundant proteins before the electrophoretic run

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3 representative examples. 1) serum sample obtained from human. 2) the bacterial sample from *Bacillus* species. 3)A plant leaf sample are now discussed

The protein extraction and solubilization methods for these 3 different types of samples should give an idea of the of methods required to employed during sample preparation.

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The first example is serum proteome analysis. Prior to discussing sample preparation, study design including subject selection, sample preparation and storage should also be discussed.

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Preanalytical issues in clinical proteome studies are first discussed.

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Proteomics aims for the simultaneous analyses of thousands of proteins of a given clinical sample, which could be serum, urine, saliva, urine, CSF or a tissue sample.

Pre-analytical factors have a tremendous impact on such clinical studies. They can occur because of biological variations or technical artefacts.

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Biological pre-analytical factors:

- These include intrinsic and extrinsic factors.
- The intrinsic include gender, age and ethnicity.
- The extrinsic influences include diet, medication, smoking and alcohol consumption.
- While designing a clinical study bias due to intrinsic factors should be avoided. The population should be segregated on the basis of age and gender into different groups.

- While comparing 2 cohorts, they need to be age and gender matched, especially in the discovery phase.
- To validate the results however, such stringency can be disallowed and the analysis should be extended to different groups of age, ethnicity and gender. Extrinsic influences such as diet, smoking, alcohol, medication should be excluded wherever possible as they can alter the proteome and the discovery process. In the large cohort of patients the number of non-disease-related biological effects can additionally alter the proteome changes induced due to the diseases.
- Thus, the study design should include age and gender matching and exclude biasing influences such as smoking, alcohol consumption, drug habits, etc. It is very useful to involve a statistician before designing these types of experiments.

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Technical pre-analytical factors:

- Sample collection: Artefacts can be introduced by this process itself. Factors such as patient posture and tourniquet application time can itself exert gross effects
- The correct sample container should be used. When you are collecting samples such as serum or plasma, the correct container should be used, to either allow or prevent coagulation.
- Sample processing is also important- hemolysis should be preferably avoided during blood processing to collect serum or plasma.

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Care should be taken during sample collection and handling. The collection and handling procedures of bio-fluids will affect the sensitivity, selectivity and reproducibility of the experiments.

- Collection tubes used can influence the analysis. Contaminating components can be shed from the tube itself, or the serum could adsorb to the tube surface. As an example, during CSF collection, concentration of different proteins such as ß-amyloid and tau proteins differed when collected in tubes of different materials. Such effects were lowest in the polystyrene tubes.

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Sample Storage

- Sample storage is another crucial factor.

- Whether you are storing your samples at -20°C or -80°C, time delays during processing could have effects. All of these small variations influence the sample analysis and the proteome analysis at a later point.
- Good procedure involves storage of sample in small aliquots to avoid freeze-thawing, avoiding long term storage under less than ideal temperatures etc. Additionally, using fresh samples is preferable if possible, to avoid the progressive degradation in long term storage.

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The discussion on how to perform the protein extraction and proteome analysis of serum and different types of biological samples will be continued in the next lecture. In summary, this lecture has covered cell lysis, protection the proteins from proteolysis by adding various types of protease inhibitors, sample, fractionation methods, how to use protein extraction and solubilization for effective protein solubilization, with specific examples. The next lecture will continue the discussion on serum proteome analysis followed by bacterial and plant protein and proteome analysis.