Proteins and Gel-Based Proteomics
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Indian Institute of Technology, Bombay
Mod 03 Lecture Number 10

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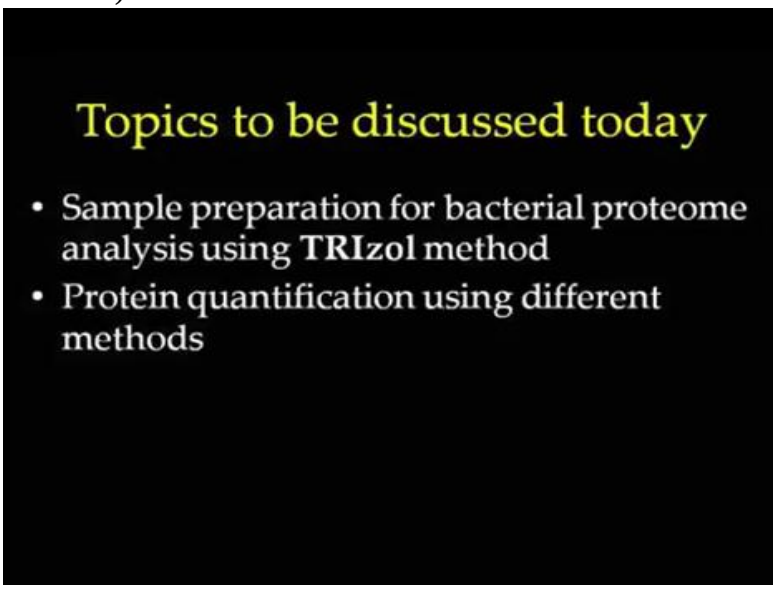

Welcome to the Proteomics course.

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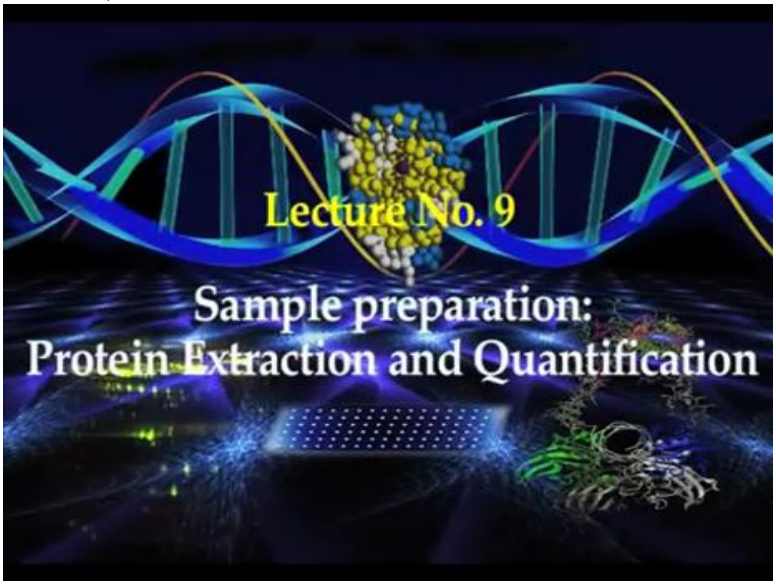

Today we will talk about sample preparation for proteomics application with special emphasis on bacterial proteome analysis and protein quantification. In the last 2 lectures, we discussed about different methods which can be used for very good protein sample preparation for proteomic applications.

We talked about different types of lysis methods. We talked about how to prevent the proteolysis during the lysis, different types of protein prefactionation methods. Then we talked about protein precipitation, protein solubilization and how to remove various types of interfering components.

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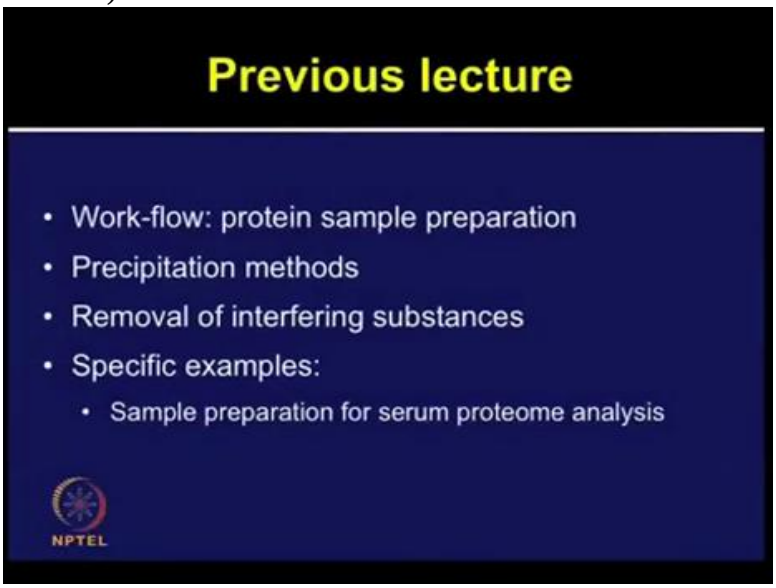

So we discussed about a workflow for protein sample preparation, different types of precipitation methods, removal of interfering substances and then in the last lecture, I started talking about specific examples. First we discussed about how to analyze serum proteome, how to prepare the sample for serum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

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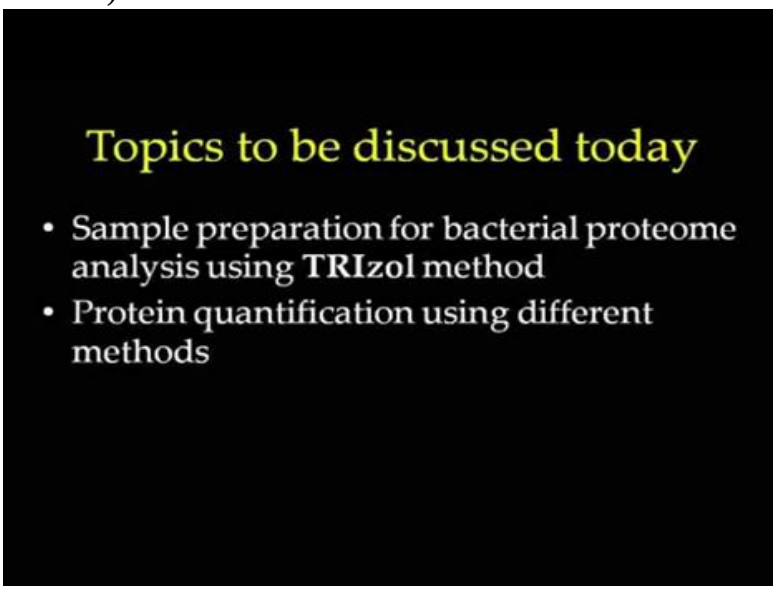

So in today's lecture, we will first talk about different types of methods available for bacterial protein extraction. I will recommend one method and elaborate on that. We will then talk about how to quantify the proteins accurately so that you are ready to perform proteomic experiments.

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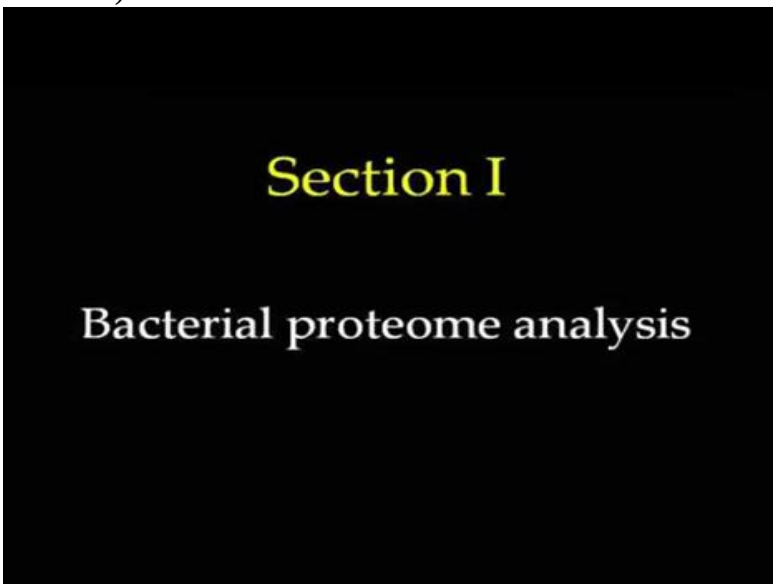

Ok, so let’s start with the bacterial proteome analysis...

