Proteomics: Principles and Techniques
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Module No. # 11
Lecture No. # 11
Sample Preparation for Proteomics Applications: Bacterial and Plant Proteome, Quantification

Welcome to the Proteomics course.

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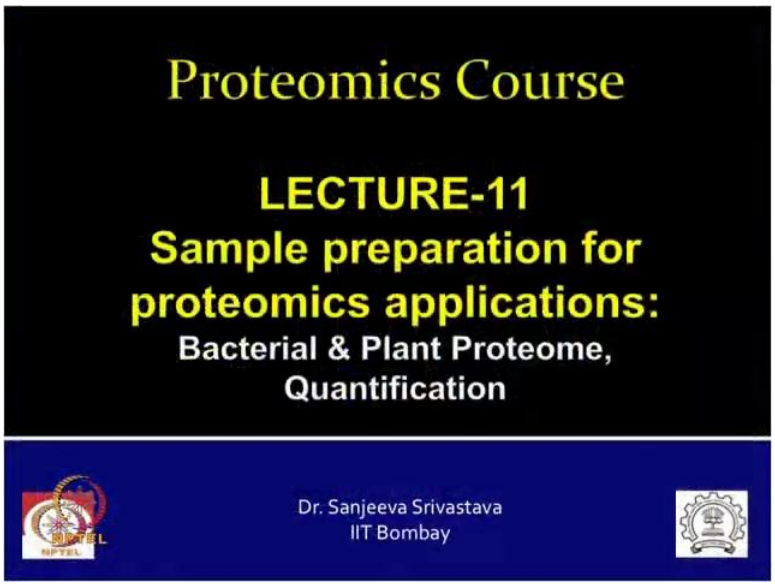

Today, we will talk about Sample Preparation for Proteomics Applications with special basis on bacterial and plant proteome analysis and protein quantification. In the last two lectures, we discussed about different methods which can be used for a very good protein sample preparation for proteomics applications, we talked about different type of lysis methods, we talked about how to prevent the proteolysis during the lysis, different type of protein free fractionation methods, then we talked about protein precipitation, protein solubilization and how to remove various type of interfering components.

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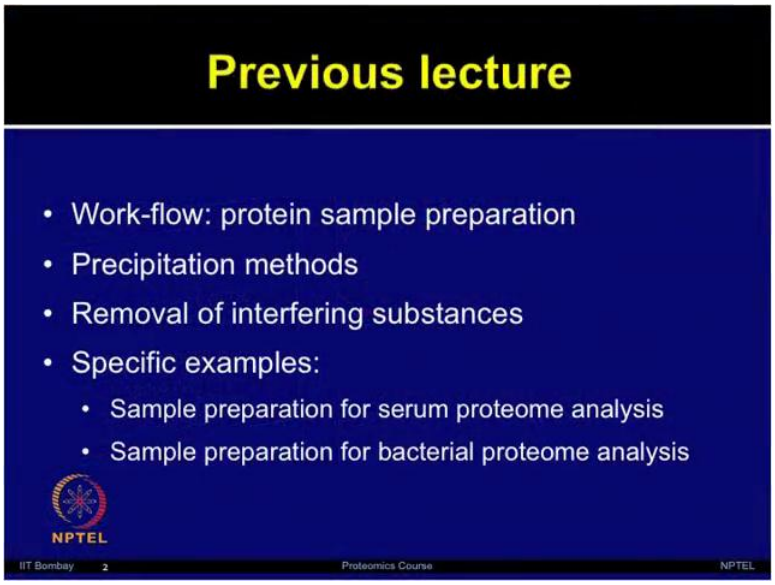

So, we discussed about a work flow for protein sample preparation, different type of precipitation methods, removal of interfering substances and then in the last lecture, I started talking about specific examples, first we discussed about how to analyze serum proteome, how to prepare the sample for serum protein analysis. And then, we moved on to serum to bacteria and we will start discussing about sample preparation strategies for bacterial proteome analysis.

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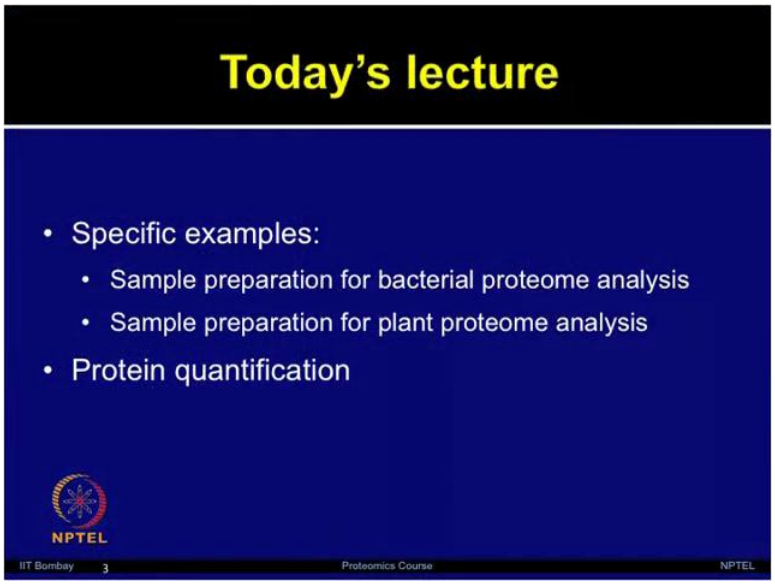

In the last lecture, I stop there and then today; we are going to continue from the bacterial proteome analysis. So, in today's lecture, we will first talk about different type of methods available for bacterial protein extraction. I will recommend one method and elaborate on that, then we will talk about how to prepare good samples for plant proteome analysis, we will then talk about how to quantify the proteins accurately so that you are ready to perform proteome experiments.

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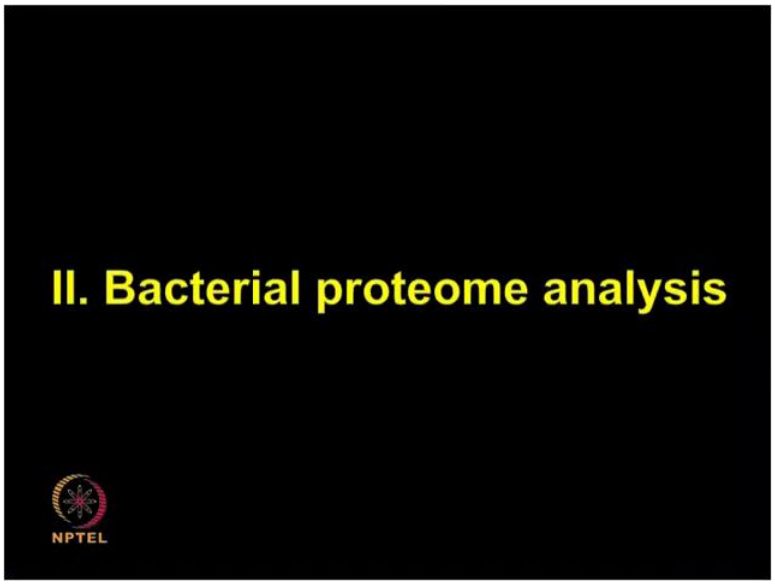

So, let us start with the bacterial proteome analysis.

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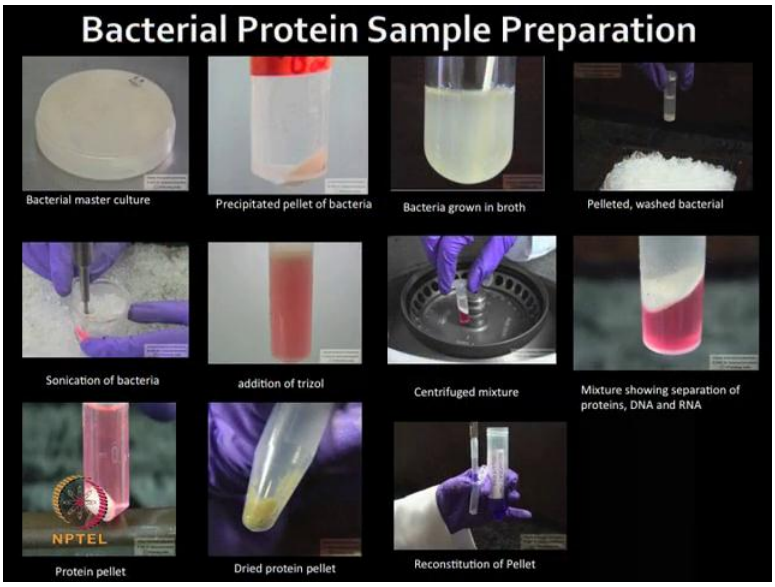

So, if remember in the last lecture, I stopped on the slide where I showed you various steps which are involved in bacterial protein sample preparation. So, now let us elaborate on this and continue different type of sample preparation strategies which people apply

for bacterial protein extraction, these are all reported methods available in the literature. I have just compiled it for your brief overview for detailed each of the protocol, you can refer to the detail publications.

