HANDOUT

LECTURE-11

SAMPLE PREPARATION FOR PROTEOMICS APPLICATIONS: BACTERIAL & PLANT PROTEOME, QUANTIFICATION

Slide 1:

This lecture will discuss sample preparations for proteomics applications with special emphasis on bacterial and plant proteome analysis and protein quantification. In the last two lectures, different methods, which can be used for very good protein sample preparation for proteomics applications were covered. Different type of lysis methods, prevention of proteolysis during lysis, different type of protein pre-fractionation methods protein precipitation, protein solubilization and removal of various interfering components were discussed.

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The work flow for protein sample preparation, different type of precipitation methods and removal of interfering substances with specific examples were covered first, followed by sample preparation methodology for human serum and bacterial proteome samples

Slide 3:
This lecture will continue the discourse on bacterial proteome analysis. In today's lecture we will first talk about different types of methods available for bacterial protein extraction, with emphasis on one method This lecture will then discuss prepare good samples for plant proteome analysis. We will then talk about quantify the proteins accurately.

Slide 4:
Let's start with the bacterial proteome analysis.

Slide 5:
This slide brings you upto date with what was discussed in the last lecture.

Slide 6:
The above is a list of different type of sample preparation strategies employed for bacterial protein extraction.

- 2% SDS and heat treatment has been applied for bacterial sample preparation

- Similarly lysozyme and acetone precipitation methods have been used.
- TCA (tri-chloro acetic acid) and acetone together have been effectively used for precipitation.
- Direct protein extraction and solubilization in the solubilization buffer has also been employed.

The Trizol method has been used for bacterial sample preparation.

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The Trizol method: The Trizol method is useful because this method provides an opportunity for recovering DNA, RNA and protein, from same sample by sequential extraction method. Trizol contains Guanidinium isothiocyanate, which is a RNAase inhibitor and provides good quality RNA. This method also excludes any possibility of nucleic acid contamination because you are already removing DNA and RNA andsalts. There is no lipid contamination because chloroform is used along with trizol which dissolves lipids . The protein content is easy to resolubilize after extraction from the trizol method.

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The below procedure can be modified for an individual experiment.

- Add 1 ml trizol reagent to thebacterial sample.
- Then add 200 µl chloroform immediately to the mixture and vortex vigorously and incubate for 15 min at the room temperature.
- Centrifuge it at 12000 g for 15 minutes.

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- After the centrifugation carefully remove upper layer containing RNA using micropipette and add 300 µl ethanol to bottom layer.
- Again you need to centrifuge at 5000 g for 5 minutes to remove DNA. In fact, this step can be used for keeping the material safe for further DNA extraction. The same applies to the RNA removed in the previous step
- Once you have removed the supernatant which contains the proteins then you can collect that in a fresh tube and in this supernatant you can add 4 volumes of chilled acetone and incubate this mixture for 4-6 hours at -20^{o} C.

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- Once incubation is done then start centrifugation step at 12000 g for 5 minutes,
- Discard the supernatant and retain pellet.

- This pellet can be washed with 95% ethanol, 3-4 times then dried at the room temperature and reconstituted in a buffer suitable for your analysis. If you are going to perform 2-D electrophoresis, you need to add the lysis buffer which contains urea, thio-urea and different other components. If you want to only run an SDS-PAGE you can add the Laemmli buffer.

Animation 1

So let me show you the bacterial protein sample preparation in the interactive animations:

For any bacterial proteome analysis, first you need to grow bacterial culture on LB media containing a suitable antibiotic, at 37°C, overnight. The overnight cultures are diluted with fresh LB and grown at 37°C for 6-8 hours. Continue growing the culture till it reaches mid exponential phase. The bacterial cells can be harvested by centrifuging at 12000 rpm for 10 minutes at 4°C. Wash the bacterial pellet with phosphate buffer of pH 7.4 for 4 times to remove the media. Resuspend the pellet with protease inhibitors and lysozyme and cells can be further ruptured by sonication on ice to prevent foaming and heating. The sonication step helps to release the content of bacterial cells. Sonication can be performed by using a sonicator for set cycles of 5 seconds pulse with 30 seconds gap in between at 20% amplitude. Sonication involves the use of high energy sound waves those are capable of opening of outer membrane of cell. Cell debris and unbroken cells can be separated by centrifugation step.

