Introduction to Proteomics
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Lecture - 08
Sample preparation: Protein extraction and quantification

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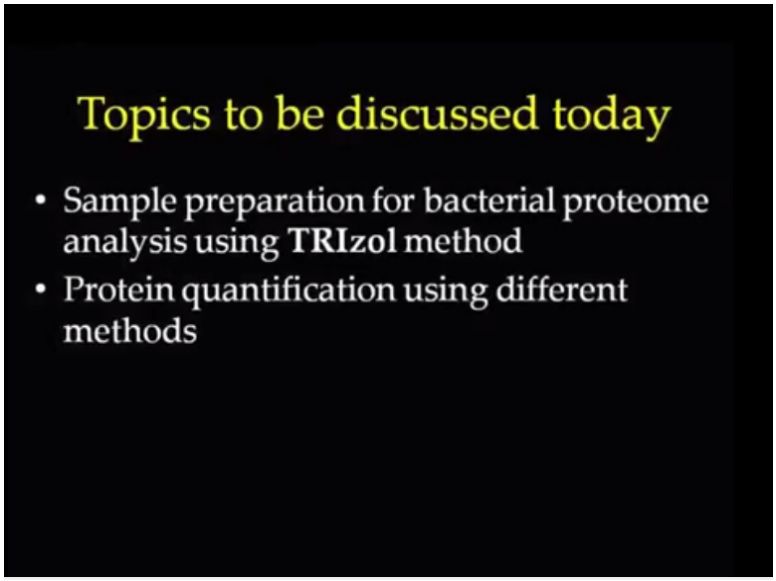

Welcome to the proteomics course. Today, we will talk about sample preparation for proteomics applications with special emphasize on bacterial proteome analysis and protein quantification. In the last 2 lectures, we discussed about different methods, which can be used for a very good protein sample preparation 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 pre-fractionation methods. Then we talked about protein precipitation, protein solubilization and how to remove various type of interfering components.

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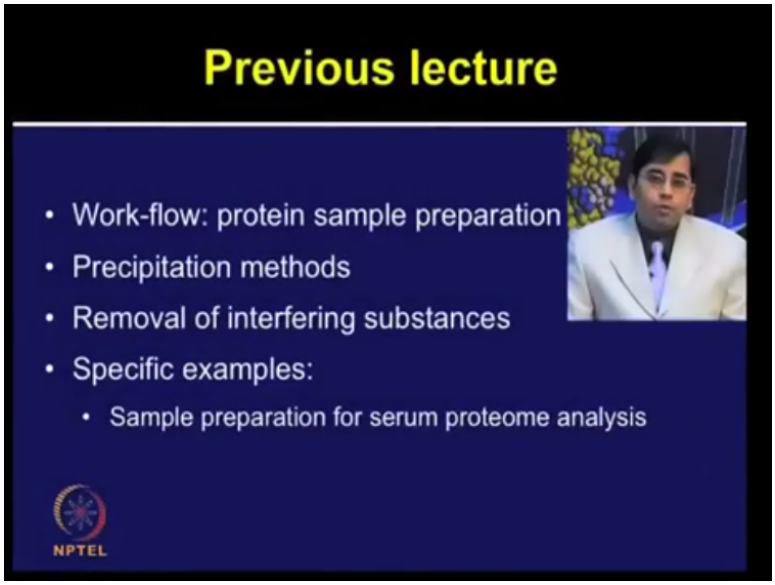

So we discussed about a work-flow for protein sample preparation, different type of precipitation methods, removal of interfering substances and then in the last lecture, I started talking about specific examples. First, we discussed about how to analyze serum proteome, how to prepare the sample for serum protein analysis.

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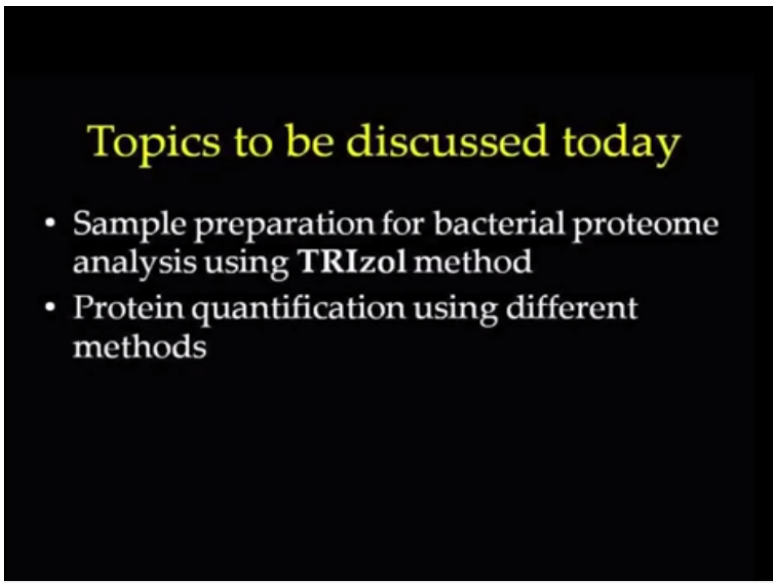

So in today's lecture will first talk about different type of methods available for bacterial protein extraction. I will recommend one method and elaborate on that. We will then talk about how to quantify the protein accurately so that you are ready to perform proteome experiments.

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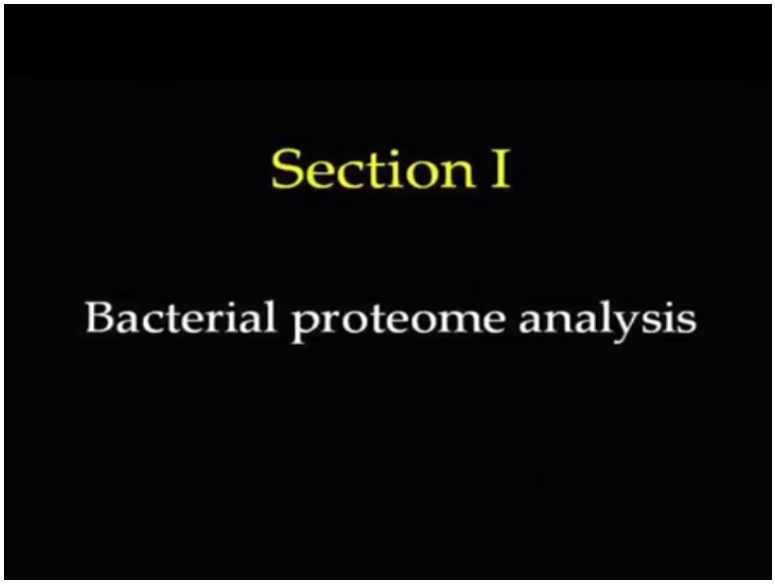

Okay so let us start with the bacterial proteome analysis.

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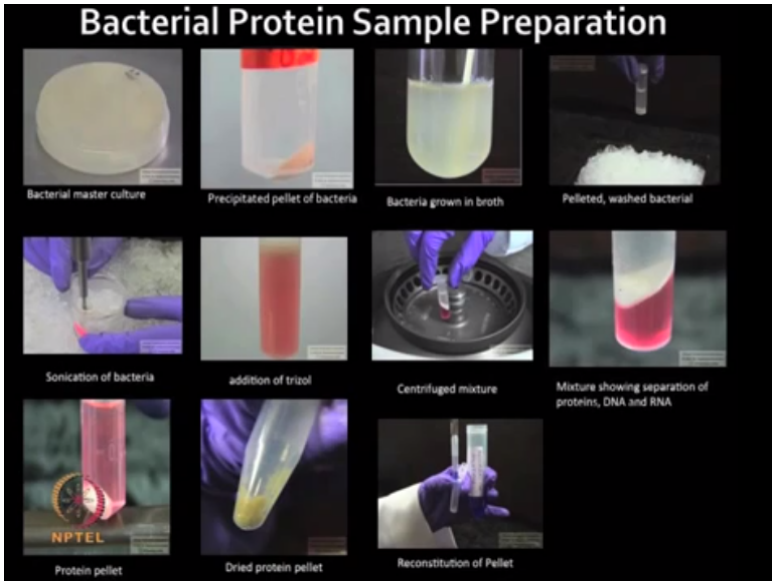

On the slide, various steps which are involved in bacterial protein sample preparation. So now let us elaborate on this and continue different type of sample preparation strategies, which people apply for bacterial protein extraction. These are all reported methods available in the literature. I have just compiled it for your brief overview. For detailed each of the protocol, you can refer to the detailed publications.

