HANDOUT

LECTURE-10

SAMPLE PREPARATION FOR PROTEOMICS APPLICATIONS

SERUM AND BACTERIAL PROTEOME

Slide 1:

The previous lectures discussed the workflow of protein sample preparation, including cell disruption or lysis, protection by addition of different protease inhibitors during the proteolysis step, fractionation of samples to reduce the complexity of the proteome, and how to extract and solubilise the proteins, with the commonly used methods for each step of the process. This lecture will discuss sample preparation for various proteomic applications. Some specific examples for serum and bacterial proteome analysis will be provided.

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As mentioned, the previous lecture discussed how to disrupt the cell using different methods, how to protect the proteins from proteolysis using various protease inhibitors, sample pre-fractionation and protein extraction and solubilisation methodology.

Slide 3:

This lecture will discuss precipitation methods used in sample preparation for proteome analysis. followed by methods of removal of interfering substances. such as salts, nucleic acids or other types of contaminants. Failure to remove these during sample preparation can sometimes cause the experiment to fail entirely. This lecture will finally cover specific examples of sample preparation for serum and bacterial proteome analysis.

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Various types of precipitation procedures will now be discussed.

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Ammonium sulfate precipitation: Historically, This is one of the classic, most commonly used methods, although it is not in wide usage today. However, it still remains a good choice. While increasing the ionic strength (or the salt concentration) initially increases the solubility of a protein, pasta certain point, increases in the salt concentration or the ionic strength of a solution causes the solubility of the protein to fall. The high salt concentration in the in ammonium sulfate solution causes the the proteins lose water in the hydration shell (also called the hydration layer) ,as a result of which they aggregate and precipitate out of the solution. Usually this can be achieved by adding>50% concentration of ammonium sulphate. .The protein precipitate can be recovered by centrifugation.

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Acetone precipitation: In this method, an excess of atleast 3 or 4 volumes of ice cold acetone are added to the sample and incubated at -20°C for 1-2 hour to allow for protein precipitation. The precipitate is subsequently removed by centrifugation. Acetone can be removed from the pellet by drying it out. In this procedure, many organic-solvent soluble contaminants such as detergents, lipids remain in solution so is an extremely effective technique.

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TCA or trichloroacetic acid precipitation: TCA is a very effective protein precipitant. 10-20% TCA is added the sample and precipitation occurs on on ice for 30-60 min. The protein pellet should be washed by adding acetone or other organic solvents such as ethanol. This method is very effective for sample recovery-- almost 99-100% sample recovery can be expected with this method.

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As TCA and acetone demonstrated efficacy in protein precipitated, protocols that combined the 2 were developed, and were shown to be more effective than either TCA alone or acetone alone Proteins can be precipitated out using 10% TCA in acetone containing 15-20 mM DTT for 1-2 hours at -20°C. The precipitate is pelleted by centrifugation, washed 3-4 times with acetone alone, or acetone supplemented with 20 mM DTT, which is more effective. Keeping the samples cold through out the procedure helps avoid protein degradation by proteolysis.At the end of the procedure the pellet is dried to remove acetone.

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Ammonium acetate in methanol: This is a less commonly used methods, useful for plant samples containing a high concentration of polyphenols and other interfering

substances. In this precipitation method proteins are extracted in phenol and subsequently precipitated by adding 0.1 M acetate in methanol. The pellet obtained is washed with acetone.

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Removal of Interfering substances: Any proteomic technique, need it be 2-DE or direct LC-MS or surface plasmon resonance label-free proteomics or protein microarrays, requires the removal of interfering substances for a successful experiment. While close to complete removal is possible but difficult, even a partial reduction can make a huge difference.

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Common contaminants include salts, small ionic components, polysaccharides, nucleic acids and lipids. Salt removal is very important during 2-DE or it can interfere with isoelectric focusing. Other contaminants such as polysaccharides, lipids and nucleic acids can form complexes with the proteins by electrostatic interaction and the formation of such complexes can clog the gel and impede efficient separation.

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Salts and buffers: Buffers used during various steps may not get removed completely during subsequent steps and can accumulate during sample preparation On the other hand, the presence of salt in the sample is partly dependant on the nature of the sample itself. For example, biological fluids such as urine, plasma or serum are already very rich in salt content. Additionally, various plant cells have also high salt levels. Different types of salt removal methods include dialysis, spin dialysis, gel filtration, precipitation and resolubilisation.