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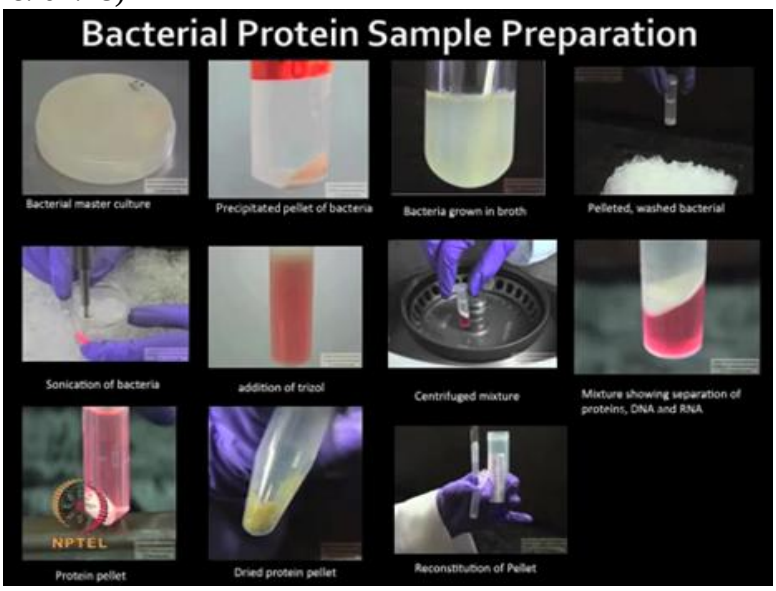

On the slide, various steps which are involved in bacterial protein sample preparation....So now let’s elaborate on this and continue. Different types of sample preparation strategies which people apply for bacterial protein extraction; these are all reported methods available

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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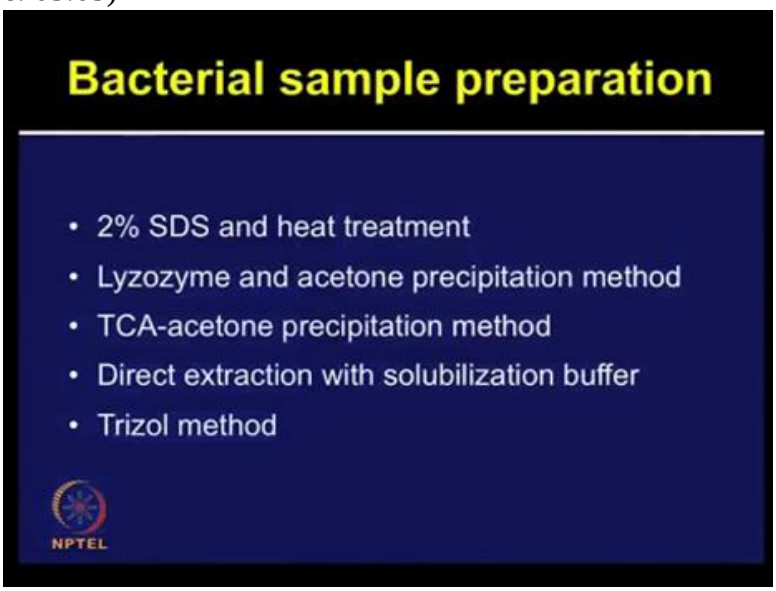

So 2% SDS and heat treatment has been applied for bacterial sample preparation. Similarly lyzozyme and acetone precipitation method have been used. Trichloroacetic acid and acetone together have been effectively used for precipitation.

Direct protein extraction and solubilization in the solubilization buffer, this 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 proteins, 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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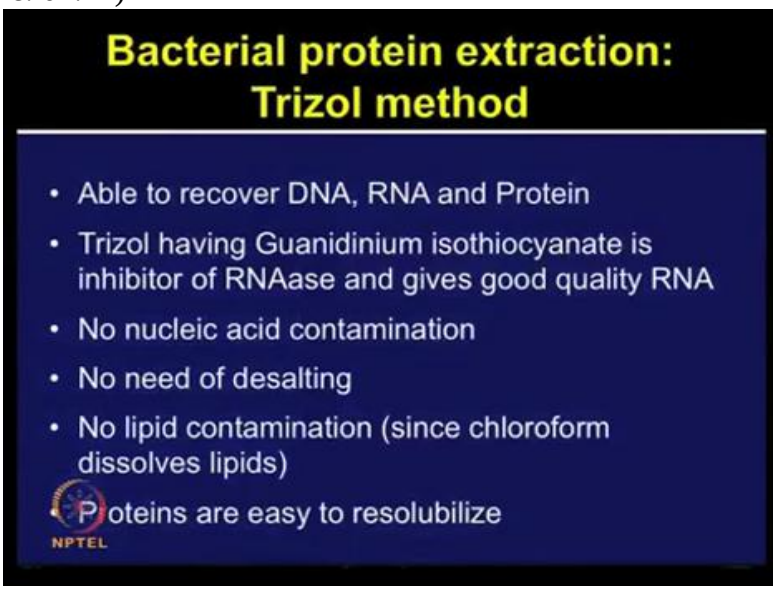

Trizol having guanidinium isothiocyanate is one of the inhibitors for RNase and it provides very good quality of RNA. This method also eliminates any possibility of nucleic acid contamination because you are already removing DNA and RNA.

Salts are also getting rid of 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 types of interfering components as well as different types of contaminants.

And finally, the proteins are easy to re-solubilize 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 on how much protein extract they want and based on their sample.

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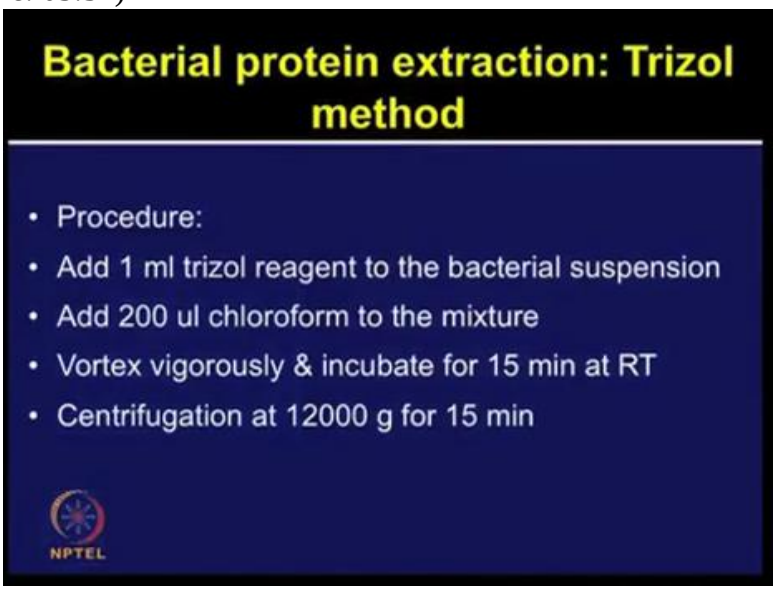

So you can start with 1 ml of Trizol reagent and add that in the bacterial suspension. Then add 200 micro liter of chloroform immediately to the same mixture. Vortex this mixture vigorously for 15 to 30 seconds; and incubate it at the room temperature for 15 minutes. Centrifuge the sample at 12000 g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