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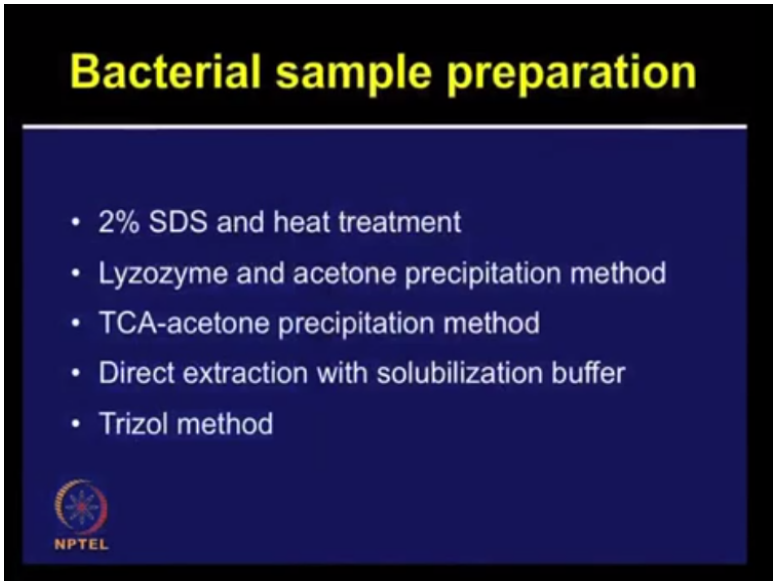

So 2% SDS and heat treatment has been applied for bacterial sample preparation. Similarly, lysozyme and acetone precipitation method have been used. Trichloroacetic acid and acetone together have been effectively used for precipitation. Direct protein extraction and solubilization in the solubilization buffer. This 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. So why we want to elaborate on Trizol method? Because this method provides you an opportunity for recovering DNA, RNA and protein. All 3 major biomolecules from the same sample in the sequential extraction method one can obtain DNA, RNA and protein all by using this method.

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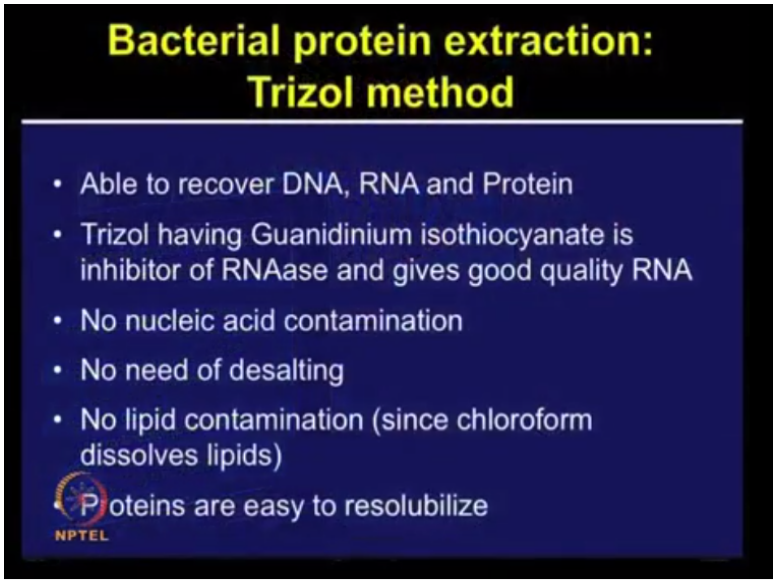

Trizol having Guanidinium isothiocyanate is one of the inhibitor for RNAs and it provides very good quality of RNA. This method also eliminates any possibility of nucleic acid contamination because you are already removing DNA and RNA, sites are also getting rid of by using this method. There is no lipid 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 these depending upon how much protein extract they want and based on their sample.

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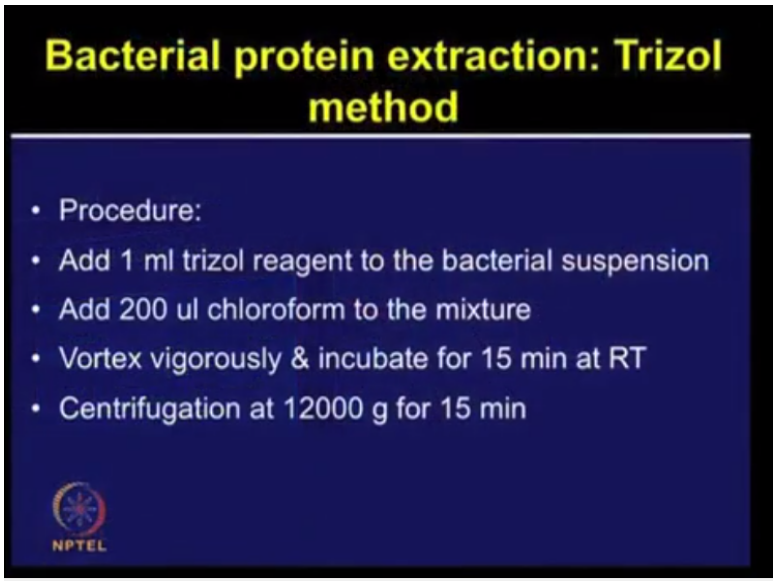

So you can start with 1 ml of Trizol reagent and add that in the bacterial suspension. Then add 200 microliter of chloroform immediately to the same mixture. Vortex this mixture vigorously for 15 to 30 seconds and incubate it at the room temperature for 15 minutes. Centrifuge the sample at 12000 g for 15 minutes.

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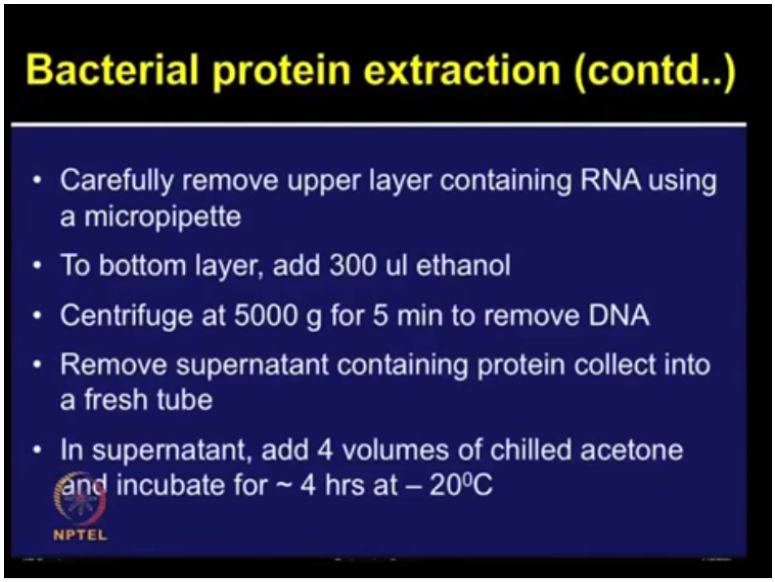

After the centrifugation, remove the upper layer which contains RNA and the bottom layer you can add ethanol. Again, you need to centrifuge at 5000 g for 5 minutes. This step will remove any DNA contamination. Now in fact, this step can be used for keeping the material safe for further DNA extraction. Same applies to the previous step where you can collect the RNA which can be further used for RNA cleanup and preparation.

So once you have removed the supernatant, which contains the protein, then you can collect that in a fresh tube and in this supernatant you can add 4 volumes of chilled acetone, incubate this mixture at -20 degrees for 4 to 6 hours. Once incubation is done, then start centrifugation step for 5 minutes.