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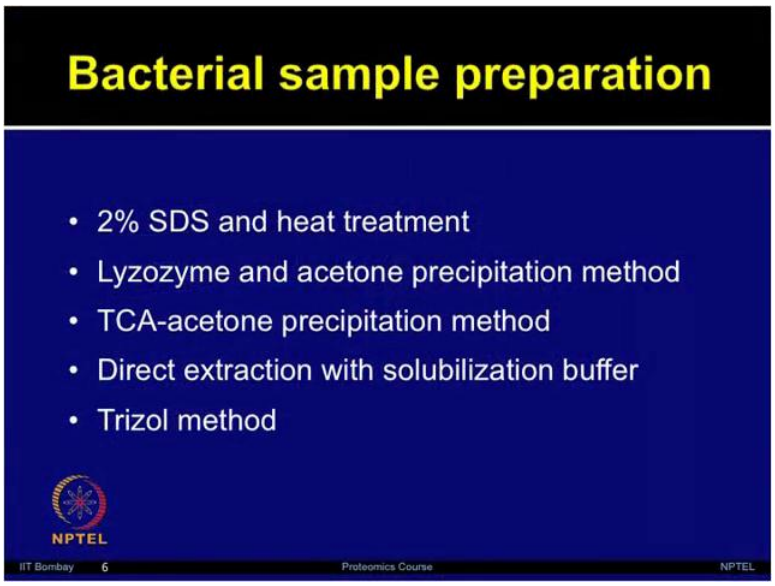

So, two percent SDS and heat treatment has been applied for bacterial sample preparation. Similarly, Lyzozyme and acetone precipitation method have been used, trichloroacetic acid and acetone together have been effectively used for precipitation, direct protein extraction and solubilization in the solubilization buffer, this is strategy has also been used. And then a method, trizol method has been used for bacterial sample preparation; I will elaborate on the trizol method.

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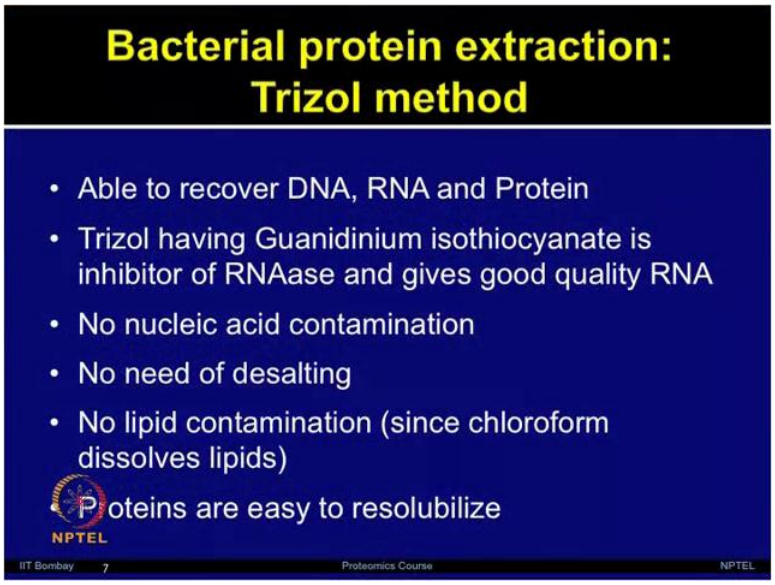

So, why we want to elaborate on Trizol method, because this methods provide you an opportunity for recovering DNA, RNA and protein, all three major biomolecules from the same sample. In the sequential extraction method, one can obtain DNA, RNA and protein, all by using this method. Trizol having Guanidinium isothiocyanate is one of the inhibitor for RNAase and it provides very good quality of RNA. This method also eliminates any possibility of nucleic acid contamination, because you are already removing DNA and RNA, sites are also rid of by using this method, there is no lipid contamination because chloroform is being used along with trizol which dissolves the lipids.

So, this method is able to get rid of various type of interfering components as well as different type of contaminants and finally, the proteins are easy to resolubilize after extraction from the trizol based method. So, this method is very useful for extracting bacterial and other proteome analysis, let me brief you about the procedure. So, first, this is not the exact detail for the protocol, just I am giving you few numbers for your reference, one can modify this depending upon their, how protein extract they want and based on their sample.

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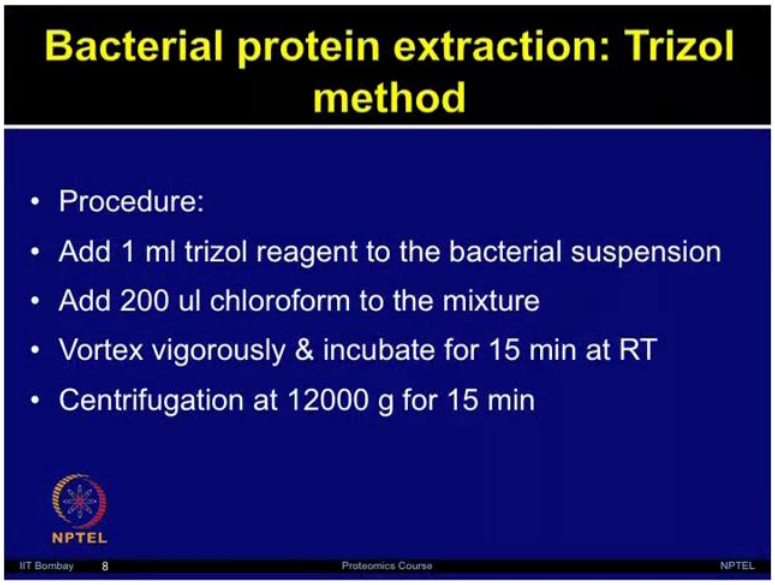

So, you can start with one ml of trizol reagent and add that in the bacterial suspension, then add 200 micro liter of chloroform immediately to the same mixture, vortex this mixture vigorously for 15 to 30 seconds and incubate it at the room temperature for 15 minutes, centrifuge the sample at 12000 g for 15 minutes.

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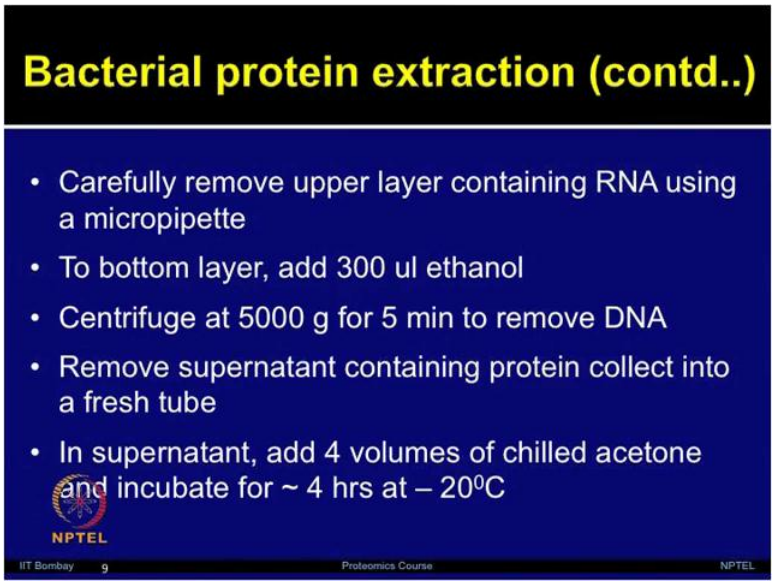

After the centrifugation, remove the upper layer which contains RNA and the bottom layer, you can add ethanol. Again, you need to centrifuge at 5000 g for 5 minutes, this step will remove any DNA contamination. Now, in fact, this step can be use for keeping the material safe for further DNA extraction, same applies to the previous step where you can collect the RNA which can be further used for RNA clean up and preparation. So, once you have removed the supernatant which contains the protein, then you can collect that in fresh tube. And in this supernatant, you can add 4 volumes of chilled acetone; incubate this mixture at minus 20 degrees for 4 to 6 hours.