Dialysis is one of the most commonly used methods used to remove salts and other unwanted components. Samples can be added to dialysis cassettes or tubing, with a range of available molecular weight cut-offs (MWCO) for the dialysis membrane. This tubing or cassette is then suspended in a suitable buffer. Any salts and contaminants in the sample below the MWCO of the dialysis membrane move out into the buffer the dialysis tubing is suspended in, and are replaced by molecules of the suspension buffer. So dialysis also achieves buffer exchange. Importantly, the overall volume of the sample does not change, and nor does the concentration of the protein molecules of interest. However, if the sample at this point is too dilute, it can be concentrated, usually by using commercially available centrifugation based concentration systems or a speed vacuum system.

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Nucleic acid contaminations can cause artefacts by various means:

- These can increase sample viscosity and cause background smear or different type of streaking on the 2DE gels.
- The high molecular weight nucleic acids such as DNA or RNA can clog the gel pores, impeding effective protein separation.
- These negatively charged nucleic acids can bind to the proteins through electrostatic interactions and cause interference in the isoelectric focusing step, resulting in severe streaking. The nucleic acids can also form complexes with the carrier ampholytes.

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To remove the nucleic acid contamination, the sample should be treated with protease-free DNase or RNase mixtures. This is achieved by a 1:10 dilution of a stock solution of 1 mg/mL DNase, 0.25 mg/mL RNase and 50 mM $MgCl_2$ during the cell lysis or tissue homogenization step, on ice.

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Polysaccharide contamination: The issues caused by polysaccharide contamination are less severere compared to nucleic acid contamination, however still need to be taken into consideration. Different types of uncharged polysaccharides such as starch and glycogen can clog the pores of polyacrylamide matrices as they are very large molecules,

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Effective removal of most polysaccharide contaminants occurs during the precipitation step itself, if TCA, ammonium sulfate and phenol/ammonium acetate precipitation methods are used.

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Lipids can also interfere in protein separation. Membrane lipids can bind to proteins that function as lipid-carriers and lead to artefactual heterogeneity. Low level contaminants can be removed by detergents added in the solubilisation step that disaggregate lipids and delipidate and solubilise the proteins.

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High level lipid contamination, present in plant seed or algal samples, can be removed by chemical delipidization prior to the sample solubilisation. This process of delipidation can be achieved by extraction with organic solvents containing chlorinated solvents or ethanol or acetone alone. This step is essential while working with samples containing a high amount of lipid contamination.

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Ionic detergents.

SDS is an ionic detergent which forms strong complexes with proteins. SDS in the context is SDS-PAGE will be discussed later. But it is also very useful during sample preparation for molecular weight separation based strategies, but due to its strong negative charge, has to be removed prior to IEF. The SDS solubilised mixture can be diluted by using high concentration of non-ionic or zwitterionic detergents such as CHAPS and Triton X-100 This ensures that the final SDS level is below 0.25% At such an amount, this detergent will not hamper isoelectric focussing.

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Nucleic acids, proteins and lipids and salts are not the only contaminants in a protein preparation. Other interfering compounds depend on the nature of the sample itself. For example, plant extracts can be contaminated with such as lignins, polyphenols, tannins, alkaloids and pigments. These will be discussed in greater detail in the module on plant proteome analysis.

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The next section discusses how to apply the concepts discussed so far in the specific applications of human serum, bacterial and plant leaf proteome analysis.

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The 3 examples of human serum, bacterial and plant leaf proteomes cover the diversity of biological samples and will give an idea of the different sets of challenges being posed by each of these unique sample types.

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The first application discussed is human serum or plasma proteome analysis.

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The blood proteome is one of the most complicated components of the human proteome. The liquid portion of the blood is referred to as plasma and the removal of fibrinogen and other clotting factors from the plasma results in serum.

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Under healthy conditions,human serum or plasma proteins originate from a variety of tissue and blood cells as a result of secretion from blood cells and the body tissues. Alteration in the expression pattern of various serum proteins can occur in response to injury, a disease state, and various treatments. The blood is thus a true reflection of the physiological state.of an individual. I. Analysis of the physiological state though the study of serum or plasma proteome is also attractive because drawing blood is minimally invasive and easy, compared to say, any kind of tissue biopsy.

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Although sample removal is easy, serum or plasma proteome analysis is not so and poses a number of challenges.

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These challenges include

Dynamic range of the proteins concentration: Serum shows a large diversity of proteins whose concentration range extends over 10 orders of magnitude. Obtaining the full spectrum of the serum or plasma proteome by applying conventional proteomic techniques is challenging because the typical dynamic range for any of these techniques will be much smaller ranging from 10^2 -10^4. To reduce interference from molecules present at much higher concentrations, abundant proteins are depleted, simplifying the proteome and reducing the dynamic range of its components. ,

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High abundance proteins: There are different high abundance proteins present in serum and plasma that complicate proteome analysis. There are almost 22 highly abundant proteins present in serum which represent about 99% of the total protein mass. These high abundance proteins prevent the detection of very low abundance proteinsthat are most often the targets of the study.