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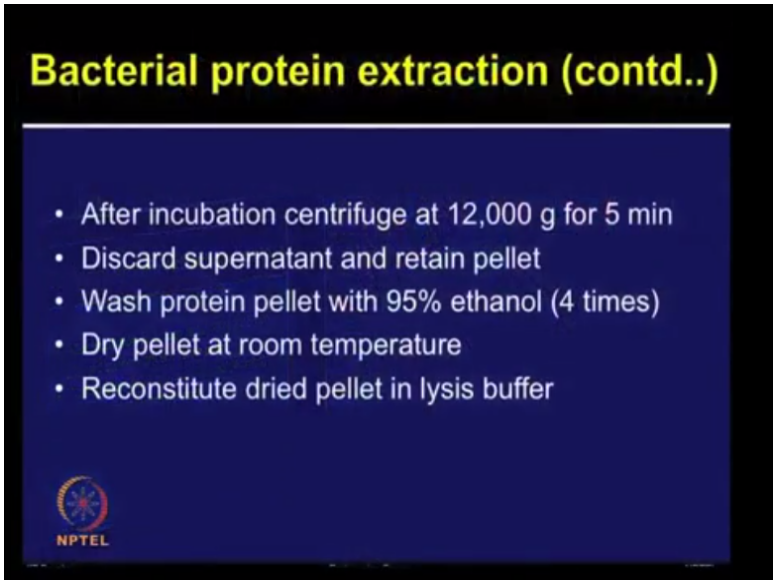

Discard the supernatant after centrifugation and retain the pellet. This pellet can be washed with 95% ethanol 3 to 4 times. Then dry this pellet at the room temperature and this pellet can be reconstituted in a buffer suitable for your analysis. If you are going to perform 2-dimensional electrophoresis 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 SDS-PAGE you can add the (()) (07:51) buffer. So depending upon your application you can reconstitute the dry pellet in the right buffer composition.

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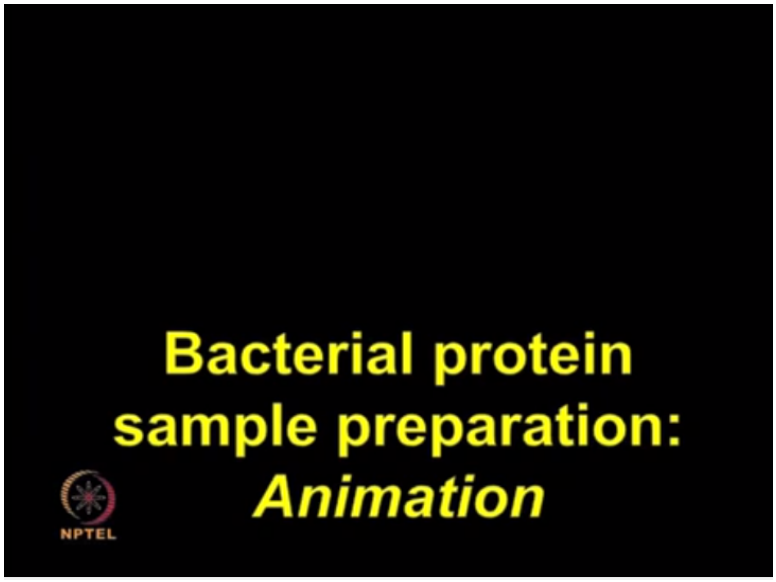

So let me show you the bacterial protein sample preparation in the interactive animations.

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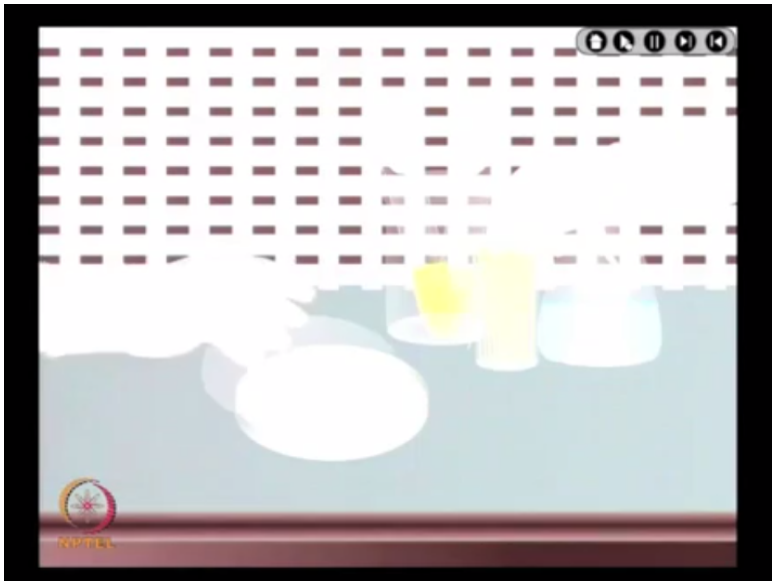

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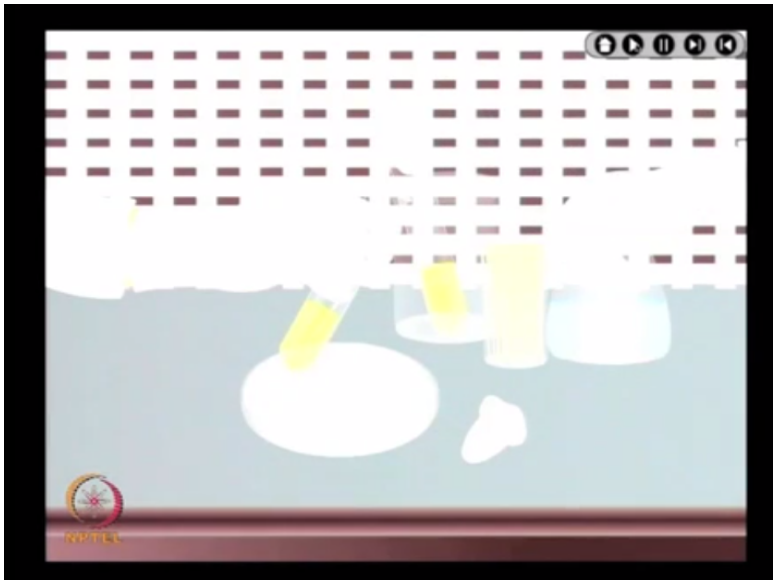

For any bacterial proteome analysis first you need to grow the bacterial culture on LB media containing suitable antibiotic.

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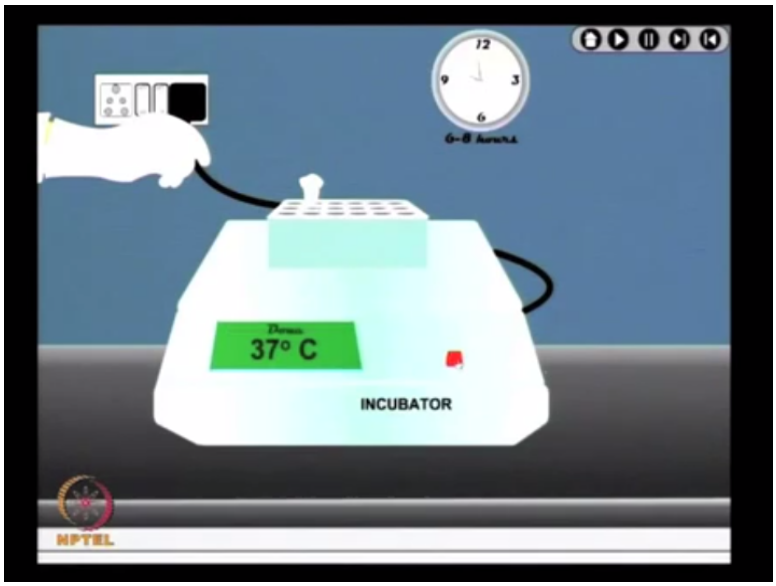

Now the proper 37 degrees temperature is provided for overnight or 6 to 8 hours of growth.