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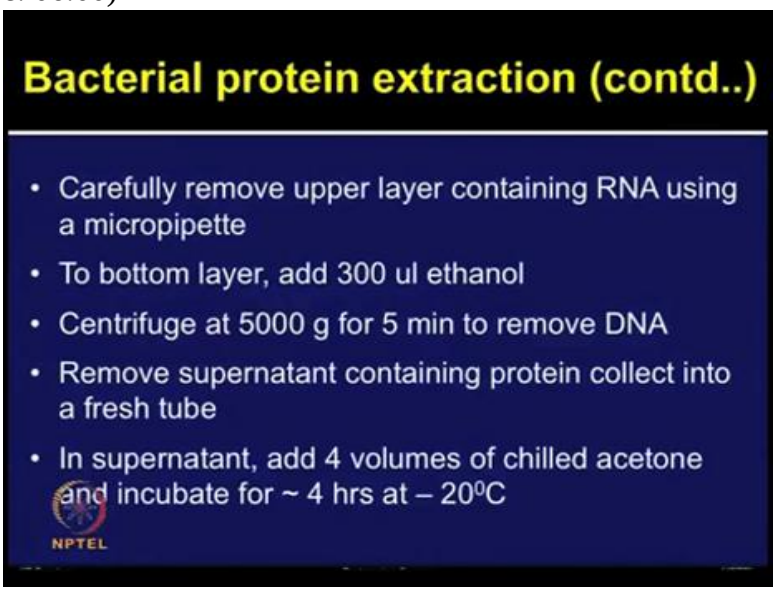

After the centrifugation, remove the upper layer which contains RNA; and the bottom layer, you can add ethanol. Again you need to centrifuge at 5000 g for 5 minutes. This step will remove any DNA contamination.

Now in fact, this step can be 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.

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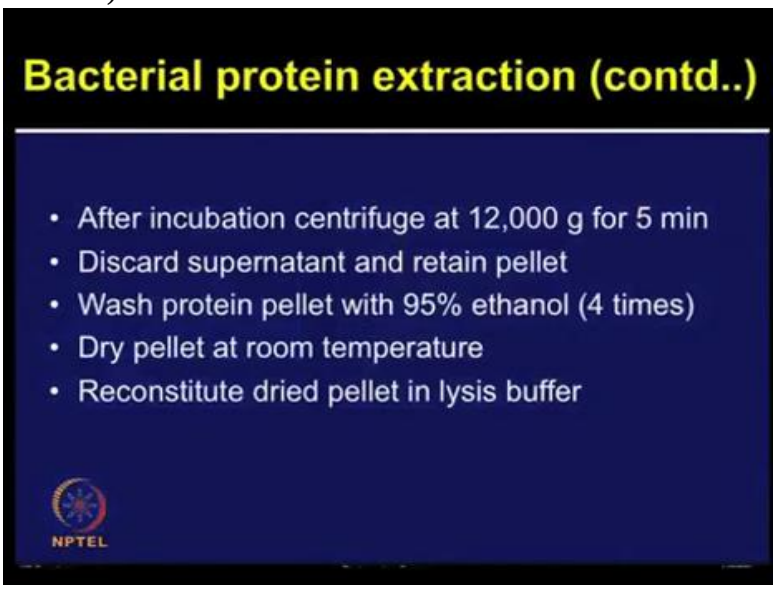

Once incubation is done, then start centrifugation step for 5 minutes. Discard the supernatant after centrifugation and retain the pellet. This pellet can be washed with 95% ethanol 3 to 4 times. Then dry this pellet at the room temperature and this pellet can be reconstituted in a buffer suitable for your analysis.

If you are going to perform two-dimensional 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 Laemmli buffer. So depending upon your application, you can reconstitute the dried 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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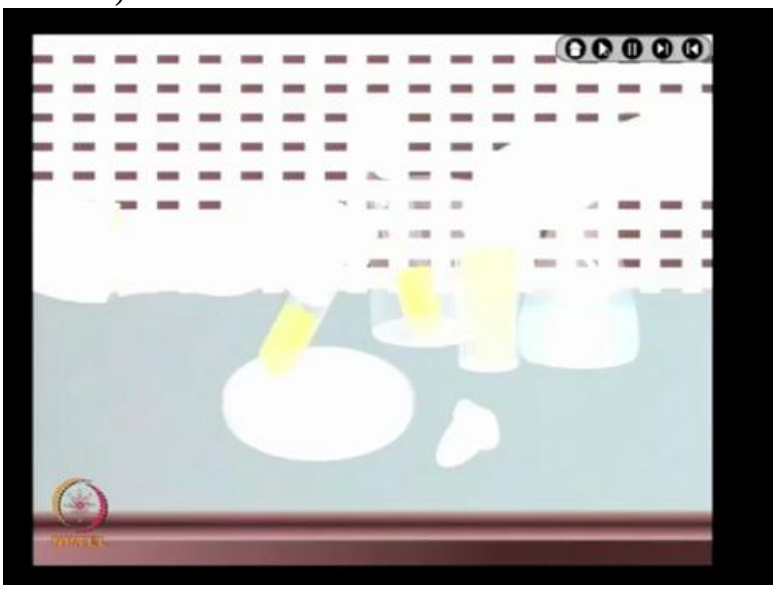

For any bacterial proteome analysis, first you need to grow the bacterial cultures on algae media containing suitable antibiotic.

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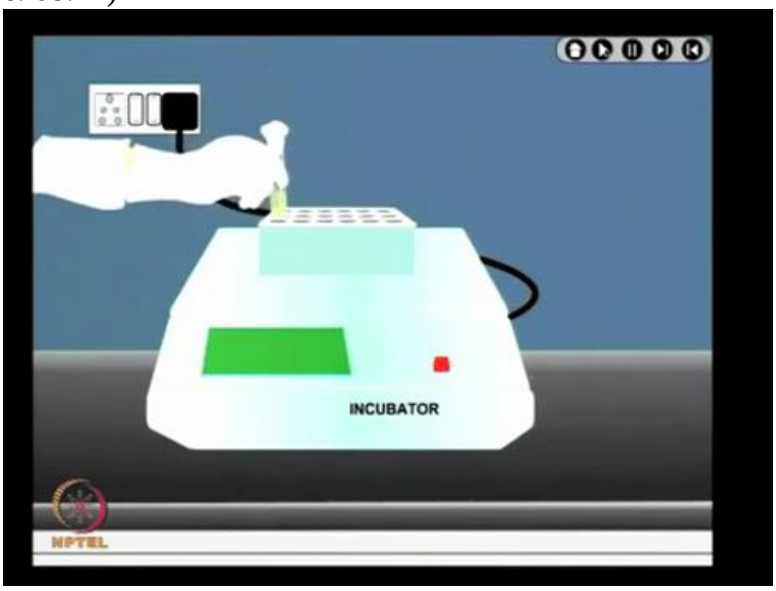

Now proper 37 degrees temperature is provided for overnight or 6 to 8 hours of growth

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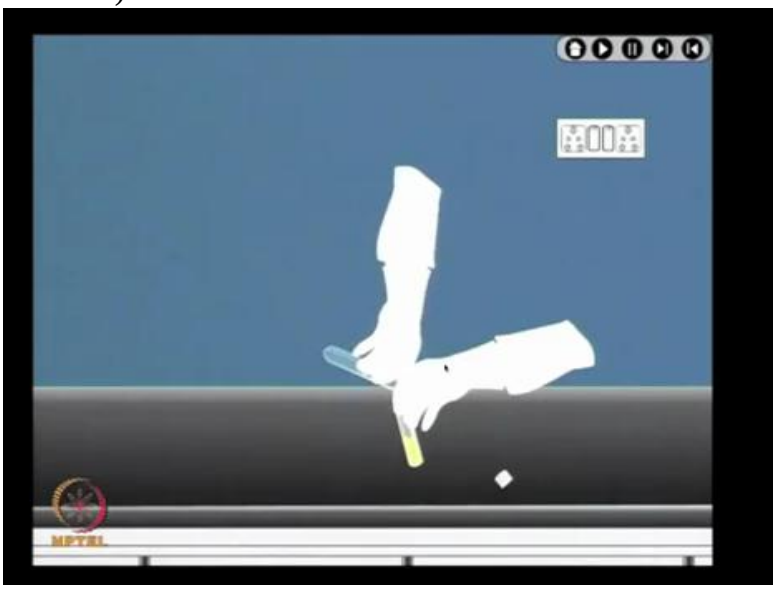

The overnight cultures are diluted with fresh LB…

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…and grown at 37 degrees for 6 to 8 hours.