To the supernatant add 1 ml of Trizol and 200 µl of chloroform and mix vigorously for 15 seconds by vortexing it at room temperature to allow the phase separation. After phase separation centrifuge the sample at 12000 rpm for 15 minutes at 4°C. The top pale yellow layer contains RNA, the middle white layer contains protein and lower phenol layer contains both proteins and DNA. Remove the upper layer and use it for RNA isolation using Isopropanol if needed. To the bottom layer, add 300 µl of absolute alcohol per 1 ml of trizol and mix gently to suspend the white precipitate and keep at room temperature for 3 minutes. Centrifuge the mixture at 12000 rpm for 15 minutes at 4°C to precipitate the DNA. To the clear pink layer add 4 volumes of chilled acetone and keep it at -20°C for 20 minutes. The protein pellet can be washed with 95% ethanol for 3-4 times, with vortexing. You need to allow the pellet to dry at the room temperature and then add the lysis buffer containing 7 M of urea, 2 M of thio-urea, CHAPS, IPG buffer, DTT and bromophenol blue.

To give further information about the detailed protocol as well as how to perform these experiments in the lab, I will show a video of the lab demonstration of bacterial protein sample preparation for proteomics applications.

Bacterial protein extraction-This process involves culturing of bacteria, harvesting and sonication of the obtained culture followed by protein extraction.

Bacterial culturing- Clean the laminar work space thoroughly with ethanol and keep the master plate containing bacterial culture ready, light the spirit lamp or Bunsen burner to maintain sterile or aseptic conditions throughout the process. Carefully remove a bacterial colony from the master plate and inoculate the autoclaved growth media. Incubate this inoculated sample at 37^{o}C for 6-8 hours with constant shaking to allow bacteria to grow. The turbidity of the culture which gradually increases indicates bacterial growth.

Culture harvesting-

Transfer the grown bacterial culture to a fresh tube under sterile condition. Centrifuge this tube at 12000 rpm for 10 minutes, maintain the temperature 4^{o}C. Transfer the pellet obtained containing intact bacterial cell in a fresh microfuge tube. Wash this pellet thoroughly with phosphate buffer to remove any unwanted debris.

Sonication- The resuspended pellet is sonicated on ice to enable the bacterial cells to break open so that contents are released. Sonication involves the use of high energy sound waves that are capable of breaking open the outer membranes of cells. All cellular contents including proteins of interest leak out of this disrupted membrane. Carry out the sonication procedure for 30 seconds with a pulse of 1 second at 40% amplitude.

Once it is complete, centrifuge the contents and collect the supernatant that is obtained.

Protein extraction-

The trizol extraction protocol allows efficient separation of not just bacterial proteins but also their DNA and RNA. Add the trizol reagent containing Guanidinium isothiocyanate, phenol and chloroform to the supernatant obtained after sonication. Mix the content thoroughly by vortexing. Add chloroform to this solution, mix the contents and place the tube on ice for few minutes. Centrifuge the tube at 2000 rpm for 5 minutes. 3 distinct layers will be obtained. The uppermost layer is aqueous layer containing RNA, at the center is the interphase containing proteins while bottom layer is organic and contails DNA. Discard the top layer containing RNA then add absolute alcohol to the remaining layer and mix the solution well. Centrifuge the contents at 2000 rpm for 5 minutes. The DNA forms a white precipitate at the bottom of the tube while the proteins remain in the clear supernatant. Collect the supernatant in a fresh tube. Then add chilled acetone to this tube and mix well by vortexing. Store this solution at -20^{o}C for at least an hour

before centrifuging it to obtain the protein pellet. Discard the supernatant and dry the pellet at room temperature. Reconstitute the dried pellet with rehydration buffer and store overnight at -20^{o}C before carrying out protein quantification.

I hope this video was informative and now you are able to appreciate the complexity involved in this procedure as well as how useful this method can be for different type of biomolecular extraction including DNA and RNA and proteins

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So now let's move on to the plant proteome analysis, the third example.

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Plants are very crucial because they provide a food source for human and animals. To understand molecular events happening inside the plant cells, one needs to study its genome, transcriptome and proteome. The plant proteome analysis is useful because it can reveal various molecular mechanisms underlying plant growth, and development.

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The analysis of plant proteome can provide information about protein abundance, protein modification, where the proteins are localized at the sub-cellular level, their 3-D structures and interactions with proteins as well as other biomolecules.

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Protein extraction for Leaf proteome analysis:.