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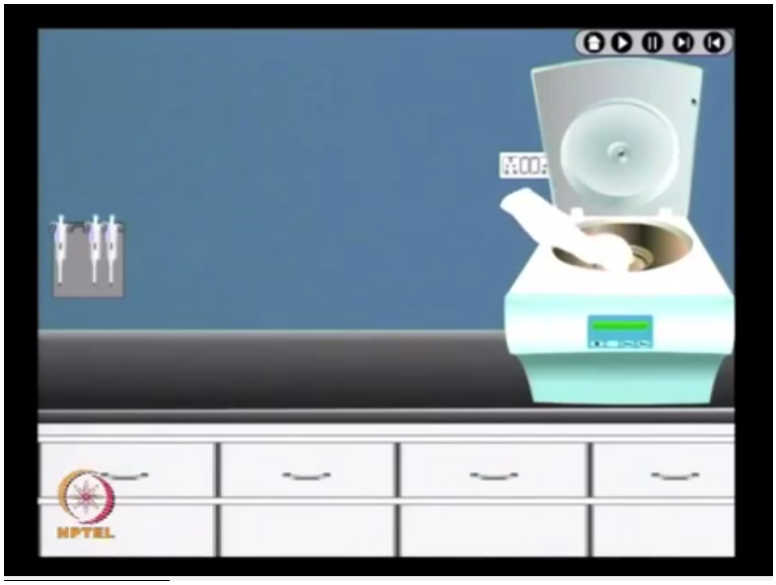

The overnight cultures are diluted with fresh LB and grown at 37 degrees for 6 to 8 hours. Continue growing the culture till it reaches the mid exponential phase. The bacterial cells can be harvested by centrifuging the cells at 12000 RPM for 10 minutes at 4 degrees.

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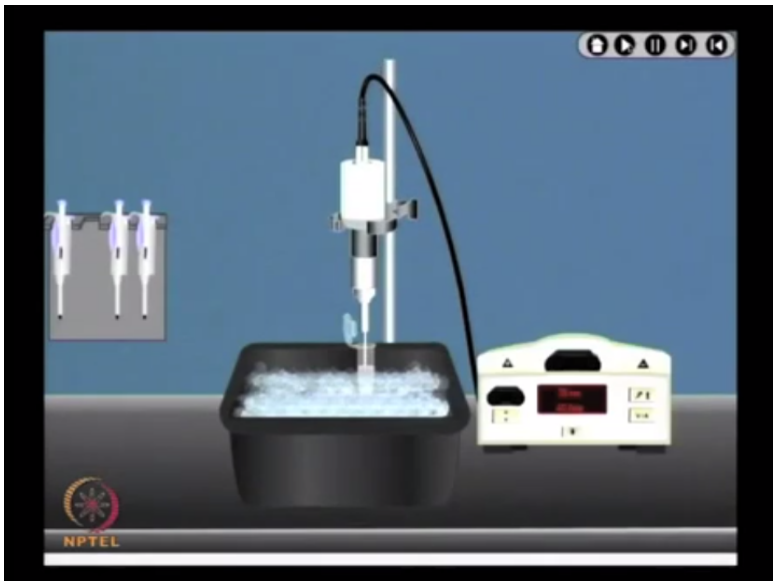

Wash the bacterial pellet with phosphate buffer of pH 7.4 for 4 times to remove the media. Re-suspend the pellet with protease inhibitors and lysozymes and cells can be further ruptured by sonication in ice to prevent forming heat. The sonication step helps to release the content of bacterial cells.

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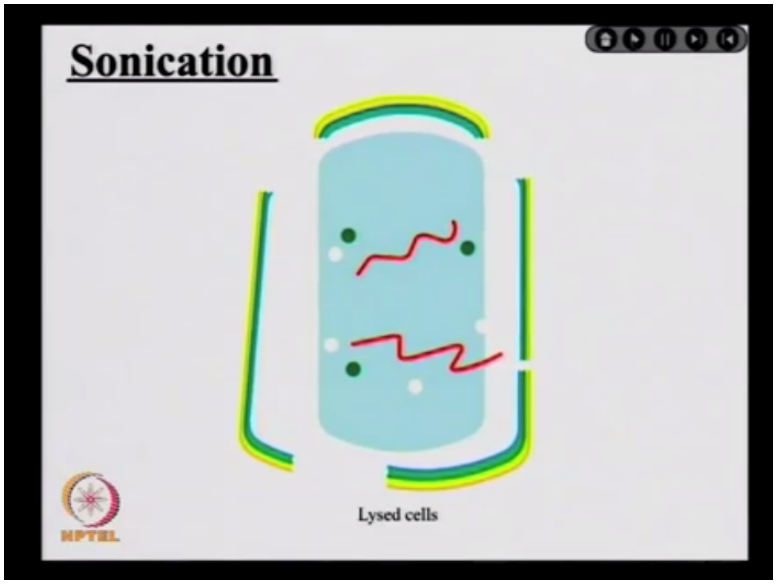

Sonication can be performed by using a sonicator for 6 cycles of 5 second pulse with 30 second gap in between at 20% amplitude. Sonication involves the use of high energy sound waves that are capable of breaking open the outer membrane of the cells.

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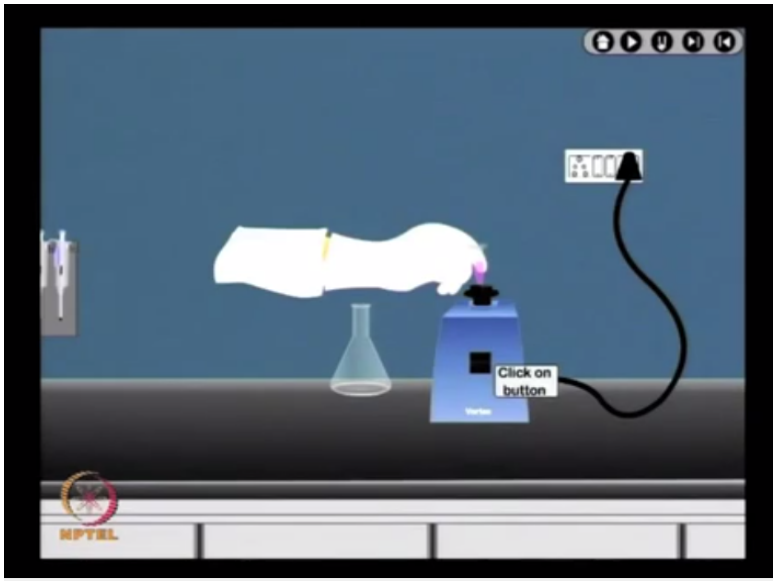

Cells debris and unbroken cells can be separated by the centrifugation step to the supernatant at 1 ml of Trizol and 200 microliter of chloroform and mixed vigorously for 15 seconds. So click on the vortexes, mix it vigorously at room temperature to allow the phase separation.

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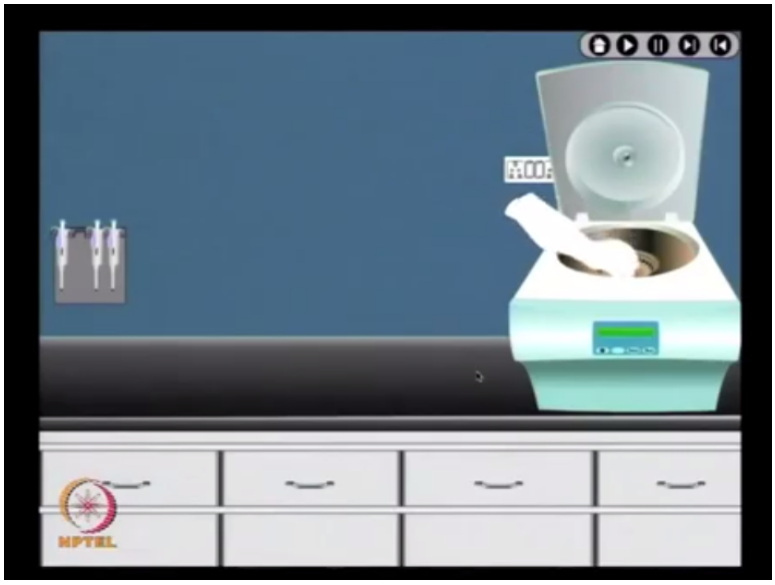

After phase separation, centrifuge the sample at 12000 RPM for 15 minutes at 4 degrees.