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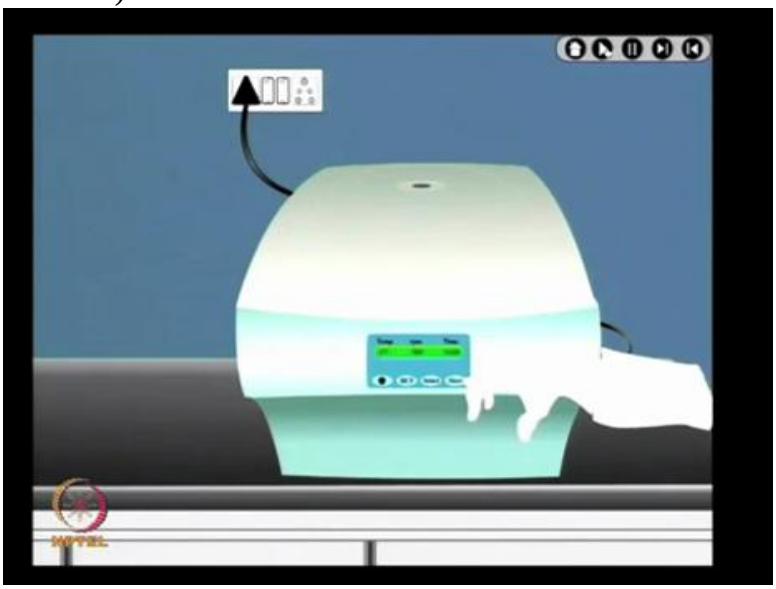

Continue growing the culture until it reaches the mid-exponential phase. The bacterial cells can be harvested by centrifuging the cells at 12000 rpm for 10 minutes at 4 degrees.

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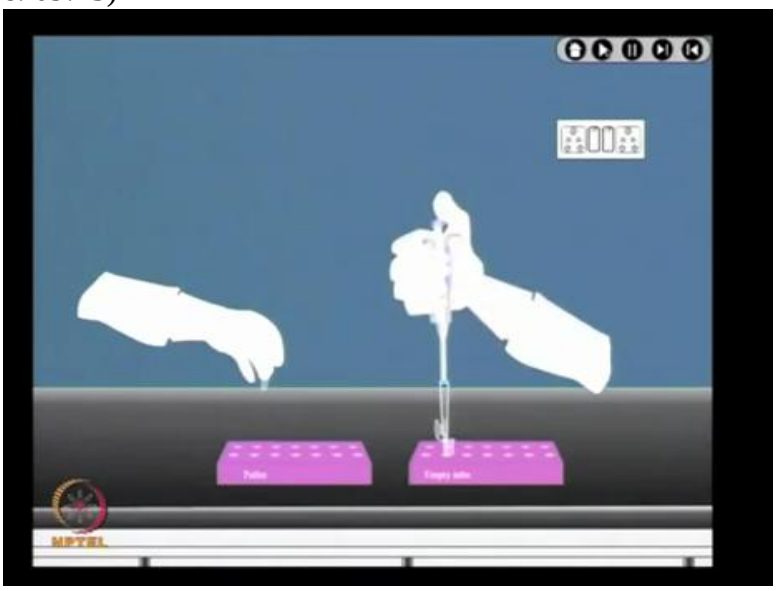

Wash the bacterial pellet with phosphate buffer of pH 7.4 for 4 times to remove the media

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Re-suspend the pellet with protease inhibitors and lysozymes and cells can be further ruptured by sonication in ice to prevent foaming and heat.

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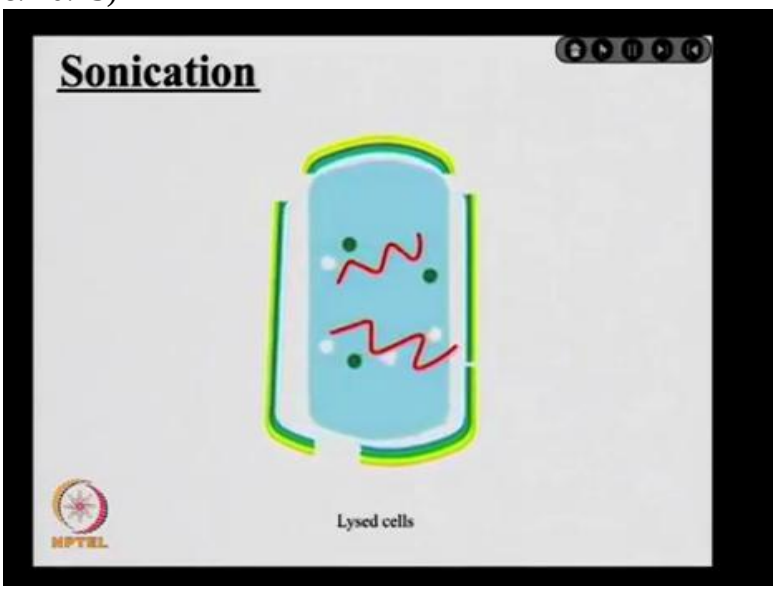

The sonication step helps to release the content of bacterial cells. Sonication can be performed by using a sonicator for 6 cycles of 5 second pulse with 30 seconds gap in between at 20% amplitude.

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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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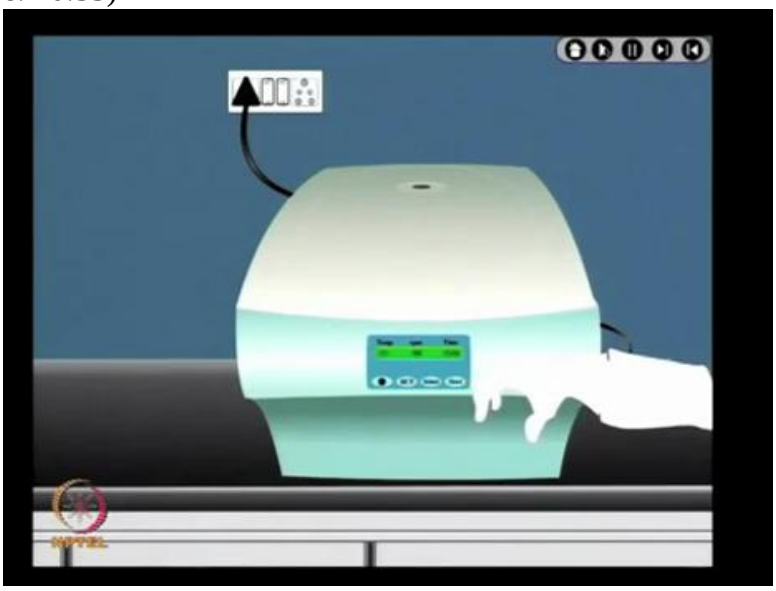

Cell debris and unbroken cells can be separated by the centrifugation step….