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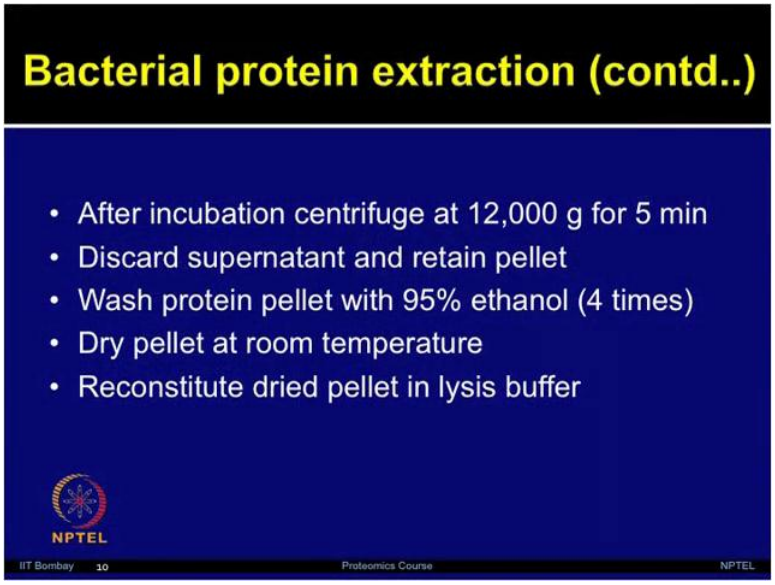

Once incubation is done, then start centrifugation step for 5 minutes, discard the supernatant after centrifugation and retain the pellet, this pellet can be washed with 95 percent ethanol 3 to 4 times. Then dry this pellet at the room temperature and this pellet can be reconstituted in a buffer suitable for your analysis. If you are going to perform two dimensional (( )), you need to add the lysis buffer which contains urea, CHAPS, thiourea and different other components which we have talked previously, or if you want to analyze on the (( )), you can add the laemmli buffer. So, depending upon your applications, you can reconstitute the dry pellet in the right buffer composition.

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

animations. For any bacterial proteome analysis, first you need to grow the bacterial cultured on (( )) containing suitable antibiotic. Now, the proper 37 degrees temperature is provided for over night or 6 to 8 hours of growth, the over night cultures are diluted with fresh (()) and grown at 37 degrees for 6 to 8 hours.

Continue going the culture till it reaches the mid exponential phase, the bacterial cells can be harvested by centrifuging the cells at 12000 rpm for 10 minutes at 4 degrees, wash the bacterial pellet with phosphate buffer of PH 11.4 for four time to remove the media, re suspend the pellet with (( )) inhibitors and lyzozymes and cells can be further ruptured by sonication in eyes to prevent forming an heat, this sonication step helps to release the content of bacterial cells. Sonication can be performed by using a sonicator for set cycles of 5 second pulse with 30 second gap in between at 20 percent amplitude.

Sonication involves the use of high energy sound waves that are capable of breaking open the outer membrane of the cells, cell (( )) an unbroken cells can be separated by the centrifugation step to the supernatant at 1 ml of trizol and 200 micro liter of chloroform, and mix vigorously for 15 second. The click on the vortex (( )), mix it vigorously at room temperature to allow the phase separation. After phase separation, centrifuge the sample 12000 rpm for 15 minutes at 4 degrees. As I mentioned earlier, the different protocols one can use for bacterial proteome analysis, here we are demonstrating the trizol method in which the top peel yellow layer contains RNA, the middle white precipitate having protein and lower phenol layer contains both protein and DNA, remove the upper layer and use it for RNA isolation using isopropanol.

If you want to use RNA for some other applications to the bottom layer at 300 micro liter 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 degrees to precipitate the DNA; to the clear pink layer as four volumes of chilled acetone and keep it at minus 20 degrees for 20 minutes.

Protein pellet can be washed with 95 percent ethanol for three to four times during the washing steps, you have to also vortex so that the pellet is properly washed, you need to allow the pellet to dry at the room temperature and then add the lysis buffer containing 7

molar of urea, 2 molar of thiourea, CHAPS, IPG buffer DTT and bromophenol blue.

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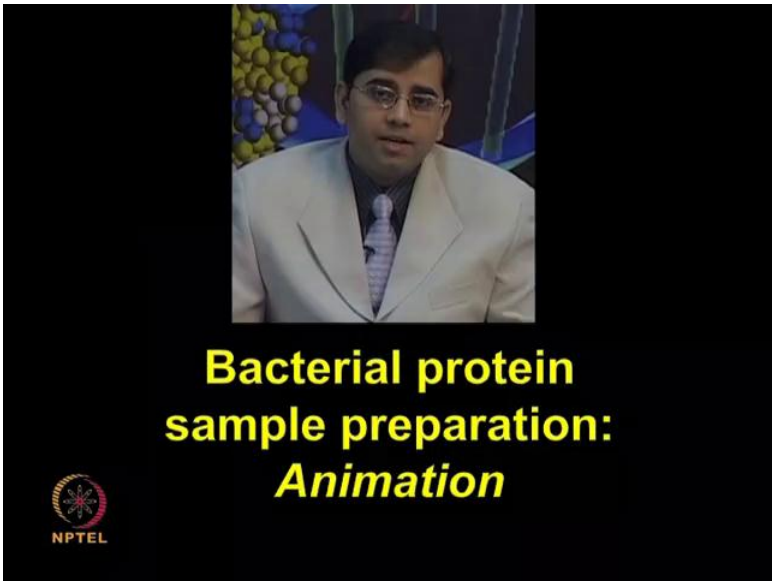

So, I hope this animation was informative, bacterial protein extraction by using trizol method and you must appreciate that in this method you can obtain RNA, DNA and protein, all protein, all the bio molecules together.

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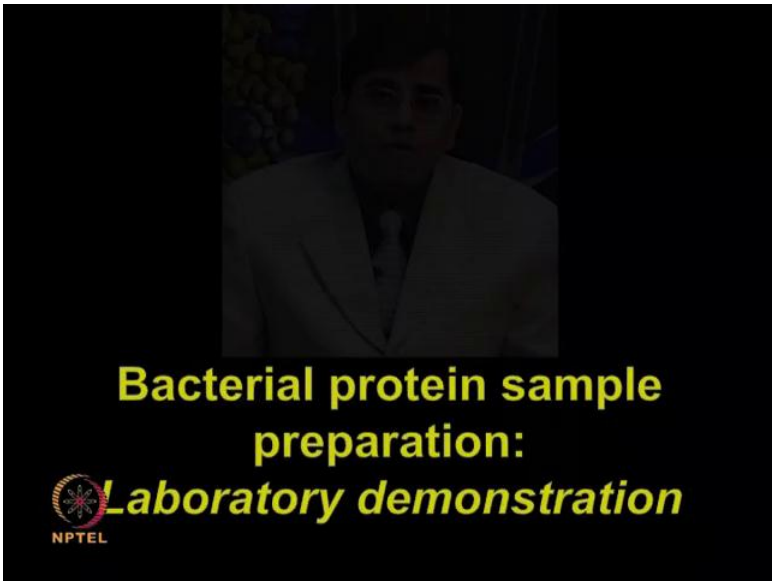

So, to give you a further information about the detailed protocol as well as how to perform these experiments in the lab, I will show you a video for the laboratory demonstration of bacterial protein sample preparation for proteomics applications, so let us watch this video.

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 workspace thoroughly with ethanol and keep the master plate having the bacterial culture ready, light the spirit lamp or Bunsen burner to maintain sterile and aseptic conditions throughout the process. Carefully remove a bacterial colony from the master plate and inoculate the auto clay of the growth media.

Incubate this inoculated sample at 37 degree Celsius for 6 to 8 hours with constant shaking to allow the 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 conditions, centrifuge this tube at 20000 rpm for 10 minutes maintaining a temperature of 4 degree Celsius. Transfer the pellet obtained containing intact bacterial cells to a fresh micro centrifuge tube, wash this pellet thoroughly with phosphate buffer to remove any unwanted deadly.

Sonication, the resuspended pellet is sonicated on ice to enable the bacterial cells to break open so that its 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 procedures for 30 seconds with a pulse of 1 second at 40 percent 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 the bacterial proteins, but also their DNA and RNA, add the trizol reagent consisting of Guanidinium thiocyanate, phenol and chloroform to the supernatant obtained after sonication, mix the contents thoroughly by vortexing. Next, 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.

Three distinct layers will be obtained, the topmost is the aqueous layer containing RNA, the centre is the interface containing proteins and the bottom layer is organic and consists of DNA. Discard the transparent top layer having the RNA, then add absolute alcohol to the remaining layers 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 the solution at minus 20 degree Celsius 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 minus 20 degree Celsius before carrying out protein quantification.

So, 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 biomolecule extraction including DNA and RNA and how protein can be get rid of various types of contaminants by using trizol method.