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The presence of high salts and other interfering compounds: Blood has a high osmolarity, with the various salts present in the blood being required for various

functions such as maintenance of osmotic balance, acid-base balance etc. A few salts such as sodium chloride or potassium chloride are also added when you are processing the serum sample. The intrinsic salt present in the blood as well as extrinsic salt added during the sample processing results in the salt levels becoming very high, which can produce artefacts at various points in various proteomic analyses.

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The presence of excessive salts, detergents and other contaminants can tremendously influence the electrophoretic separation of proteins and also later it also effects the direct determination of the proteins and peptides by mass spectrometry based techniques. So regardless of whether gel-based or gel-free techniques are used, efficient removal of salt components should be ensured.

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Variations among individuals and lack of reproducibility: Inter- and intra-individual variations pose major issues in clinical studies. Intra-individual variations are obviously more expected but even with in one person, temporal variations are possible due to diet, medication or various unpredictable factors So the drastic heterogeneity introduced by factors such as gender, age, genetic factors, dietary considerations, environmental factors and irrelevant medications are going to affect reproducibility in any study group. While these effects can't be removed entirely, they can be minimized by correct study design, as covered in the previous lecture.

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An overview of serum sample preparation is now provided. The various steps include withdrawal of intravenous blood, blood collection in the tube, centrifugation of whole blood, serum preparation from the whole blood, and sample aliquotting and storage, followed by serum proteome analysis. . For the serum proteome analysis, sonication for the disruption of high abundant proteins and improvement of resolution of separation, depletion strategies to remove abundant proteins, precipitation of remaining proteins by adding acetone and then drying out the pellet and reconstituting in suitable buffer for the proteomic applications.

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So let me show you the details of serum protein sample preparation in this animation.

Animation

Serum proteome analysis:

This interactive animation demonstrates step wise procedure of serum collection and the procedure of proteome sample preparation.

1. Blood collection

The blood proteome is one of the most complex components of the human proteome. It fluctuates depending on the physiological and pathological conditions of the patient.

Blood samples (5.0 mL) collect from the antecubital or cephalic vein of healthy or diseased participants using serum separation tubes

2. Immediately after blood collection the tubes should be kept in ice for 30 minutes for clotting. After clotting, the samples are centrifuged at 2500 rpm at 20^0C for 10 minutes

3. Serum is separated and stored in multiple aliquots at -80^0C.

4. Crude serum is diluted five fold with phosphate buffer (pH 7.4) and uniformly mixed by vortexing the tubes for 30 sec.

5. **Mild sonication** is performed using a sonicator for 6 cycles of 5 second pulses with 30 second gap in between; at 20% amplitude. Mild sonication of serum sample is found to be effective in improving the gel quality and resolution.

6. There are commercially available depletion columns. Affinity binders (ligands) are immobilized to a solid support (i.e., chromatographic medium) and used to specifically bind abundant proteins from a complex protein solution. The **depletion strategy** effectively enhances the resolution and maximizes maximum coverage of the serum proteome.

7. Depleted serum samples are mixed with ice-cold **acetone containing 10% TCA** and vortexed for 15 sec for uniform mixing. The mixture is incubated at -20^{0}C for 2 hrs for protein precipitation.

8. After incubation, tubes were **centrifuged** at 1000g for 15 min at 4^{0}C. Supernatant was separated and pellets were dissolved in **rehydration buffer** (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4-7; Linear), 40mM DTT and traces of bromophenol blue).

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The next part details how to remove high abundant proteins which are present in the serum.

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Tere are more than 20 abundant proteins present in the serum and albumin alone constitutes about 50% of these abundant proteins, IgG makes upto 15-25% of the abundant proteins followed byIgA, haptoglobin, transferrin and anti-trypsin.

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Different methods of removal of high abundant proteins such as use molecular weight cut-off membranes and chromatography methods have been tried. MWCO-based methods are risky because most of the abundant proteins have high molecular weights and other high molecular weight proteins which are non-abundant and of interest may be lot. The affinity chromatography methods have emerged as the most efficient for the specific removal of these abundant proteins and target albumin, IgG and other abundant proteins. Ligands with high affinity for these are immobilized on a protein resin and specifically bind abundant proteins, removing them from the sample.

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By applying the affinity based depletion stretegies, the high-abundance proteins can be removed, as demonstrated by the SDS-PAGE, wherein the left lane is loaded with untreated serum and the right lane is loaded with treated serum. As you can see, the high abundance proteins such as albumin, IgG are efficiently removed which allowed the emergence of some of the low abundance proteins on the gel.