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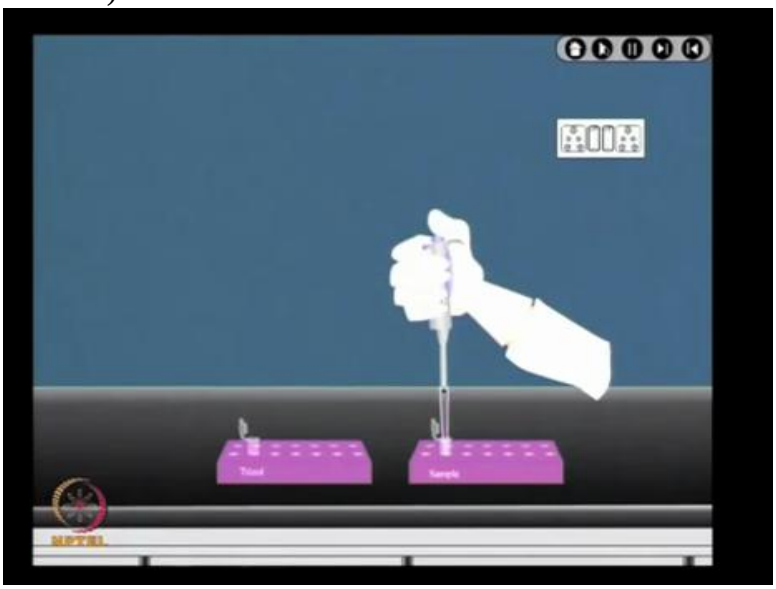

…to the supernatant add 1 ml of Trizol and 200 micro liter chloroform…

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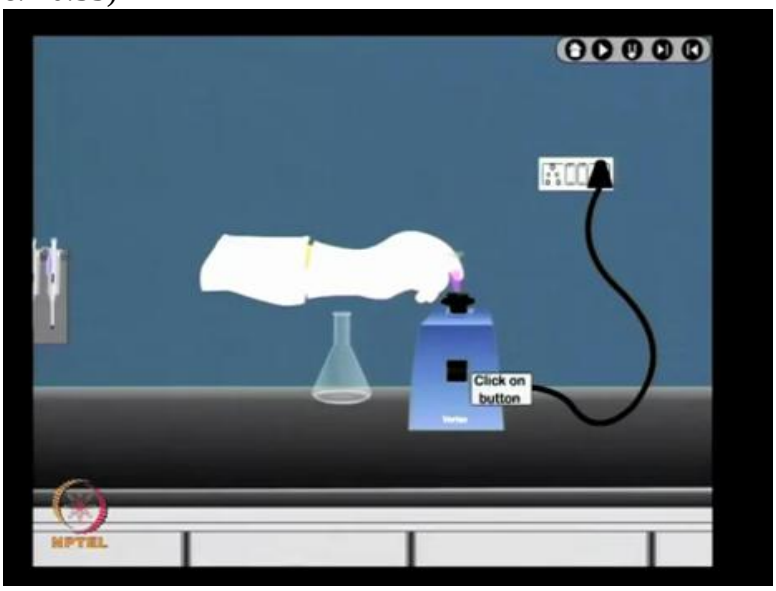

… and mix vigorously for 15 seconds, so click on the Vortex here. Mix it vigorously at room temperature…

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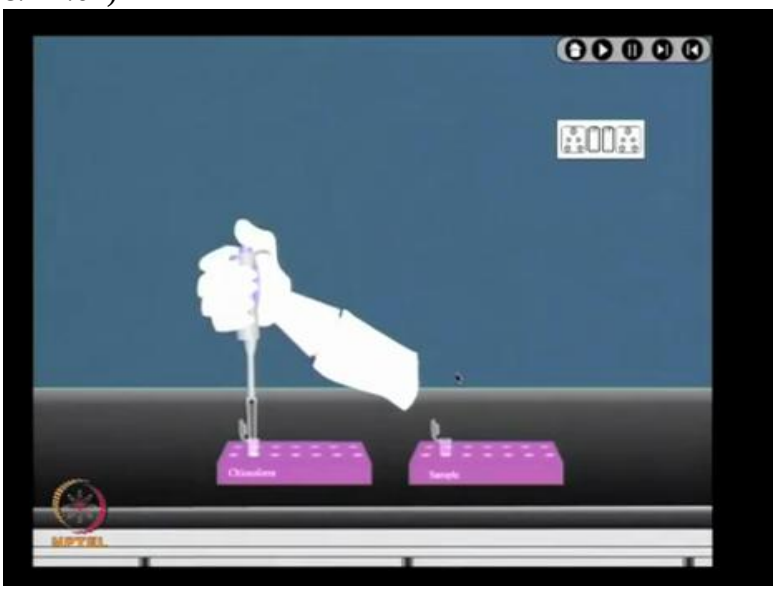

… To allow the phase separation

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After phase separation, centrifuge the sample at 12000 rpm…

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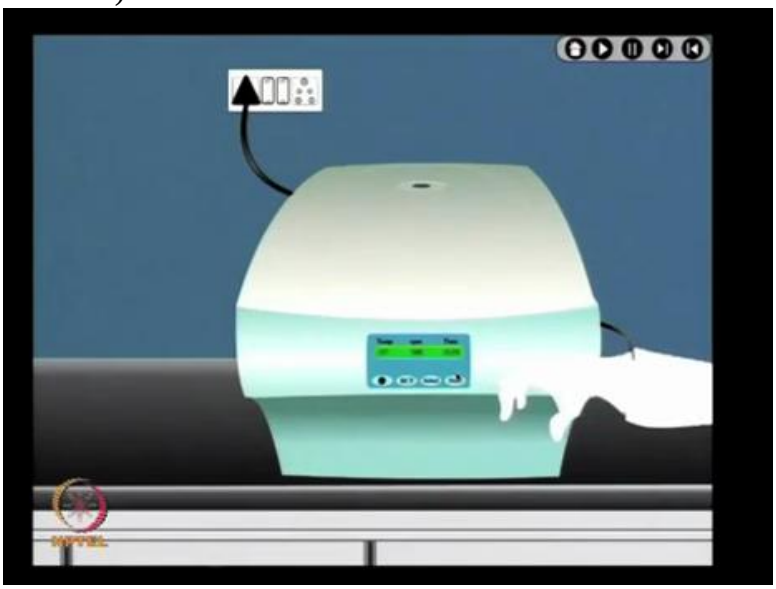

…for 15 minutes at 4 degrees.

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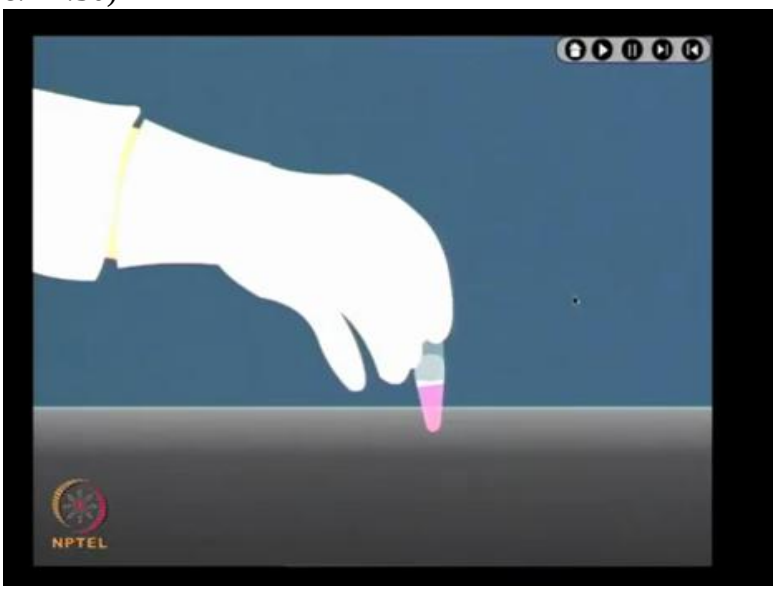

As I mentioned earlier, that different protocols one can use for bacterial proteome analysis. Here we are demonstrating the trizol method in which the top pale yellow layer contains RNA, the middle white precipitate having protein and lower phenol layer contains both protein and DNA.

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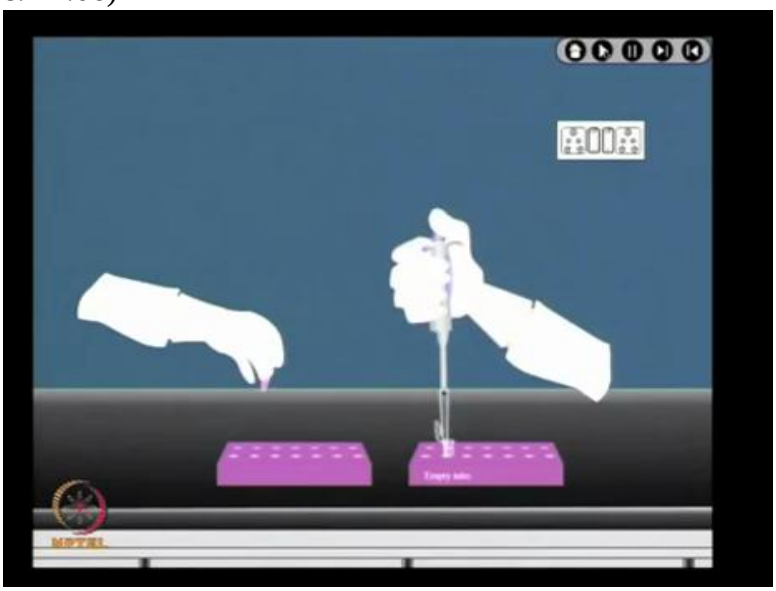

Remove the upper layer and use it for RNA isolation using isopropanol if you want to use RNA for some other applications.