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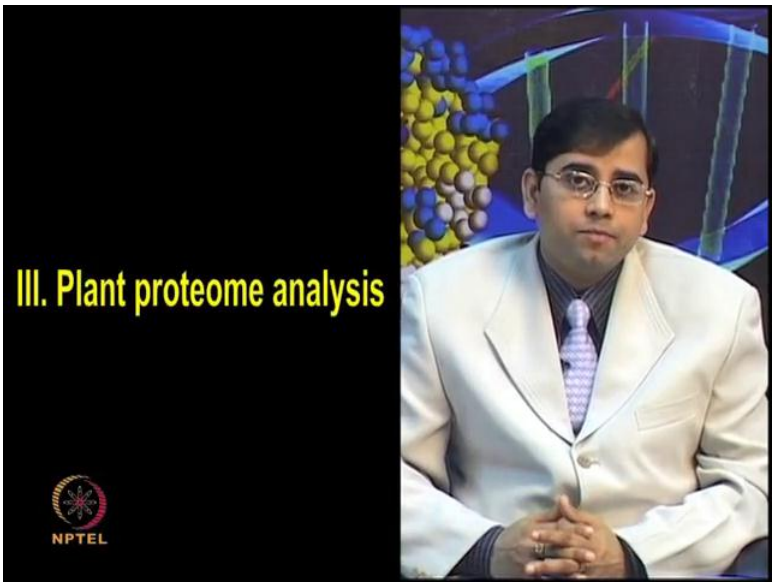

So, now let us move on to the plant proteome analysis, the third specific example. So, in the flow of the sample preparation strategies, I tried to give you a feel of different type of

samples available and the challenges which are associated with each sample type, we talked about human serum, we talked about bacteria and now let us talk about plant leaf, how to analyze a plant proteome.

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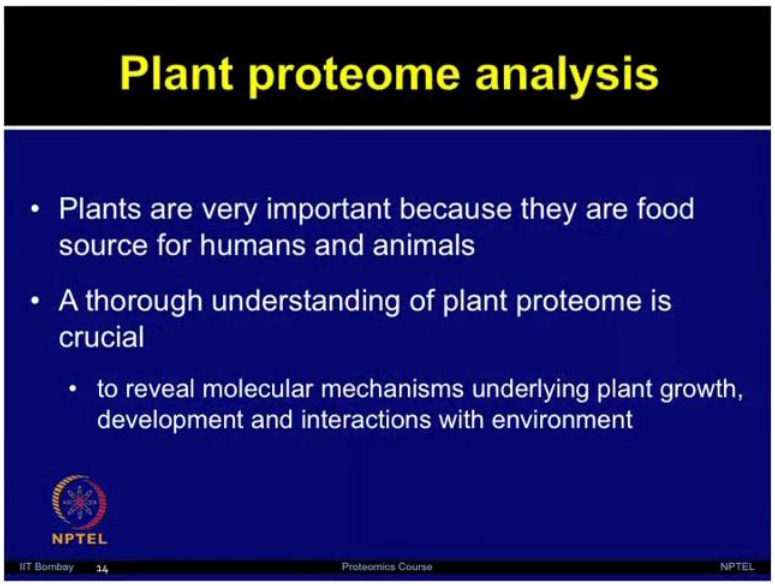

So, as you are aware, plants are very crucial, because they provide food source for human and animals. To understand the detail of molecular events happening inside the plant cells, one needs to study its genome, transcriptome and proteome to understand the events in the systems approach. The plant proteome analysis is very crucial because it can reveal various molecular mechanisms which are underlying plant growth, development, its interactions with their biomolecules as well as with the environment.

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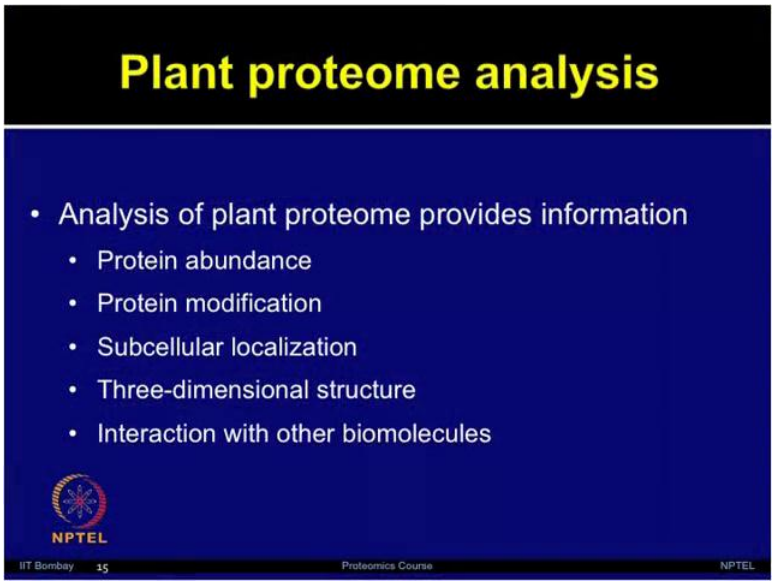

The analysis of plant proteome can provide information for various properties such as the protein abundance, protein modification where the proteins are localized, their sub cellular localization, the three dimensional structure of the proteins, its interaction with the proteins as well as other bio molecules. So, similar to other type of proteome analysis, the plant proteome analysis is very crucial.

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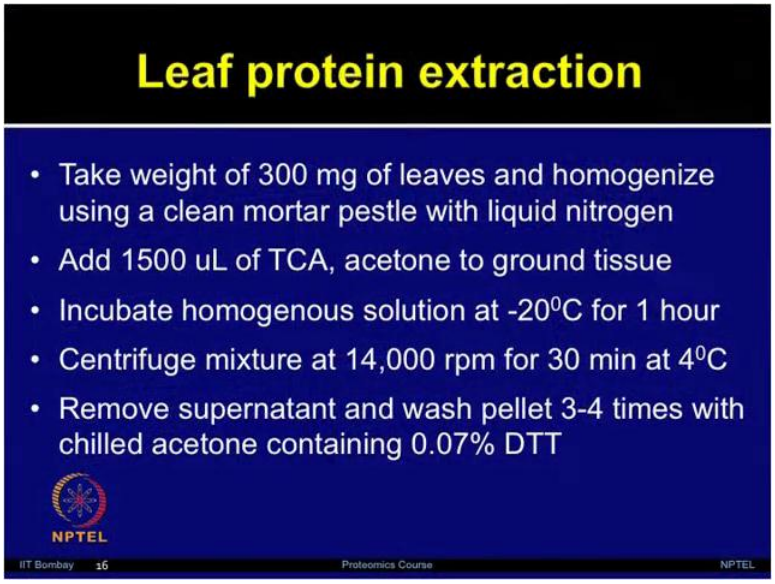

So, let me explain you a protocol, a method to show you how to perform the leaf proteome analysis, let us start with the leaf protein extraction. So, if you want to analyze the leaf proteome, you first need to extract all the proteins present in the leaf. So, you can take almost 300 milligrams of leaves and obviously, you can add just the weight depending upon your experimental requirement, just to begin with one can start with 300 milligrams, homogenize by using a mortar pestle or you can use a homogenizer or different type of lysis methods can be used.

So, homogenize this 300 milligram leaf in the liquid nitrogen in the liquid nitrogen by using mortar pestles, add almost 1.5 m l of TCA in acetone, one can use 10 percent TCA in acetone with minor addition of DTT 0.07 percent of DTT and add this to the ground tissue, again grind it so that it can become very very fine. And then incubate this homogeneous solution at minus 20 degrees, let your incubate for an hour or 2 hours, but please keep it consistent, whatever incubation timing you use, then centrifuge this mixture at 14000 rpm for 30 minutes at 4 degrees. After centrifugation, remove the supernatant layer and wash the pellet, few times, 3 to 4 times, repeat this step with acetone wash containing 0.07 percent of DTT.

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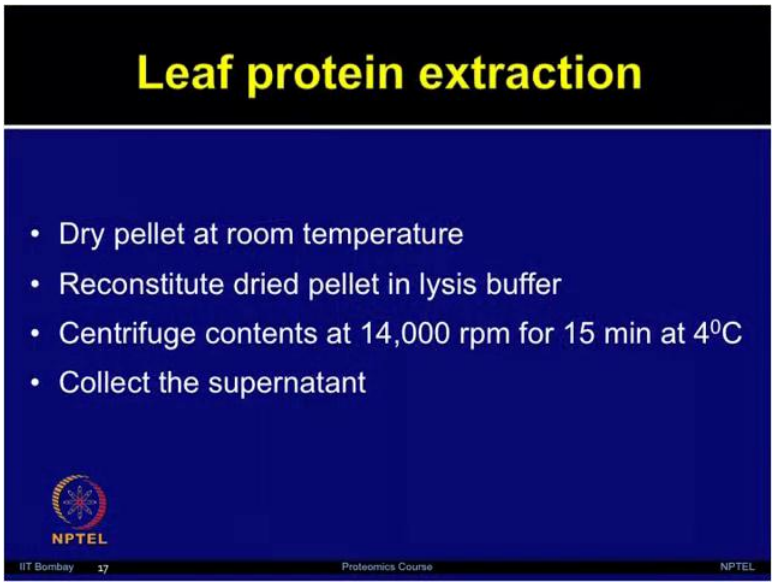

So, in the leaf protein extraction, then you need to dry the pellet at the room temperature,

reconstitute the dried pellet in lysis buffer or buffer which is suitable for your proteomics application, centrifuge the contents at 14000 rpm for 15 minutes at 4 degrees and then collect the supernatant for further proteomic applications.