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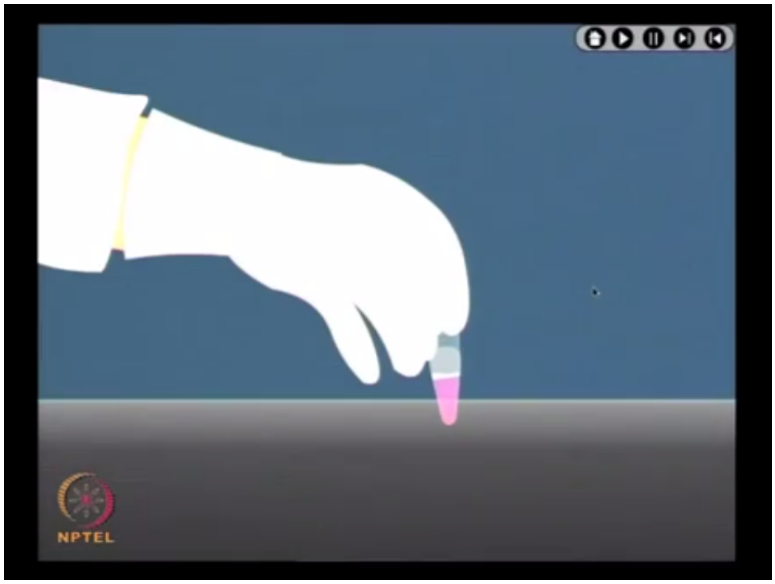

As I mentioned earlier, the different protocols one can use for bacterial proteome analysis. Here we are demonstrating the Trizol method in which the top pale yellow layer contains RNA, the middle white precipitate having protein and lower phenol layer contains both protein and DNA.

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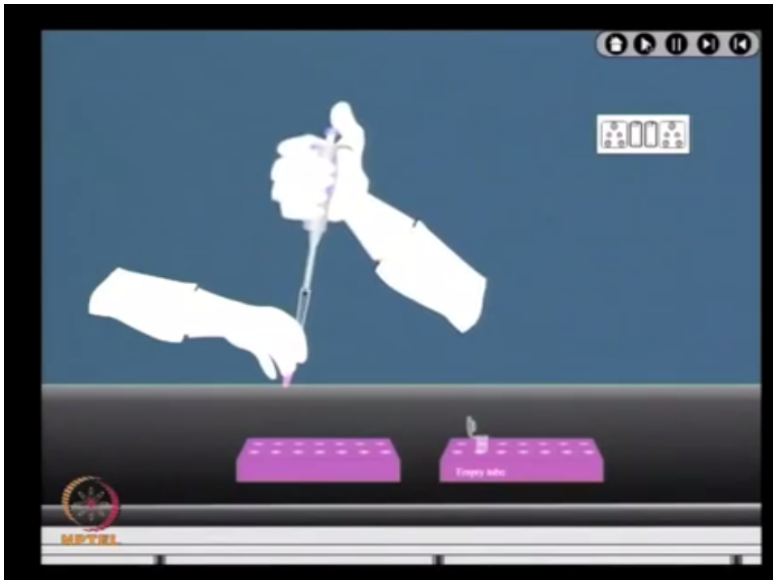

Remove the upper layer and use it for RNA isolation using isopropanol if you want to use RNA for some other applications.

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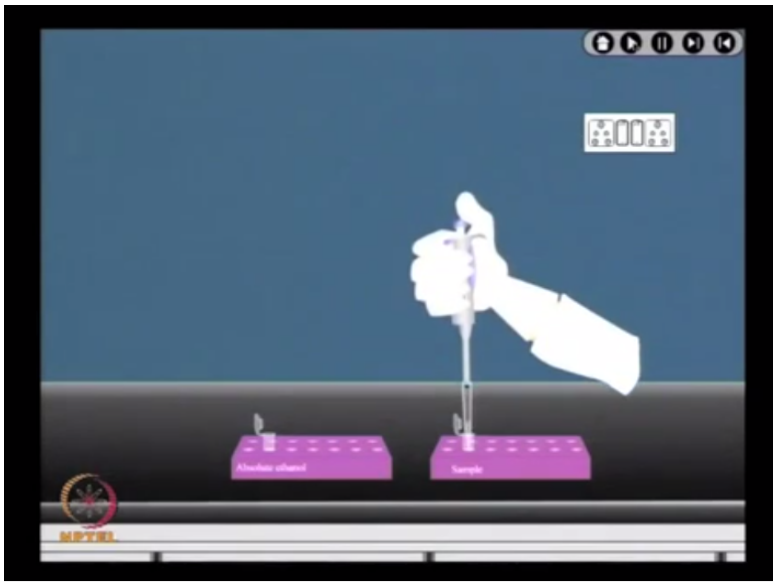

To the bottom layer add 300 microliter of absolute alcohol per 1 ml of Trizol and mix gently to suspend the white precipitate and keep at room temperature for 3 minutes. Centrifuge the mixture at 12000 RPM for 15 minutes at 4 degrees to precipitate the DNA. To the clear pink layer, add 4 volumes of chilled acetone and keep it at -20 degrees for 20 minutes. Protein pellet can be washed with 95% ethanol for 3 to 4 times.

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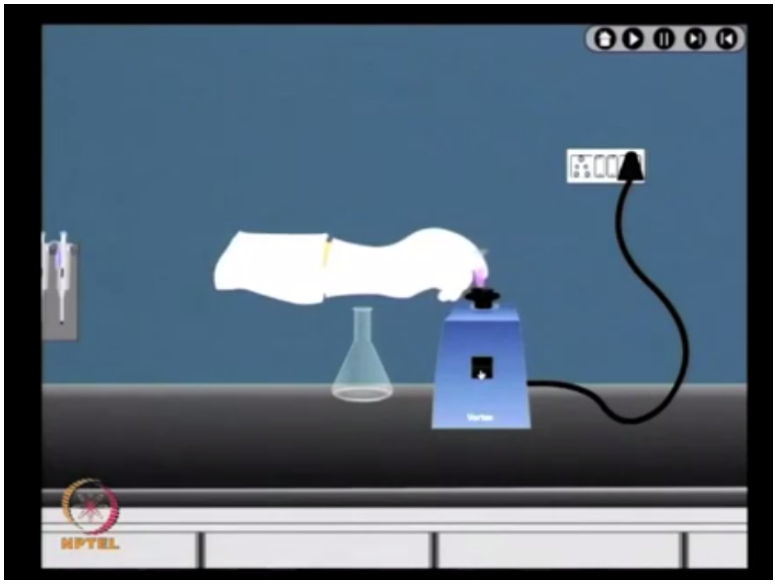

During the washing steps you have to also vortex so that the pellet is properly washed. You need to allow the pellet to dry at the room temperature and then add the lysis buffer containing 7 molar of urea, 2 molar of thiourea, CHAPS, IPG buffer, DTT and Bromophenol blue. Okay so I hope this animation 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 the biomolecules together.

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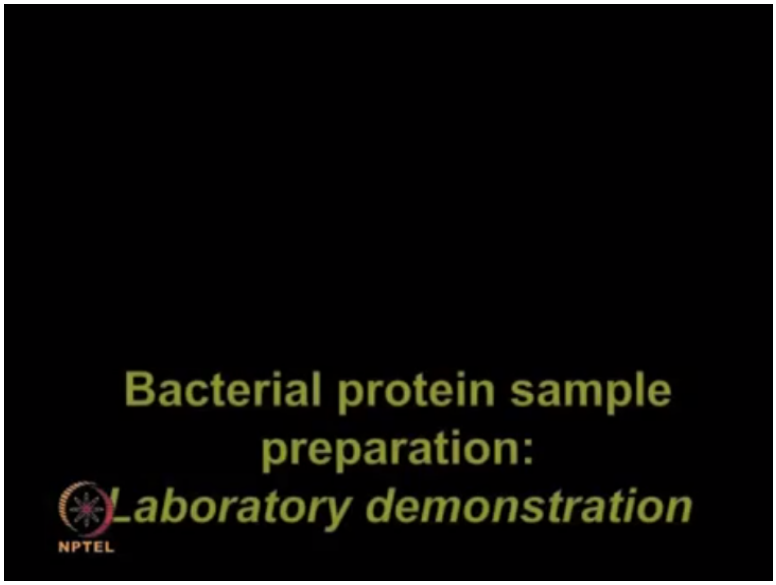

So to give you further information about the detailed protocol as well as how to perform these experiments in the lab, I will show you a video for the laboratory demonstration of bacterial protein sample preparation for proteomics applications. So let us watch this video.