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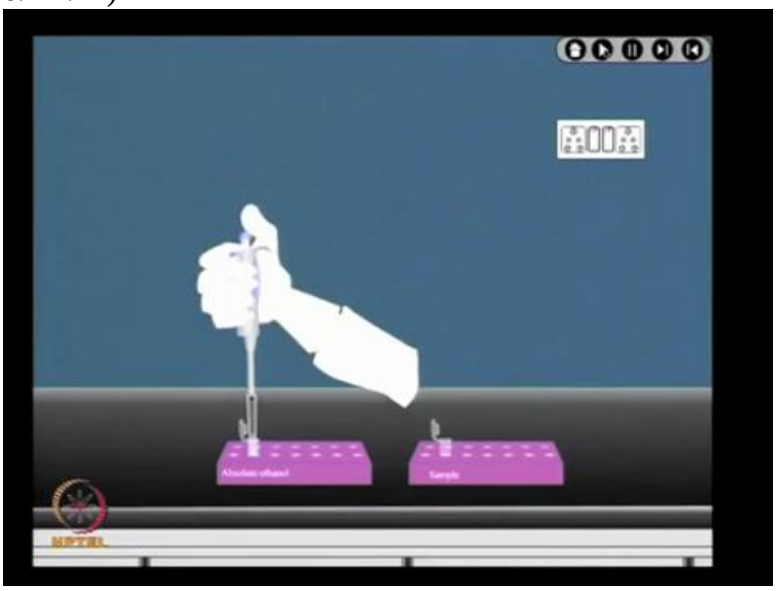

To the bottom layer add 300 micro liters of absolute alcohol per 1 ml of Trizol…

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… and mix gently to suspend the white precipitate and keep at room temperature for 3 minutes.

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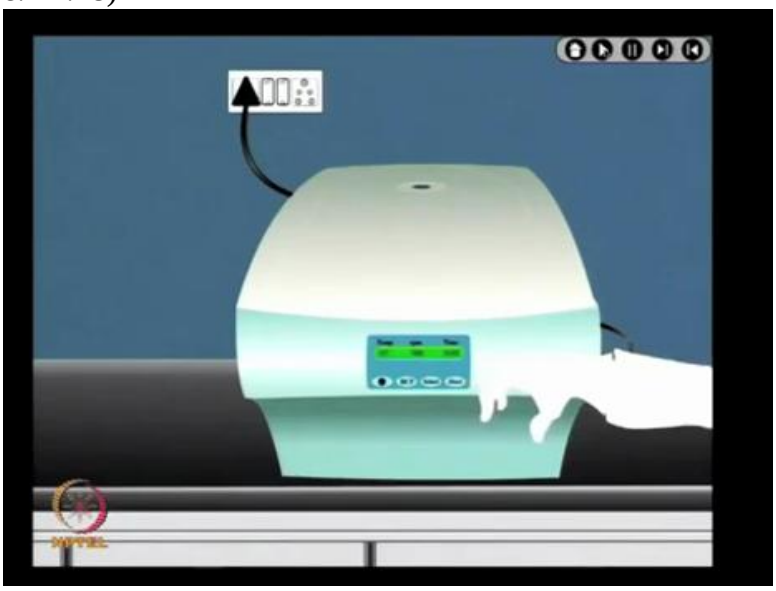

Centrifuge the mixture at 12000 rpm for 15 minutes at 4 degrees to precipitate the DNA.

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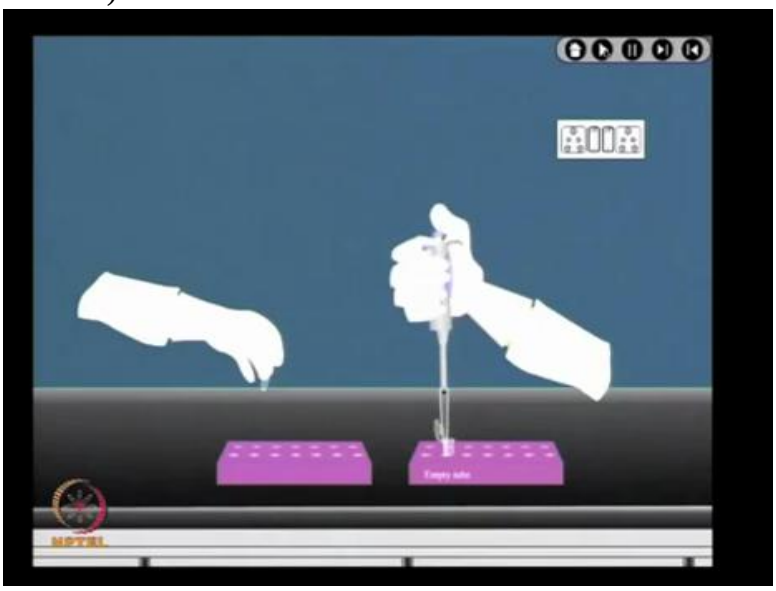

To the clear pink layer, add 4 volumes of chilled acetone and keep it at -20 degrees for 20 minutes.

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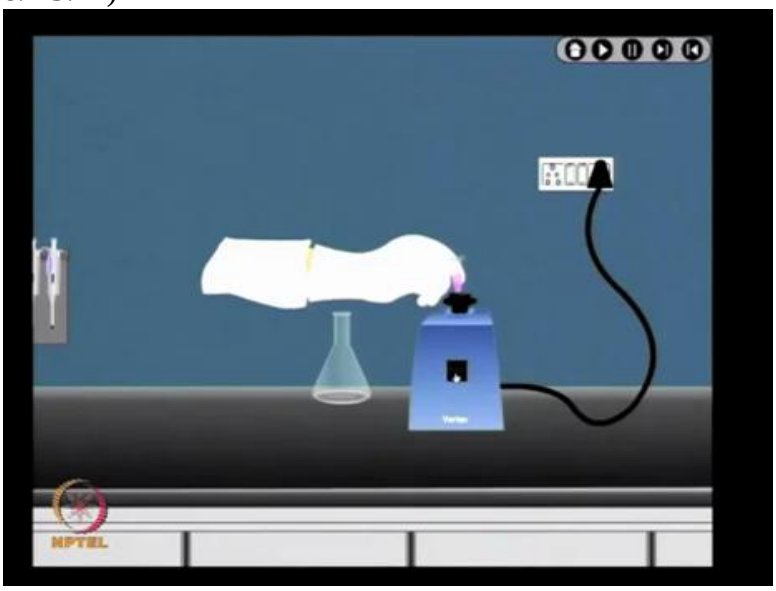

Protein pellet can be washed with 95% ethanol for 3 to 4 times. During the washing steps, you have to also Vortex so that the pellet is properly washed.