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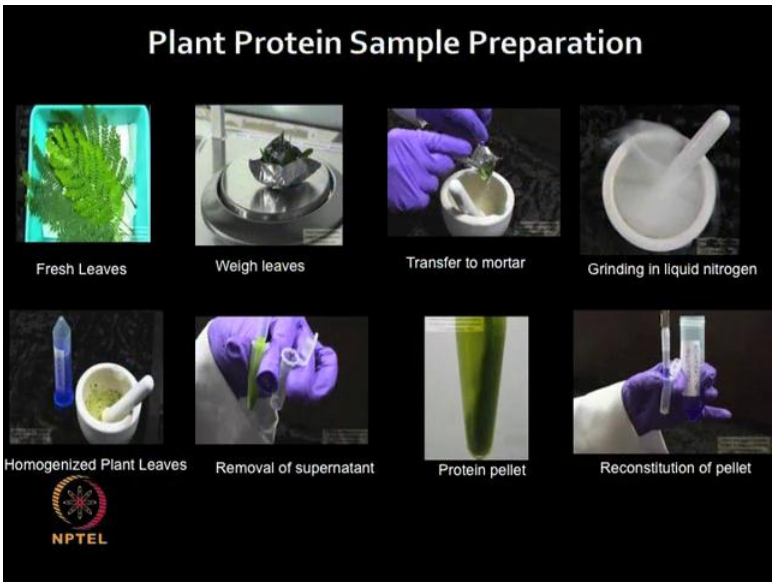

So, here in the pictorial form, I am giving you an overview of steps involved in the plant protein sample preparation starting from the leaf collection, weighing the leaves, transferring to the mortar, grinding by using liquid nitrogen with the pestle, homogenization in the presence of TC (( )) acetone, after centrifugation removal the supernatant, how the protein pellet is formed, then it need to be washed couple of times with acetone and then the pellet can be reconstituted in lysis buffer.

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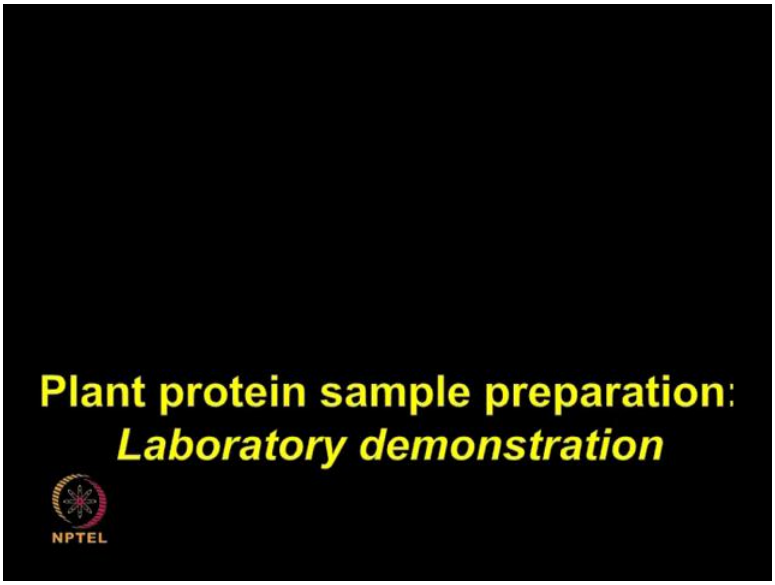

So, let me give you laboratory demonstration, a video for the plant protein sample preparation. Plant protein extraction, this process involves homogenization of the plant leaf sample followed by acetone precipitation for protein extraction. Leaf homogenization, select the plant leaf sample of interest and weigh around 300 mg of the leaf on an aluminium foil, transfer these leaves to a chilled mortar and carefully add liquid nitrogen to it which helps in drying up the leaves instantaneously. Grind the leaves well using a pestle to obtain a fine powder, to this powder, add around point 5 ml of lysis buffer containing trichloroacetic acid, acetone and diethyl ether 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 1ml of lysis buffer to the paste and then transfer the solution into 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 degree Celsius 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 minus 20 degree Celsius for an hour.

Protein precipitation, remove the pellet from minus 20 degree Celsius and add chilled acetone to it, mix the sample well by a vortexing to obtain a uniform solution. Centrifuge the contents at 4degree Celsius for 5 minutes at a speed of 10000 rpm, discard the supernatant and repeat the acetone washing, at least 3 times to remove all plant pigments, to obtain protein pellet at room temperature, reconstitute this pellet with rehydration buffer. Vortex the sample to obtain a uniform solution, centrifuge the contents the following morning at 10000 rpm for 5 minutes, collect the supernatant containing proteins in a fresh tube and store it at minus 20 degree Celsius until protein quantification is performed. So, by watching this video, now you must be familiar with the TC acetone precipitation method, how it can be effectively used for plant protein sample preparation.

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So, now let us move on to the next topic, which is protein quantification. So, by now, you have prepared your protein sample by using different type of methods, by keeping a very careful eye on different type of interfering substances and contaminants and you have tried to eliminate all of those components. Now, you want to know how much protein quantity you have in your sample, whether you can perform your experiment with that protein or not, so one need to know the protein quantity present in the protein sample. So, there are different types of protein quantification methods are available, we

will talk about protein quantification in detail now.

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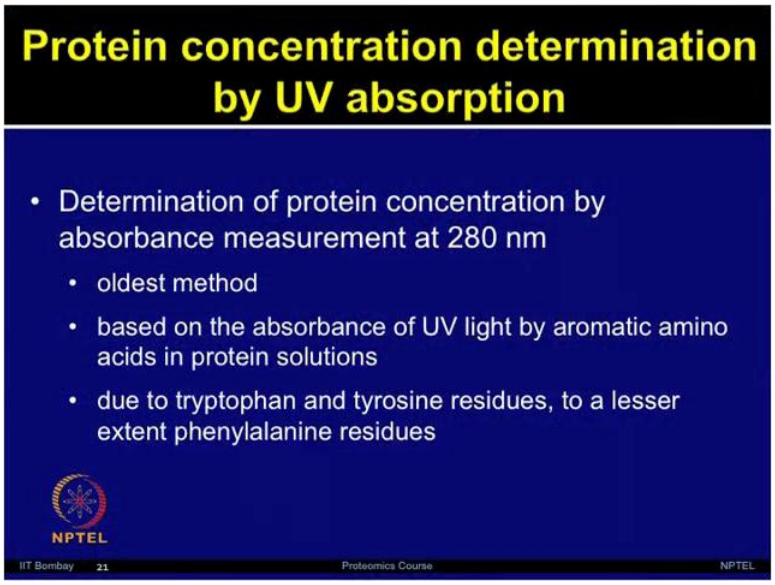

So, the protein concentration determination by the UV absorption that is one of the very commonly used method, in fact oldest method used to determine the protein concentration by the absorbance at 280 nanometers, this method is based on the absorbance of ultraviolet light by aromatic amino acids such as tryptophan and tyrosine residues which are present in the protein solutions, phenylalanine, it is also aromatic amino acid, but up to a lesser extent, it is responsible for this absorbance.