- Weigh out about 300 mg of leaves (you can adjust the weight depending upon your experimental requirement) and homogenize using a mortar pestle containing liquid nitrogen.
- Add 1.5 µl of TCA in acetone containing 0.07 % DTT.,
- Grind it so that it becomes very fine and then incubate this homogeneous solution at -20°C for 1 hour.
- Then centrifuge this mixture at 14000 rpm for 30 minutes at 4°C. Remove supernatant layer and wash pellet 3-4 times with chilled acetone containing 0.07% DTT.

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- Dry the pellet at room temperature.

- Reconstitute the pellet in lysis buffer or a buffer which is suitable for your proteomic sample, centrifuge the contents at 14000 rpm for 15 minutes at 4°C and then collect the supernatants for further proteomic applications.

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Here in the pictorial form, an overview of steps involved in the plant protein sample is provided; preparation starting from the leaf collection, weighing the leaves, transferring into the mortar, grinding by using liquid nitrogen with a pestle, homogenization in the presence of TCA and acetone, after centrifugation, removal of the supernatant, formation of the protein pellet and washing with acetone and reconstitution with lysis buffer.

Animation 2

The following is a laboratory demonstration; a video for the plant protein sample preparation.

Plant protein extraction-This process involves homogenization of the plant leaf sample followed by acetone precipitation for protein extraction.

Leaf homogenization-

Select the plant leaf sample of interest and weigh around 300 mg of the leaf on an aluminum foil. Transfer these leaves to a chilled mortar and carefully add liquid nitrogen to it which helps in drying up of leaves instantaneously. Grind the leaves well using a pestle to obtain a fine powder. To this powder add around 0.5 ml of lysis buffer containing TCA, acetone and DTT and grind it well until a fine paste is obtained. The lysis buffer causes the plant cells to swell and finally break open thereby disrupting the membrane and releasing all its intracellular contents. Add another 1 ml of lysis buffer to the paste and then transfer the solution to a fresh tube after grinding thoroughly to obtain a uniform mixture. Centrifuge the sample at 10000 rpm for 5 minutes and maintain a temperature of 4°C during the process to ensure that there is no denaturation of the proteins. Discard the supernatant and retain the pellet containing plant proteins along with various other intracellular components. Incubate the pellet at -20°C for 1 hour.

Protein precipitation- Remove the pellet from -20°C and add chilled acetone to it. Mix the sample well by vortexing to obtain a uniform solution. Centrifuge the contents at 4°C for 5 minutes at speed of 10000 rpm, discard the supernatant and repeat the acetone washing at least three times to remove plant pigments to obtain a protein pellet at room temperature. Reconstitute the pellet with rehydration buffer. Vortex the sample to obtain

a uniform solution, centrifuge the contents following morning at 10000 rpm for 5 minutes. Collect the supernatant containing proteins in a fresh tube and store at -20°C until protein quantification is performed.

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So now let's move on to the next topic which is protein quantification. There are different protein quantification methods available.

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The protein concentration determination by UV absorption is one of the very commonly used methods, in fact the oldest method to determine the protein concentration by the absorbance at 280 nm. This method is based on the absorbance of UV light by aromatic amino acids such as tryptophan and tyrosine which are present in the protein solutions. Phenylalanine is also aromatic amino acid but demonstrates lower UV absorbance than its counterparts.

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Determination of the protein concentration at A_{280} method requires that your protein contain tryptophan and tyrosine aromatic residues. Since protein samples will have variability in aromatic amino acid content so the absorptivity at 280 nm will be variable, and this method also requires a high protein concentration in the extract. To overcome these limitations, various alternate methods have emerged which include Lowry assay, BCA assay and Bradford assay.

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So let's talk about different types of colorimetric methods to determine protein concentration.

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The Lowry assay:

This is one of the very common methods used for quantification of soluble proteins. There are two main steps involved in performing Lowry assay- first- the alkaline cupric tartrate forms a complex with peptide bond of protein, second- after that a reduction step with Folin and Ciocalteu's reagent yields purple colored solution whose absorption can be measured between 500-800 nm.

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This method is very simple, precise and sensitive. However, this assay is unsuitable for those proteins which do not contain the aromatic amino acid, tyrosine, as this assay depends on the reaction of tyrosine residues with the reagent. Furthermore, this method is sensitive to interfering reagents such as Tris, EDTA etc. These limitations can be overcome by addition of a precipitant such as TCA

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BCA assay

In the BCA assay, proteins form a complex with Cu^{2+} ion in alkaline solution. Now these are reduced to Cu^{+} ions in a Biuret reaction. The protein thus produces a solution of violet in complex with BCA and then the amount of this reduction is proportional to protein present. By using this chemistry, one can know how much protein is present in the sample.