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Bacterial protein extraction.

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

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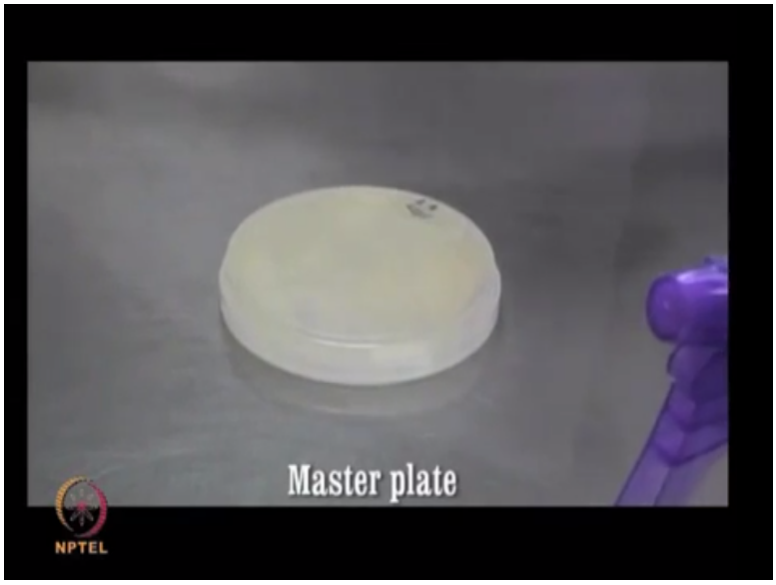

Bacterial culturing, clean the laminar workspace thoroughly with ethanol and keep the master plate having the bacterial culture ready.

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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 autoclaved growth media.

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Incubate this inoculated sample at 37 degrees Celsius for 6 to 8 hours with constant shaking to allow the bacteria to grow.

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The turbidity of the culture, which gradually increases indicates bacterial growth.

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Culture harvesting, transfer the grown bacterial culture to a fresh tube under sterile conditions. Centrifuge this tube at 12000 RPM for 10 minutes maintaining a temperature of 4 degrees Celsius.

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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 debris.

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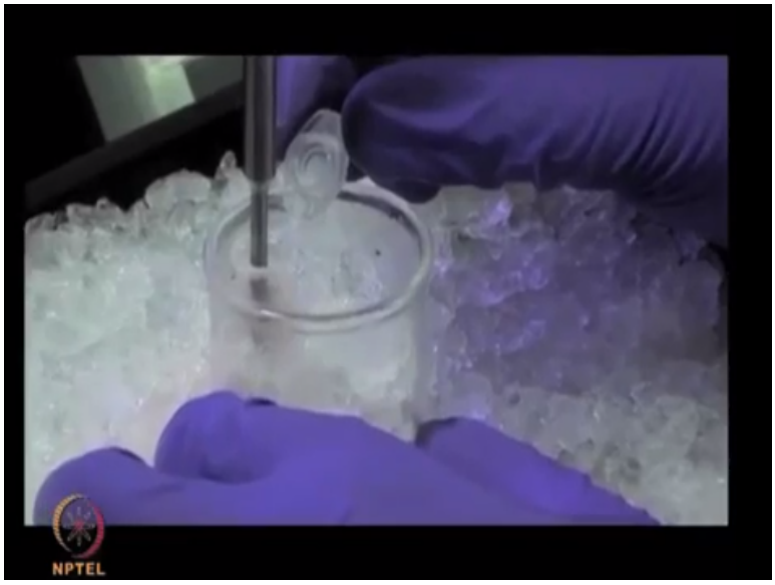

Sonication, the resuspended pellet is sonicated on ice to enable the bacterial cells to break open so that it contents are released.

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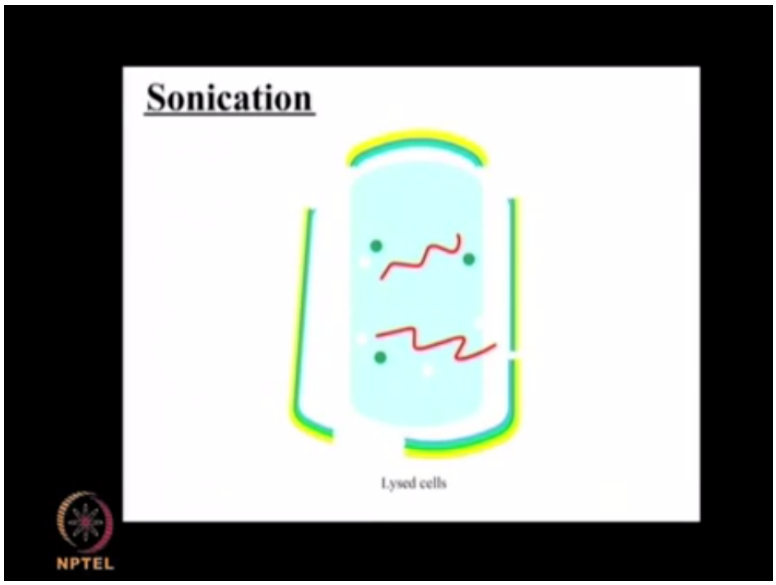

Sonication involves the use of high energy sound waves that are capable of breaking open the outer membranes of cells. All cellular contents including proteins of interest leak out of this disrupted membrane.

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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 the bacterial proteins but also their DNA and RNA.

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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.

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Three distinct layers will be obtained, the topmost is the aqueous layer containing RNA, the center is the interphase containing proteins and the bottom layer is organic and consists of DNA.

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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 200 RPM for 5 minutes.

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The DNA forms the 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 -20 degrees 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 -20 degree Celsius before carrying out protein quantification.

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Okay 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 type of contaminants by using Trizol method.

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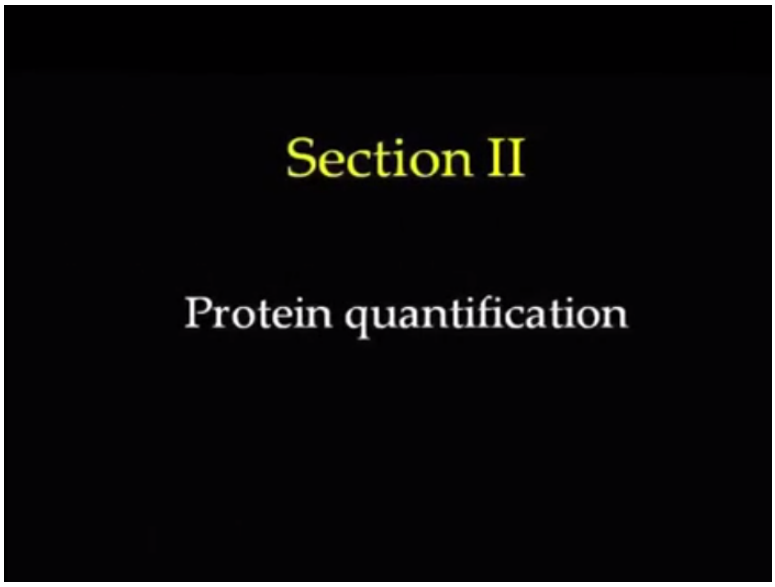

So now let us move on to the next topic, which is protein quantification. So by now you have prepared your protein sample by using different type of methods by keeping a very careful eye on different type of interfering substances and contaminants and you have tried to eliminate all of those components. 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 type of protein quantification methods are available. We will talk about protein quantification in detail now. 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 absorbents at 280 nanometers.