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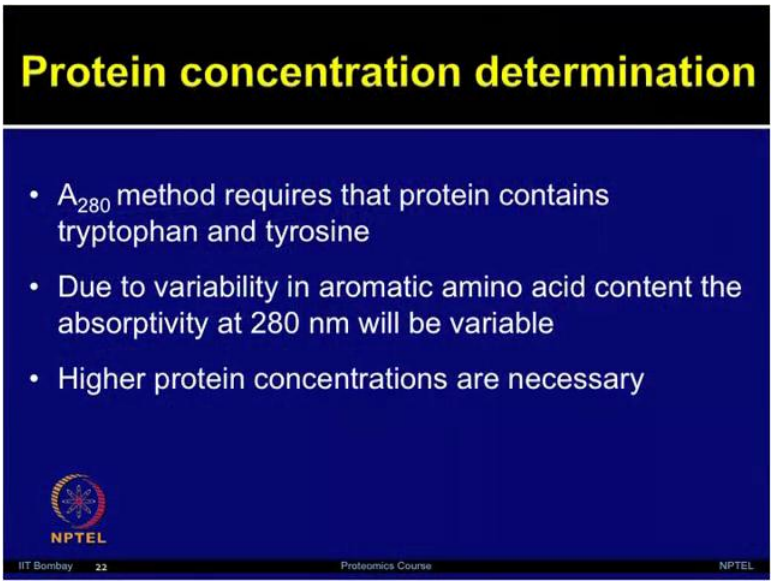

So, to determine the protein concentration at A 280 method requires that your protein contains tryptophan and tyrosine aromatic residues. Since, the protein samples will have variability in overall amino acid contents. So, the absorptivity at 280 nanometers will be a variable factor for this type of method. Also if you need very high protein concentration for your protein preparation, for your proteomic applications, then this method is again a limiting factor.

So, higher protein concentration is not always possible for you to obtain from your protein sample. So, you need to quantify the proteins even if you have the lower protein concentration. So, this method has some limitation; one, the aromatic residues has to be there to obtain the absorption at 280 nanometers and the protein concentration should be in very high amount.

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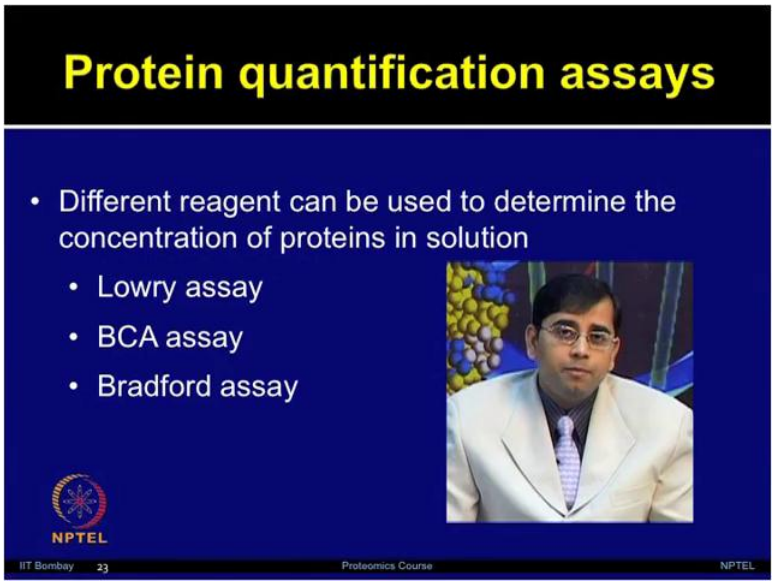

To overcome these limitations, there are different reagents, different quantification assays have immerged and these reagents can be used to determine the concentration of proteins in the given solution, these methods include Lowry assay, BCA assay and Bradford assay. There are several assays as well, but we will talk about these three, more commonly used methods.

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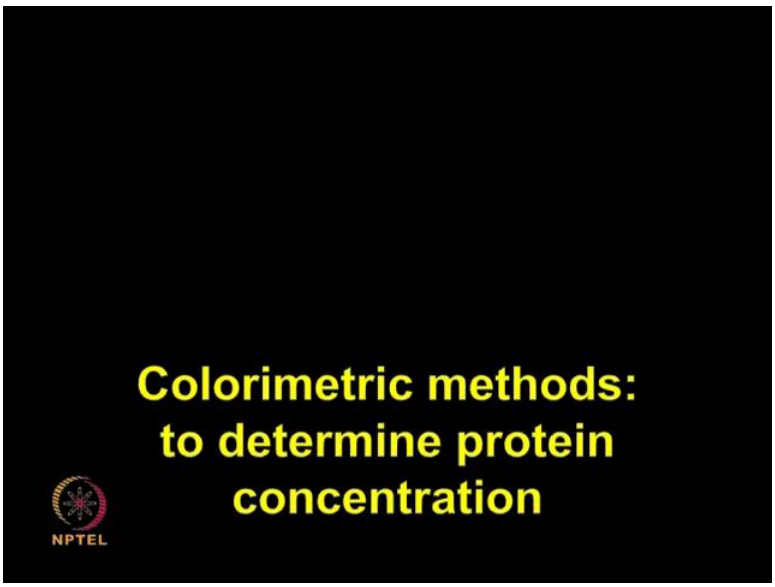

So, let us talk about different type of colorimetric methods to determine the protein concentration.

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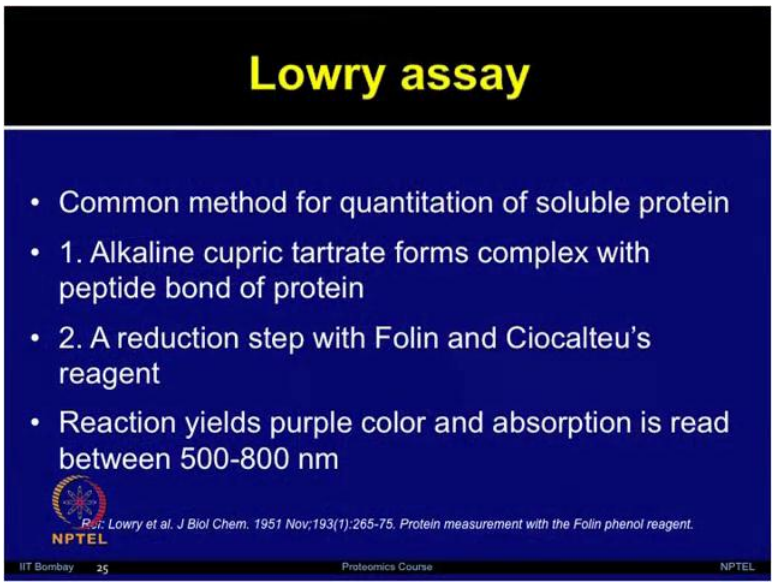

Let us first talk about Lowry, assay this is one of the very common method which has been used for quantification of soluble proteins, there are two main steps which are involved in performing Lowry assay. First, the alkaline cupric tartrate forms a complex with peptide bond of the protein. After that, a reduction step is performed by adding Folin and Ciocalteu's reagent, there this reaction yields purple colour and absorption can be measured between 500 to 800 nanometers.

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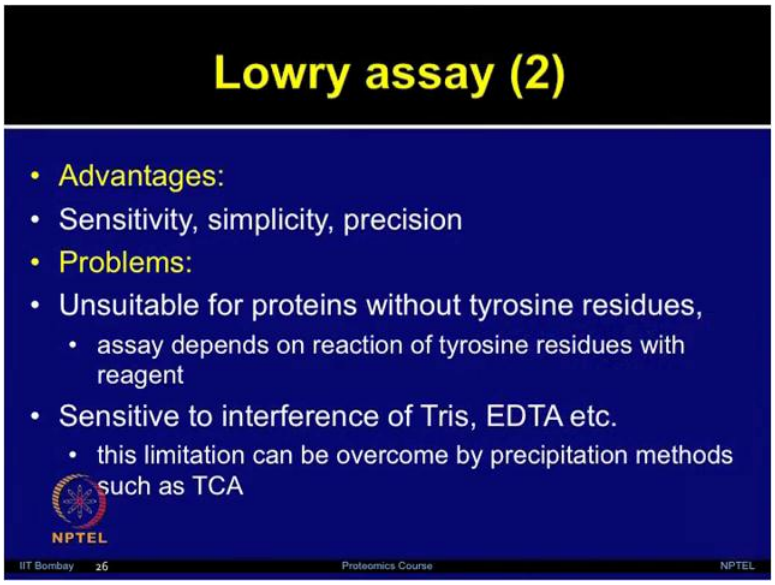

This method is very simple, it is very precise and it is very sensitive, these are various advantages of using Lowry assay and that is why, when people have abundant, enough proteins in their samples, they have used conventionally the Lowry methods. But if you are using the clinical samples, or you are using the samples with very less yield, probably this may not be the best method to use and that is why, there are different other methods are also available.

So, one of the problem of using this assay is that, it is unsuitable for those proteins which do not contain the aromatic residues such as tyrosine, this assay depends upon the reaction of tyrosine residue with the reagent. So, if you do not have tyrosine amino acid in your protein sample, then it will be not suitable, the sensitivity to the interfering components such as Tris, or EDTA that is another limitation of this method. But it can also be overcome, if you can add precipitant such as TCA, trichloroacetic acid, addition of TCA, one can overcome this limitation.