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The image shown above compares a 2DE gel where the samples were crude serum (left) and deplted serum (right) In the crude serum albumin and other abundant proteins have resulted into very much masking of other proteins and the isoelectric focusing and the gel quality is also not so good. So streaking and different types of artefacts can be seen with crude serum are eliminated by sample depletion

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Salt removal is one of the very crucial steps and can be achieved by dialysis, gel filtration and precipitation. The overall salt level needs to be below 10 mM for isoelectric focusing. Shown above are untreated(left) and desalted(right) sample gels and as you can see that there is lot of streaking in the untreated gel which is eliminated by desalting.

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Shown below is a laboratory demonstration of serum proteome sample preparation.

Laboratory demonstration

Serum protein extraction

Serum processing: Processing of serum involves blood and separation of serum, sonication of serum, depletion of high abundant proteins and precipitation of proteins.

Blood collection

1. Carefully withdraw around 4 mL of intravenous blood into a vacutainer tube.
2. Place the tube on ice for an hour immediately after collection to allow the blood to coagulate.

   Centrifuge the tube for 10 min at 2500 rpm to separate the coagulated blood. The blood cell along with the clotting factors form the pellet while the serum containing proteins of interest forms a clear supernatant.
3. Transfer the supernatant into fresh tubes. These can be stored at -80°C until required for further use.
4. Remove the serum samples from the freezer atleast 15 min before performing the experiment.
5. Place it on ice and allow it to thaw before use.
6. Then transfer the required amount of serum into fresh clean tube.

Sonication

1. Dilute the serum sample 5 fold with phosphate buffer, pH 7.4.
2. Vortex the sample to allow complete and uniform mixing.
3. Place the sample on ice to provide sonication of 6 cycles of 5 sec pulses at 20% amplitude with 59 sec gap in between each cycle.

Depletion of high abundant proteins

Serum contains several proteins in a wide range of concentrations. Of these albumin and IgG are found to be most abundant. These proteins may interfere with the gel pattern in the experimental analysis while trying to detect other proteins at lower concentrations. It is, therefore, preferred to remove these high abundance proteins before electrophoresis.

1. Depletion columns for the removal of high abundant proteins are now available. Open the cap of the column and place it a collection tube and centrifuge it after the addition of phosphate buffer.
2. Discard the flow-through in the collection tube.
3. Then add the binding buffer provided in the kit to the column and centrifuge the tube.
4. Add the serum sample to the column and incubate on ice for 5 min. This allows the high abundant proteins to bind to the affinity matrix of the column while the rest of the proteins remain unbound.
5. Centrifuge the column and collect the serum at the bottom of the collection tube for further processing.

Proteins precipitation

1. Transfer the serum depleted of high abundant proteins into a fresh tube.
2. Add a mixture of trichloro acetic acid in acetone to the depleted serum and mix the contents well. The solution gradually becomes turbid due to precipitation of proteins.
3. Place the tube at -20°C for atleast 4 hours before centrifugation to enable complete precipitation of proteins.

4. Then remove the tube and centrifuge the contents to allow the proteins to settle down as a pellet.
5. Discard the supernatant and dry the pellet at room temperature.
6. Reconstitute this dried pellet with rehydration buffer.
7. Vortex the tube for a uniform mixing.
8. Store the reconstituted protein mix at -80°C until performing the next step of protein quantification.

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The second example; bacterial proteome analysis is now discussed.

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Similar to other samples types, the overall aim is to solubilise all the proteins and achieve maximal coverage of the proteome, either by a gel based or LC-MS approach. Bacterial lysis is often very challenging for different bacteria. But each experiment can be optimized, either by variation in lysis buffer constitution, or by introduction of methods such as sonification or enzymatic digestion.

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Let's talk about sonication which is very commonly used if lysing the bacterial cells. Sonication is a physical method of disruption which can break very complex inter- and intra-protein interactions. The ultrasonic waves generated by a sonicator lyse the cells through application of shear forces. One has to take care while performing this step and it should be performed in cold conditions so that the heating and the foaming effects can be minimized.

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This is a pictorial overview of bacterial sample preparation. First you need to inoculate the culture and after obtaining the right colonies you need to grow the culture to obtain the bacterial pellets. Then you need to wash the bacterial pellet and sonicate the bacterial pellets in the preferred lysis buffer and then add trizol followed by centrifugation which results in formation ofdifferent layers of DNA, RNA and proteins. If the correct layer isn't removed properly, contaminations such as phenol or interfering substances like DNA or RNA will be present,

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This lecture discussed the sample preparation workflow which was continued from the last lecture. Dfferent types of precipitation methods which can be used for different types of applications ,removal of interfering components and specific examples of serum and bacterial proteome sample preparations were covered. The next lecture will cover plant proteome sample preparation.