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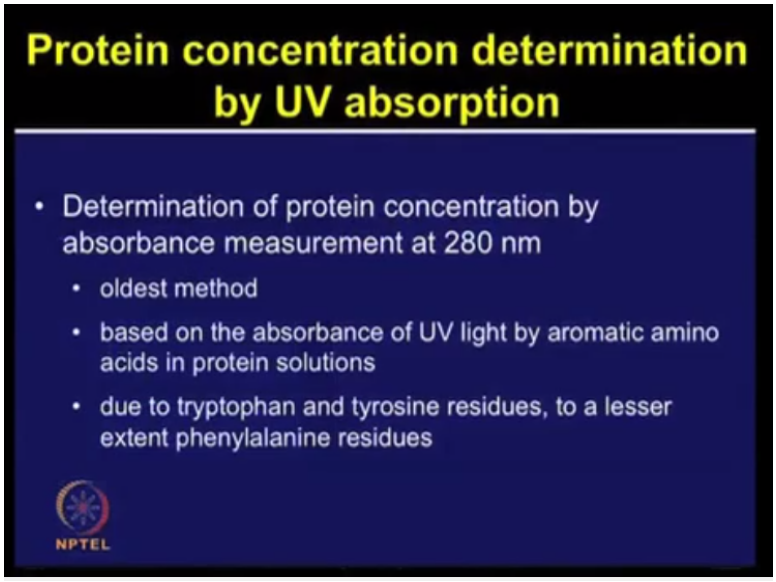

This method is based on the absorbance of ultraviolet light by aromatic amino acids such as tryptophan and tyrosine residues, which are present in the protein solutions. Phenylalanine, it is also aromatic amino acid but up to a lesser extent it is responsible for this absorbance. So to determine the protein concentration at A280 method requires that your protein contains tryptophan and tyrosine aromatic residues.

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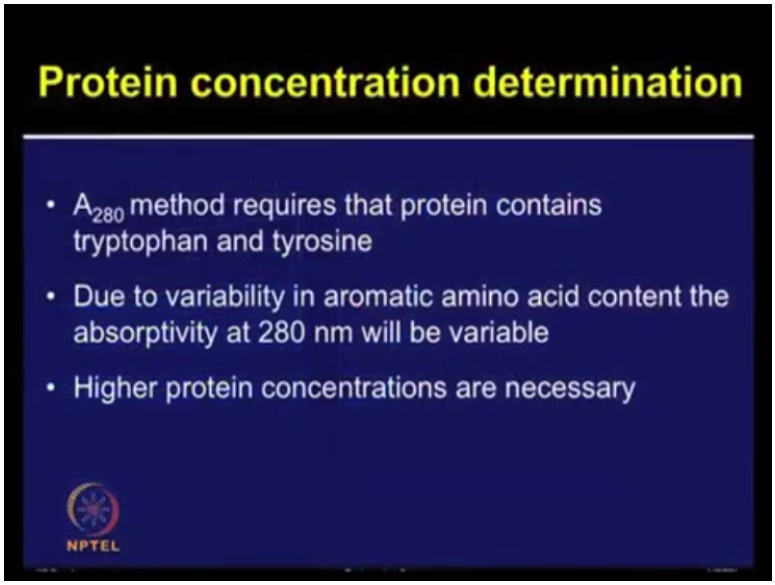

Since the protein samples will have variability in overall amino acid contents so the absorptivity at 280 nanometers will be a variable factor for this type of method. Also if you need very high protein concentration for your protein preparation for your proteomic applications then this method is again 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 nanometer and the protein concentration should be in very high amount.

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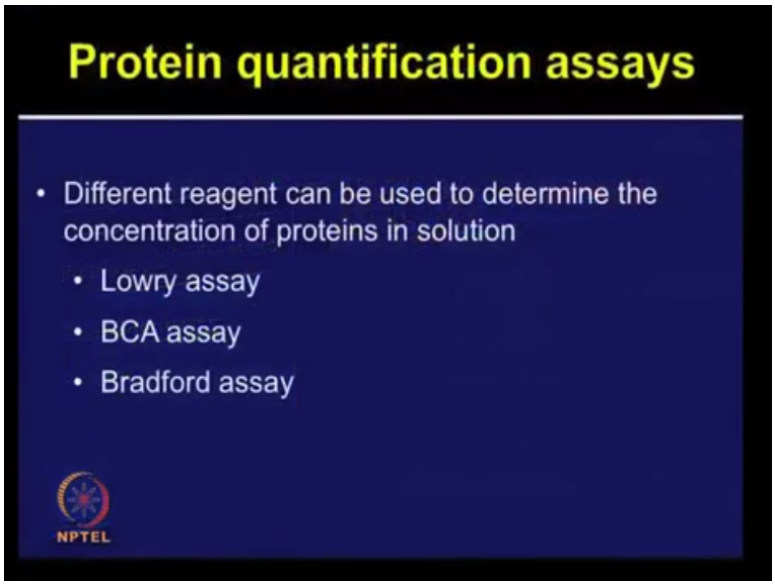

To overcome these limitations, the different regions, different quantification assays have emerged and these reagents can be used to determine the concentration of proteins with the given solution. These methods include Lowry assay, BCA assay and Bradford assay. There are several other assays as well, but will talk about these 3 more commonly used methods.

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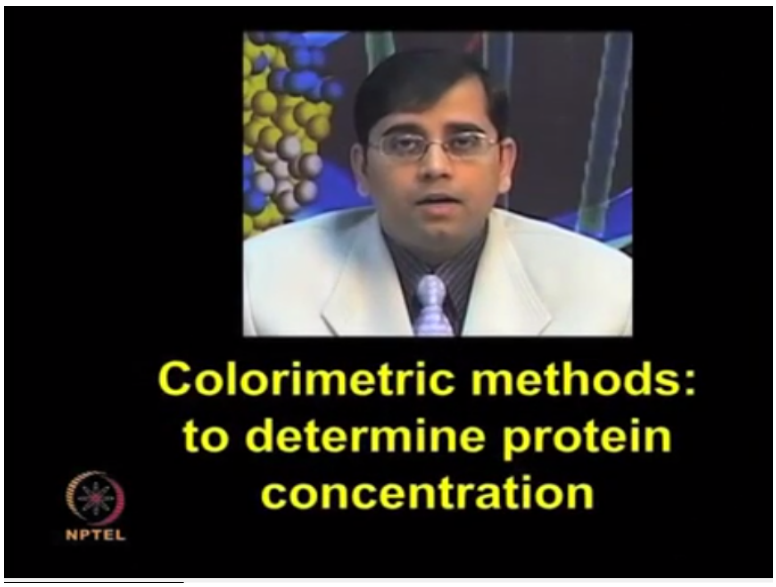

So let us talk about different type of colorimetric methods to determine the protein concentration. So how to perform the quantification? So let me show you one video, a laboratory demonstration of how to perform protein quantification.

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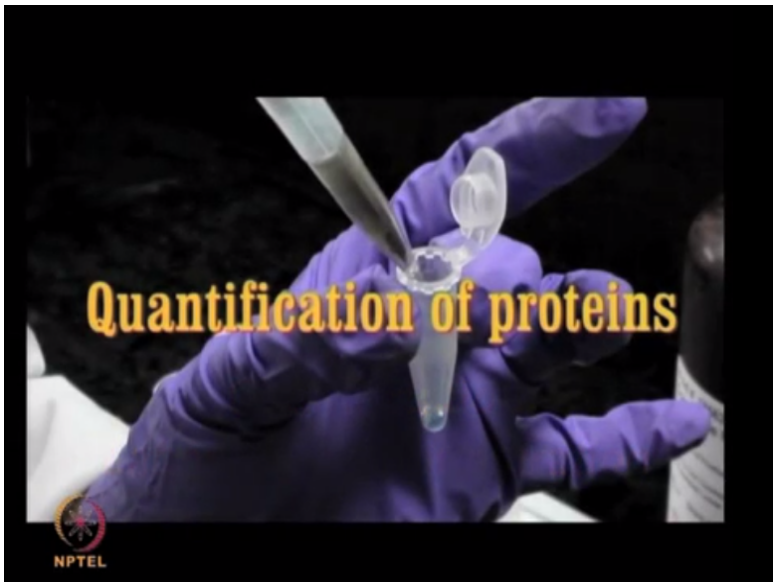

Quantification of proteins, after the protein sample has been extracted from its source, it must be quantified to determine the protein content before any further processing. Label the tubes suitably for standard and test samples. Thaw the protein samples to be quantified by gently rubbing it between the palms. Prepare the sample buffer required for diluting samples during the assay and mix it well.

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Add an increasing concentration of standard protein sample to each of the designated tubes. Bovine serum albumin 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.

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Then add the Bradford color reagent to each tube and mix well.