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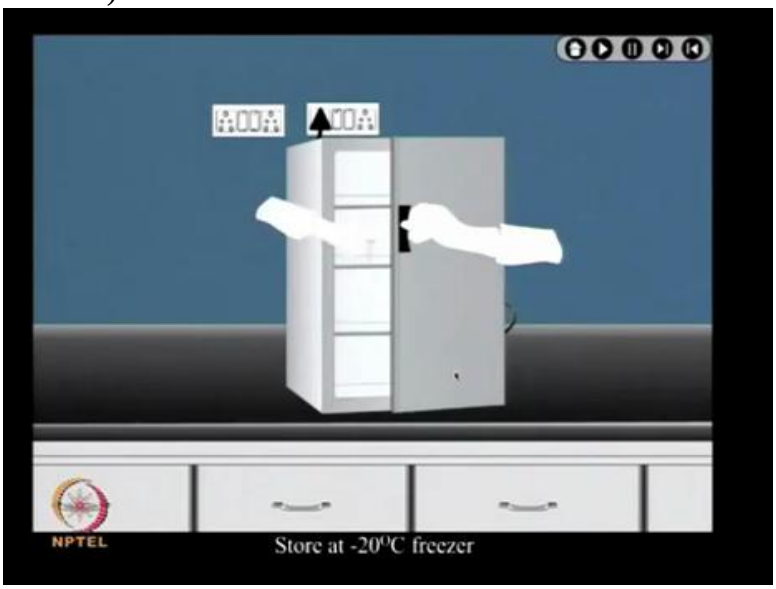

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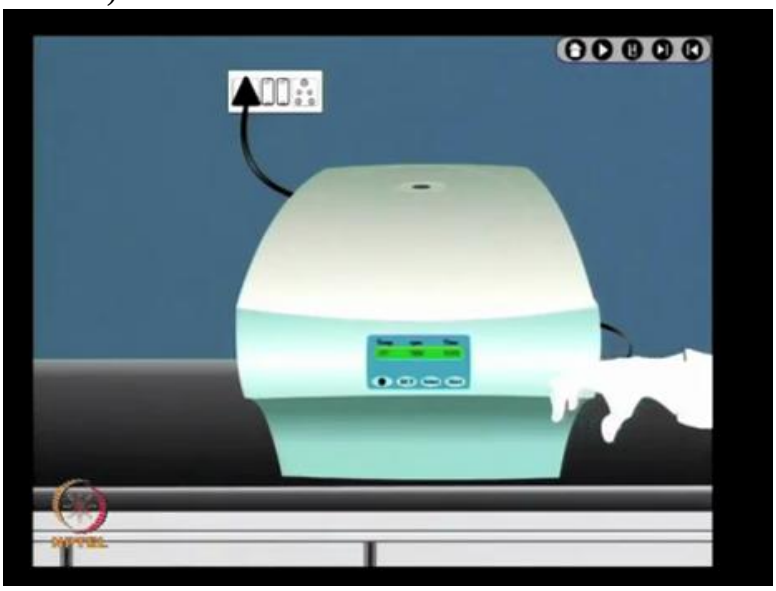

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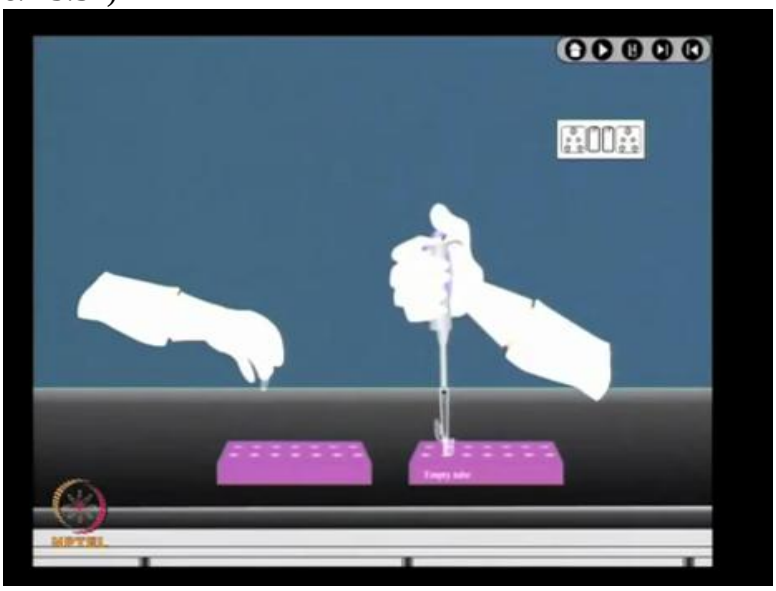

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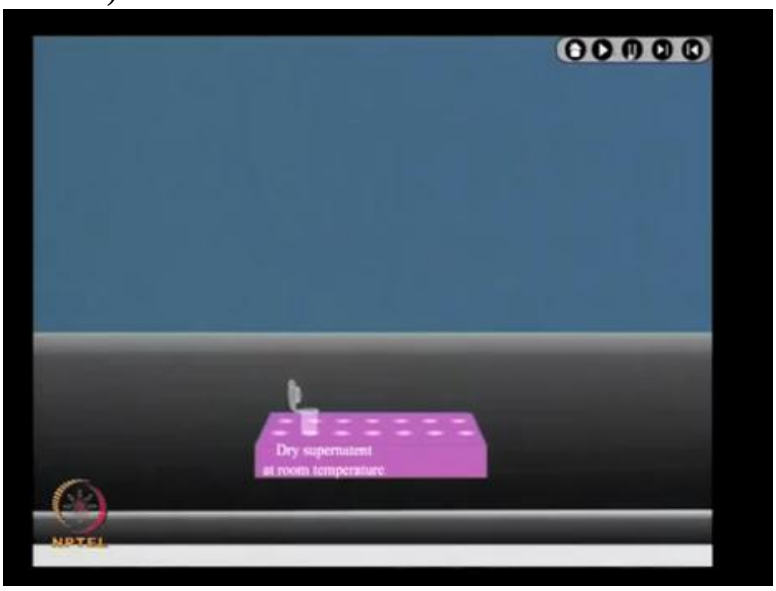

You need to allow the pellet to dry at the room temperature…

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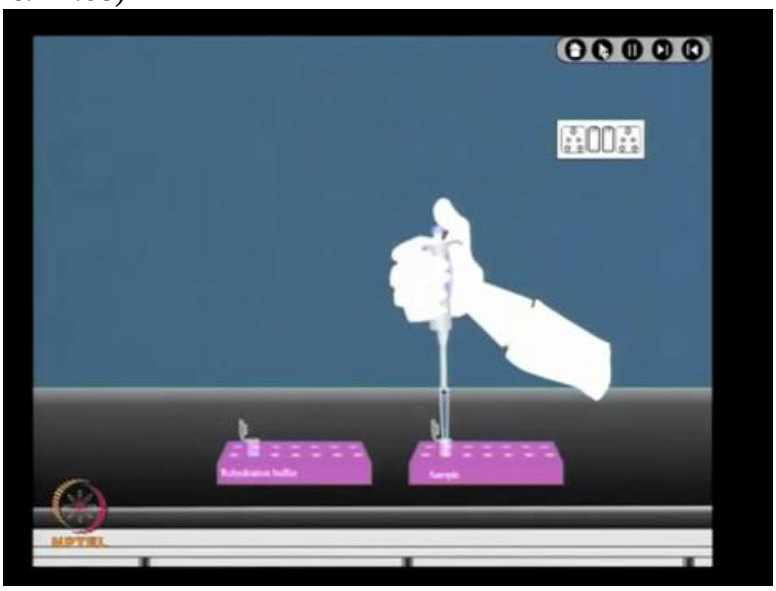

…and then add the lysis buffer containing 7 molar of urea, 2 molar of thiourea….

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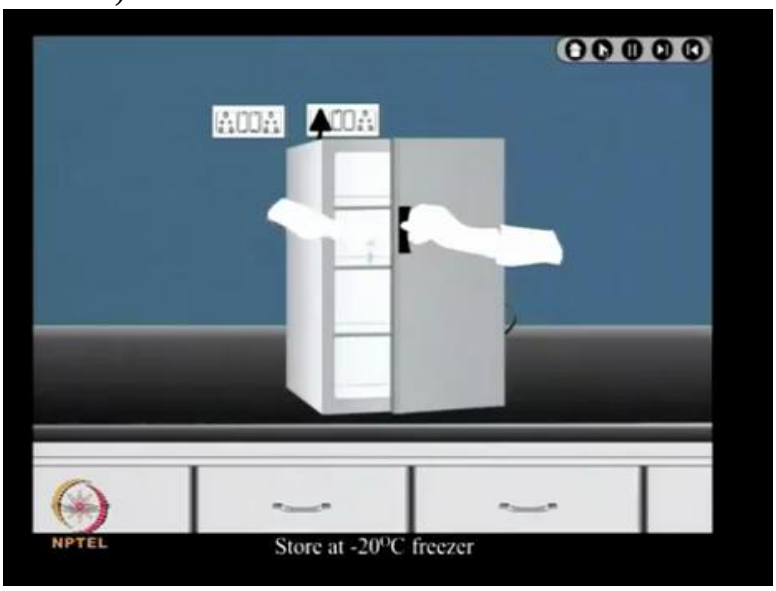

CHAPS, IPG buffer, DTT and No phenol blue. Ok, so I hope this animation was informative.