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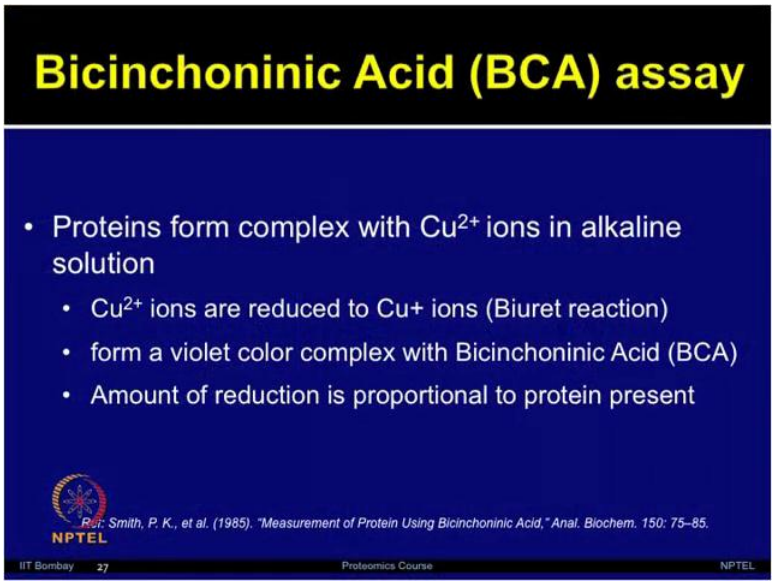

So, now let us move on to the next assay, which is BCA assay. In the B C A assay, the proteins forms complex with cupric ions in the given alkaline solution. Now, these are reduced to cuprous ions in a Biuret reaction, it forms a violet colour complex with BCA and then, the amount of this reduction is proportional to the protein present. So, by using this chemistry, one can know how much protein is present in the given sample.

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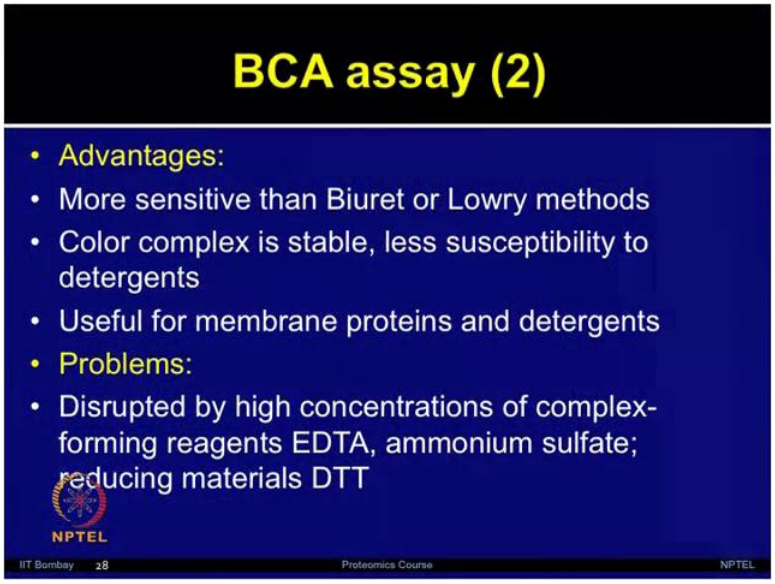

The different advantages of using BCA method, it is more sensitive than the Lowry method or Biuret, this colour complex is quite a stable, it is less acceptable to the detergents, this method is also used for those samples which are rich in detergents as well as for the TDS samples which contain membrane proteins. So, this method has definitely lot of advantages as compared to the Lowry method, but it is still has some problem, this reagent can disrupt by a very high concentration of a reagent such as EDTA, ammonium sulphate and different types of reducing material such as DTT. So, people have tried different methods of assay methods for protein quantification, because there are many interfering components are there, which could give you the false reading.

And if you are unable to accurately determine the protein concentration, then your whole analysis will going to be depending upon that and it will not lead to the right results, right comparison, many times you want to compare the controls and the treatments your 50 controls with the 50 disease samples, knowing all are those if you are starting with equal amount of protein 100 microgram for example, but your quantification has some multi facts, because you have some components which are interfering and which are giving the false reading. So, your quantification may not be accurate and rather than measuring only 100 micrograms from the controls and the treatments, you are actually measuring 90 versus 100 or 100 versus 110. So, there will be some (( )), if you do not start with the exact protein amount.

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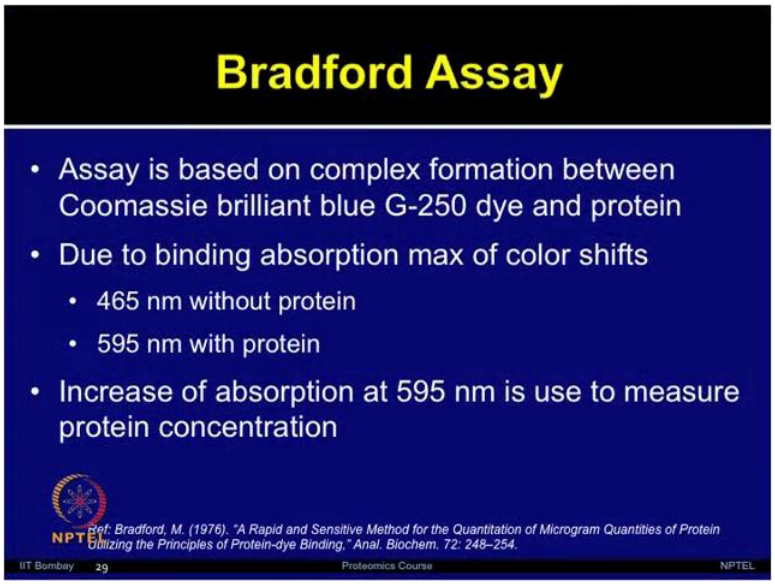

So, that is another popular method available which is Bradford assay, this assay is based on the complex formation between the Coomassie dye G 250 and protein. Due to the binding absorption max, the color shift from the 465 nanometer to 595 nanometers, this increase of absorption at 550 595 nanometer is used to measure the protein concentration.

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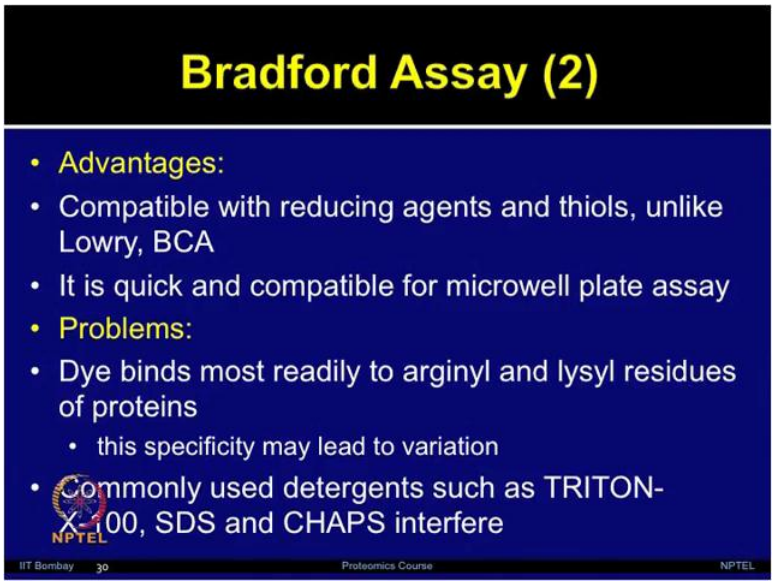

The Bradford assay has various advantages as compared to the Lowry or BCA method, because it is compatible with reducing agents and thiols, which was not the case when we talked earlier about Lowry method and BCA method. This method is also very quick and it is compatible for the microwell plate assays which can be performed in the 96 well formats, but there are different problems with Bradford assay as well, such as dye binds most readily to the arginyl and lysyl residues of the protein and this specificity may lead to the variations. Now, there are various detergents such as TRITON X 100, SDS and CHAPS, they also interfere with the Bradford assay. So, researchers have come up with various type of modified Bradford assay for a specific applications, again you have to keep an eye, what is the component of your buffer and you need to ensure that it is compatible with the assay conditions or not.