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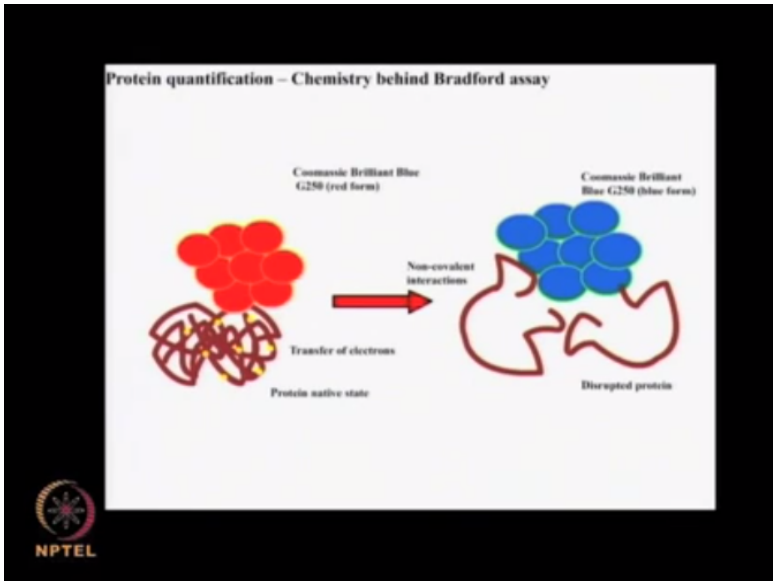

An electron transfer reaction takes place between the red form of the Coomassie dye of the reagent and the native protein. This disrupts the protein structure and establishes several non-covalent interactions between the dye and protein. The transfer of electrons converts the dye into its blue form thereby 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.

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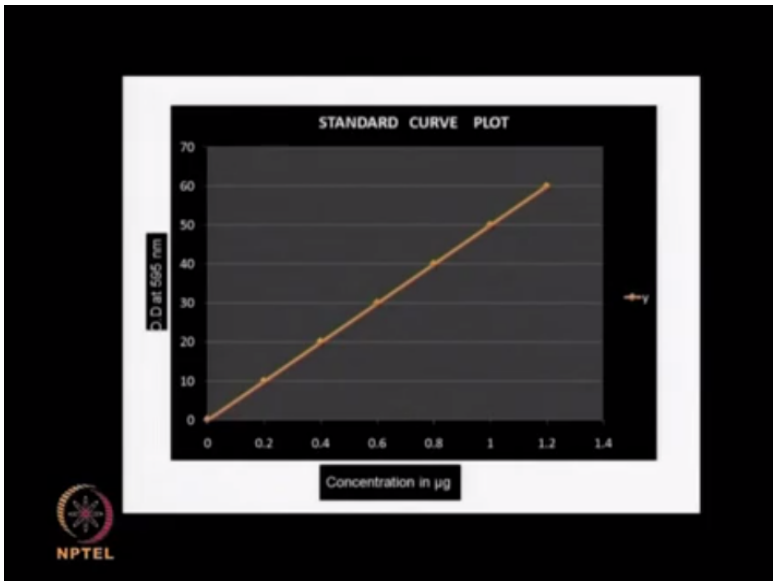

Okay so I hope it was useful for you to watch the video for protein quantification and we got a feel how to perform this experiment in the laboratory.

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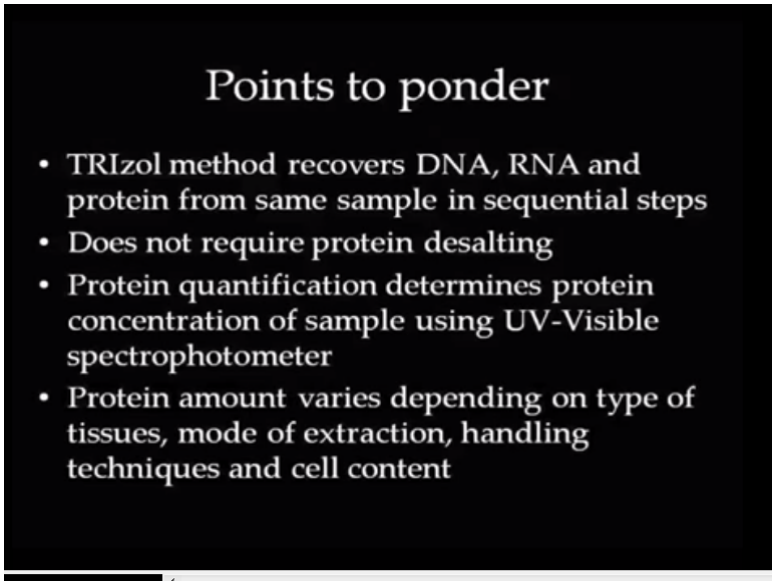

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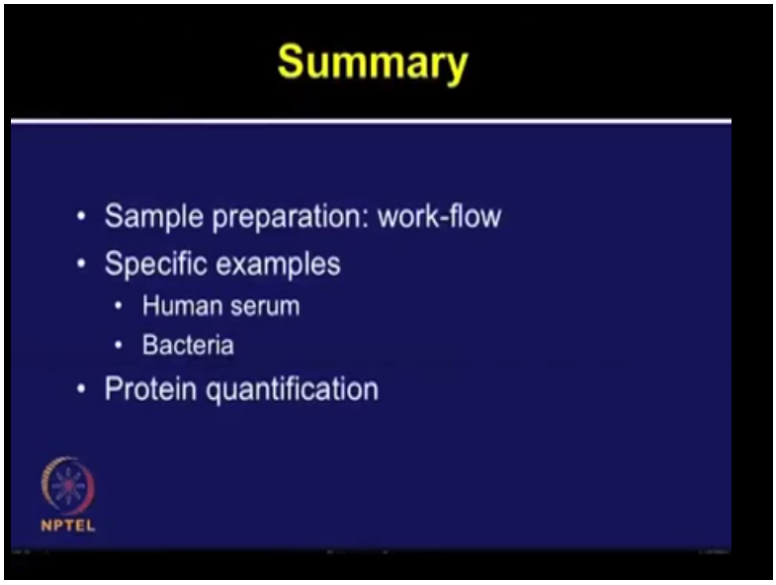

So summary, in the last 3 lectures, we talked about strategies for sample preparation, I gave you a work flow where we talked about different type of lysis methods, we talked about how to pre-fractionate the samples. Then how to precipitate the samples, how to remove the interfering substances and then we 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. Finally, we talked about protein quantification, how to determine the protein quantity present in your given sample? So we will continue our discussion on the proteomics.

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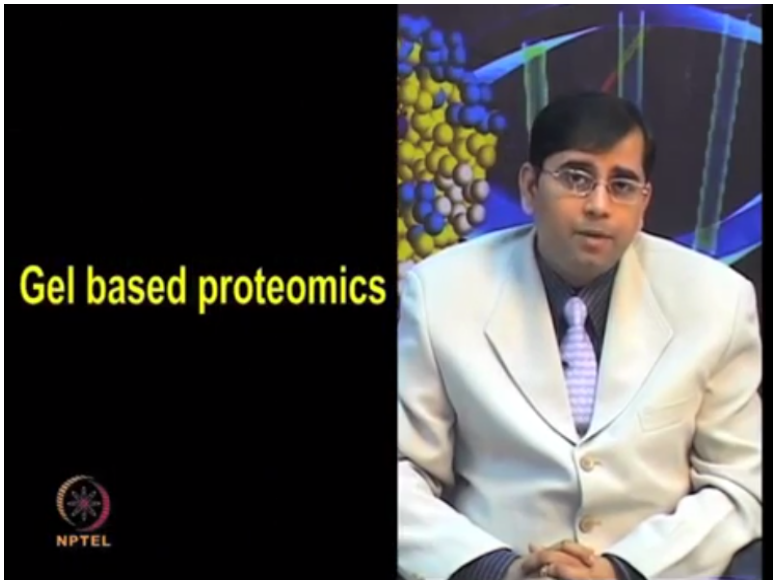

And now since we have prepared a good sample it can be applied either for using the applications on gel based proteomics approaches such as 2-dimensional electrophoresis or it can be used for gel free proteomic approaches such as mass spectrometry. So we will continue our lecture on gel based proteomics.

And 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.

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