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There are different advantages of using BCA assay. It is more sensitive than Lowry method or Biuret. The violet colored complex is quite stable. It is less susceptible to the detergents and is useful for samples containing membrane proteins. However, this reagent can be disrupted by high concentrations of complex-forming reagents such as EDTA, ammonium sulphate and reducing materials such as DTT. Accurate protein quantification is extremely imporatant especially in large studies, or there will be some artifacts if you do not start with an accurate protein measurement.

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The Bradford assay.

This assay is based on complex formation between Coomassie brilliant blue G-250 dye and protein. Due to the binding absorption max the color shifts from 465 nm to 595 nm. This increase of absorption at 595 nm is used to measure protein concentration.

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The Bradford assay has various advantages as compared to the Lowry or BCA method because it is compatible with reducing agents and thiols unlike the Lowry and BCA methods. This method is also very quick and compatible for microwell plate assays. But there are different problems with the Bradford assay as well: the dye binds most readily to arginyl and lysyl residues of proteins and this specificity may contribute to variations based on amino acid composition of the proteins. e Various detergents such Triton-X

100, SDS and CHAPS also interfere with the Bradford assay. So researchers have come up with various types of modified Bradford assays for specific applications.

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Components of the Bradford assay: include one standard let's say BSA, a salt solution, coomassie brilliant blue solution and cuvettes. For standard preparation, you can take BSA and prepare a standard range of concentrations. Then dilute this sample with 0.15 M NaCl to a total volume make to 100 µl. One sample without protein serves as a blank and can be used to autozero the spectrophotometer. For the unknown samples for which you want to determine the protein concentration, 10 or 15 µl of sample is diluted with NaCl.

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1 ml of coomassie brilliant blue solution is then added to sample, standard and blank tubes and vortexed., After that one can incubate it for 2 minutes so that color can be developed and then it can be measured for absorbance at 595 nm then the standard curve is used to determine concentration of unknown protein sample. If the sample does not fall in range of the standard curve, it should be diluted and the experiment repeated.

Animation 3:

The video elaborates how to perform protein quantification.

Quantification of proteins-

- After the protein sample has been extracted from its source, it must be quantified to determine the protein content before any further processing.
- Label the tubes suitably for standard and test samples. Thaw the protein sample to be quantified by gently rubbing it between the palms.
- Prepare the sample buffer required for diluting samples during the assay and mix it well.
- Add an increasing concentration of standard protein sample to each of the designated tube. BSA is often used as a standard. Then add the unknown protein sample whose concentration is to be determined to the appropriately labeled tube. Dilute all the samples uniformly using the sample buffer then add the Bradford color reagent to each tube and mix well.
- An electron transfer reaction takes place between the red form of the coomassie dye of the reagent and the native protein. This disrupts the protein structure and establishes several non-covalent interactions between the dye and the protein.

The transfer of electrons converts the dye into its blue form thereby giving the solution a blue color.

- Set the wavelength of the UV spectrophotometer to 595 nm. Adjust the reading to zero using a blank solution containing only the dye solution and no protein then measure the absorbance of all the standard protein sample of known concentrations followed by the unknown sample.
- Protein concentration of the unknown can be determined from the standard curve based on the absorbance values obtained.

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So in summary, in the last three lectures we talked about strategies for sample preparation. I gave you a work flow where we talked about different type of lysis methods. We talked about how to pre-fractionate the samples and then how to precipitate the samples, how to remove the interfering substances and then we moved on to specific examples, we discussed in greater detail different type of criteria being used for analysis of clinical samples and then we talked about human serum samples. Then we discussed about bacterial protein extraction and how one can analyze bacterial proteome. Then we talked about plant protein extraction for the plant proteome analysis. Finally we talked about protein quantification, how to determine the protein quantity present in given sample.

So we will continue our discussion on proteomics and now since you have prepared a good sample. It can be applied either for using the applications on gel based proteomics approaches such as 2-D gel electrophoresis or it can be used for gel free proteomic approaches such as mass spectrometry. So we will continue our lecture on gel-based proteomics and demonstrate you how you can use your prepared sample further for analysis of complex proteome such as serum proteome, bacterial proteome and plant proteome.