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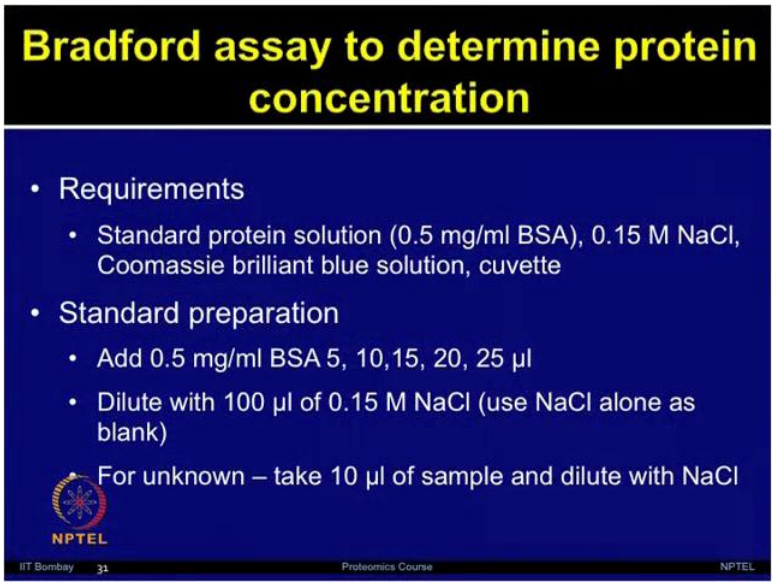

So, let us talk about the Bradford assay in little bit more detail so that one can use that to determine protein concentration. If you want to perform this assay, what are the requirements, say first of all you need one standard, let us say you can take BSA, you need some salt solution, Coomassie brilliant blue solution and cuvette. So, for the standard preparation, you can take BSA and add different concentration of this 5, 10, 15, 20, 25 micro liters or you can go even more standard preparation theories, then dilute the sample with 0.15 molar of sodium chloride and total volume make it to 100 micro liters,

one sample where there is no protein there, that can be used as the blank which can be used to auto zero the value in spectrophotometer for as a blank. And then further you can measure your samples, for the unknown samples for which you want to determine the protein concentration, you can take 10 or 15 micro liter of the sample and dilute with the sodium chloride, then same treatment can be performed for this whole experiment. Now, if your unknown is giving you absorbance higher than the, your standard preparation, whatever series you have made. So, it is better idea that you can try different dilutions of your unknown sample, because it has to fall within the standard curve of the standard dilutions what you have taken.

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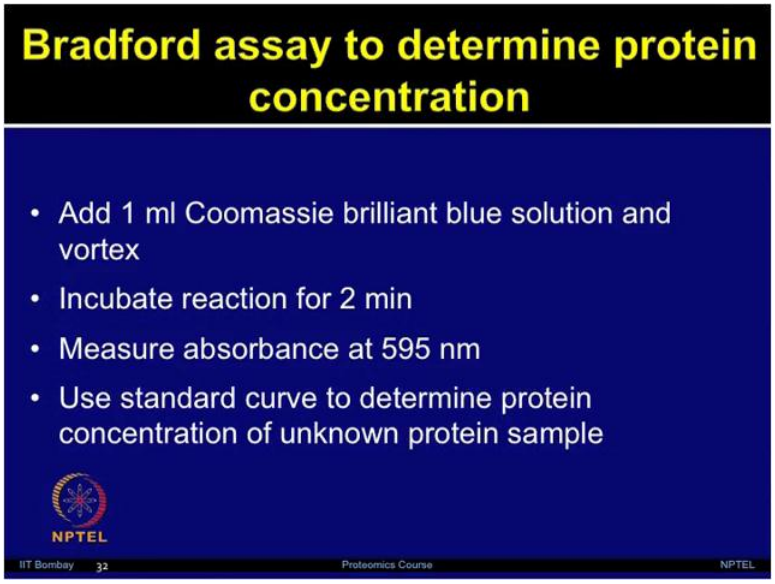

Now, you can add one m l of Coomassie brilliant blue solution and vortex it, after that one can incubate it for 2 minutes so that color can be developed and then it can be measured for absorbance at 595 nanometers, then you can use this standard curve to determine protein concentration of unknown protein sample. But often once you plot your unknown protein sample, you may realize your values for the protein sample are either too low or too high to be plotted in the standard curve. So, then you may need to adjust your unknown sample, you may have to make dilution of it and use less volume or you may need to increase the volume of your sample so that it can fall within the range of the standard curve.

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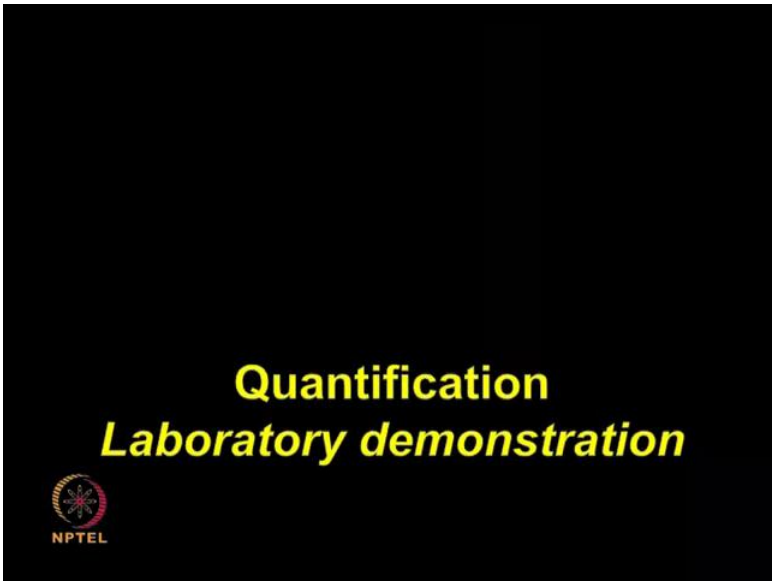

So, how to perform the quantification so let me show you one video, a laboratory demonstration of how to perform protein quantification. Quantification of proteins, after the protein sample has been extracted from its source, it must be quantified to determine the protein content before any further processing, label the tubes suitably for standard and test samples, (( )) for the protein samples 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, at an increasing concentration for standard protein sample to each of the designated tubes, bovine serum albumin is often used as a standard.

Then at 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 protein, the transfer of electrons converge the dye into its blue form thereby giving the solution a blue color, set the wavelength of the UV spectrophotometer to 595 nanometers.

Adjust the reading to 0 using a blank solution containing only the dye solution and no

protein, then measure the absorbance of all the standard protein samples of known concentrations followed by the unknown sample. Protein concentration of the unknown can be determined from the standard curve based on the absorbance value obtained. So, I hope it was useful for you to watch the video for protein quantification and we got a feel how to perform this experiment in the laboratory.

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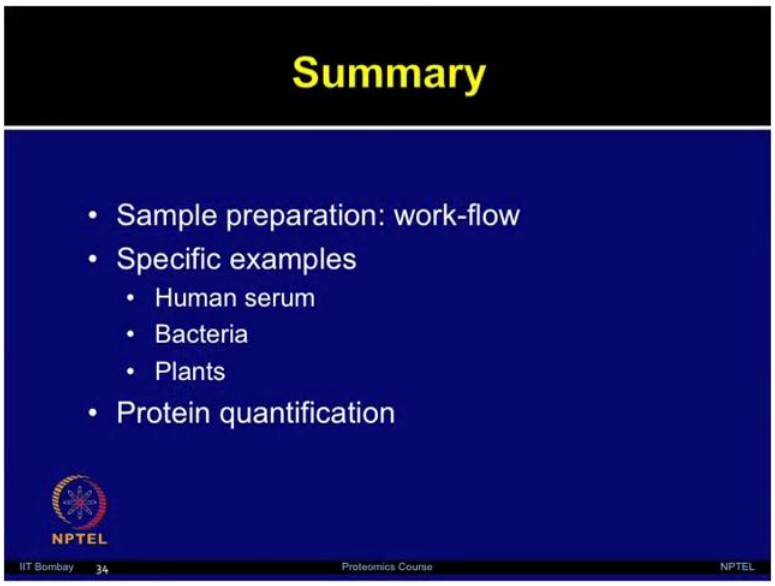

So, in summary, in the last three lectures, we talked about strategies for sample preparation, I gave you a workflow where we talked about different type of lysis methods, we talked about how to prefractionate the samples, then how to precipitate the samples, how to remove the interfering substances and then you move on to the specific examples. We discussed in more detail, different type of criteria being used for analysis of clinical sample and then we talked about, how to analyze human serum sample, then we discussed about bacterial protein extraction and how one can analyze the bacterial proteome, then we talked about plant protein extraction for the plant proteome analyses.

Finally, we talked about protein quantification, how to determine the protein quantity present in your 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 two dimensional

electrophoresis, or it can be used for gel free proteomic applications such as mass spectrometry. So, we will continue our lecture on gel based proteomics and then demonstrate you, how you can use your prepared sample further for analysis of complex proteome such as serum proteome, bacterial proteome and plant proteome, thank you.