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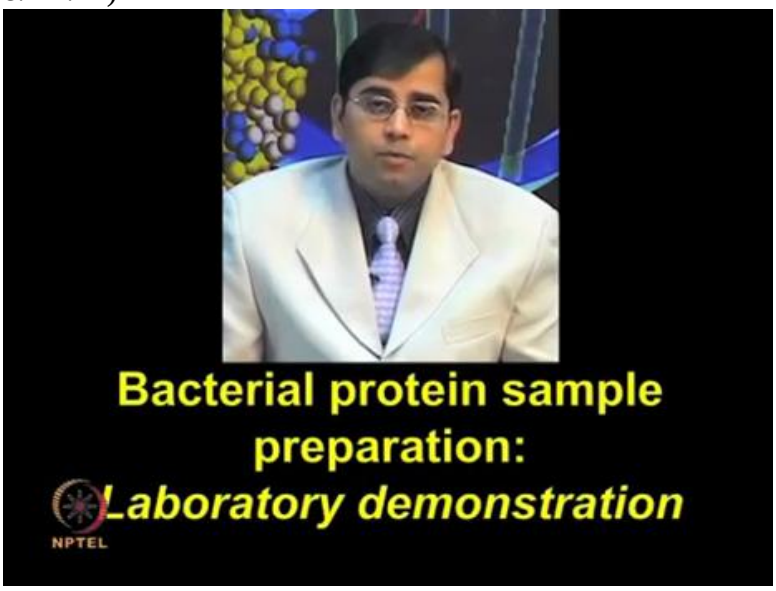

Bacterial protein extraction by using Trizol method and you must appreciate that in this method, you can obtain RNA, DNA and protein all the biomolecules together.

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's watch this video.

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Bacterial proteins extraction

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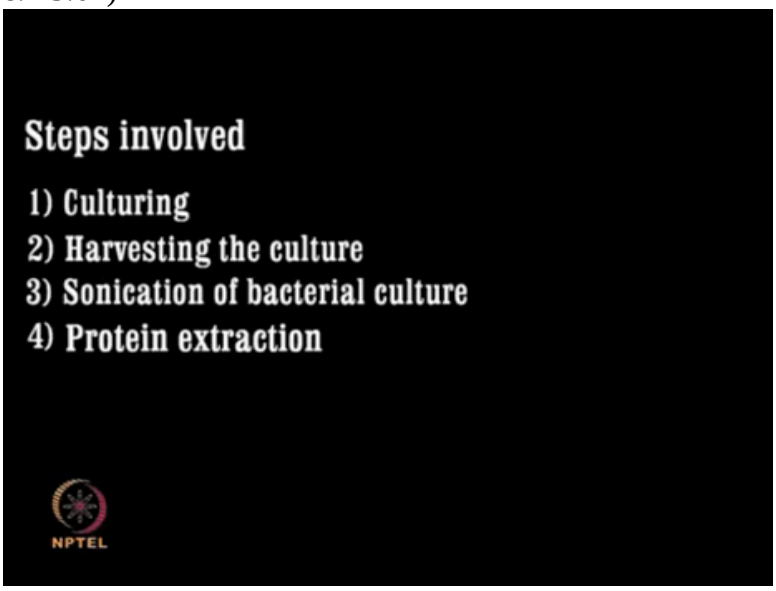

This process involves culturing of bacteria, harvesting and sonification of the obtained culture followed by protein extraction

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Bacterial culturing…

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Clean the laminar workspace thoroughly with ethanol and keep the master plate having bacterial culture ready

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Light the spirit lamp or Bunsen Burner to maintain sterile and aseptic conditions throughout the process.

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Carefully remove a bacterial colony from the master plate….

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…and inoculate…

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… the autoclaved growth media

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Incubate this inoculated sample at 37 degrees Celsius for 6 to 8 hours…

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…with constant shaking to allow the bacteria to grow. The turbidity of the culture which gradually increases indicates bacterial growth.

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Culture harvesting

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Transfer the known bacterial culture to a fresh tube under sterile conditions.

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Centrifuge this tube…

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…at 12000 rpm for 10 minutes maintaining a temperature of 4 degrees Celsius

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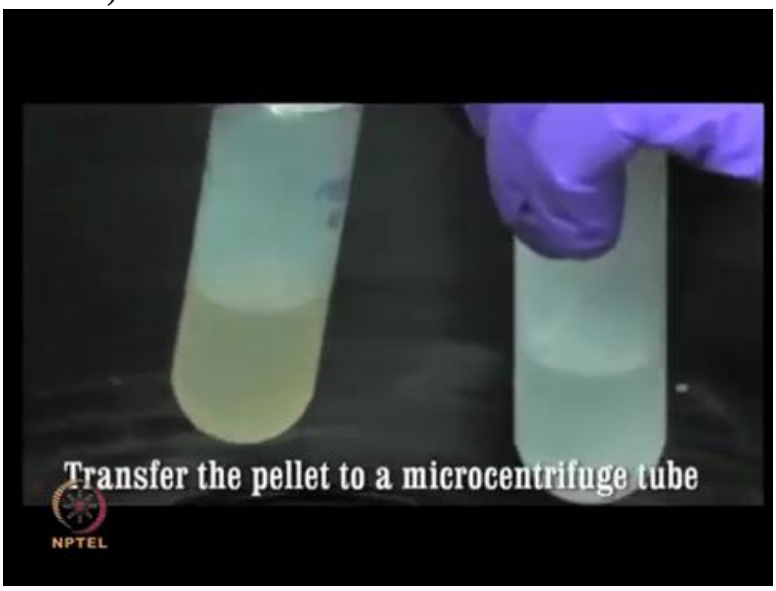

Transfer the pellet obtained containing intact bacterial cells…

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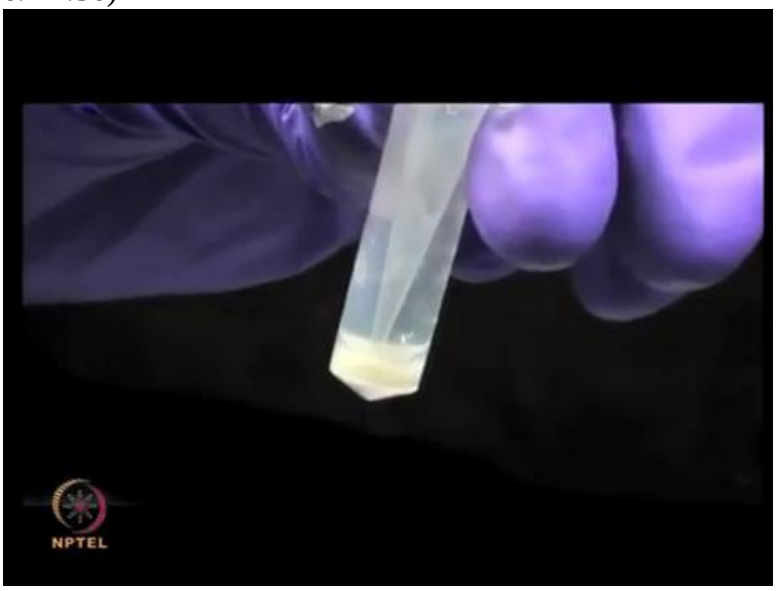

…to a fresh, micro-centrifuged tube. Wash this pellet thoroughly with phosphate buffer to remove any unwanted debris.

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Sonication

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The re-suspended pellet is sonicated on ice to enable the bacterial cells to break open ….

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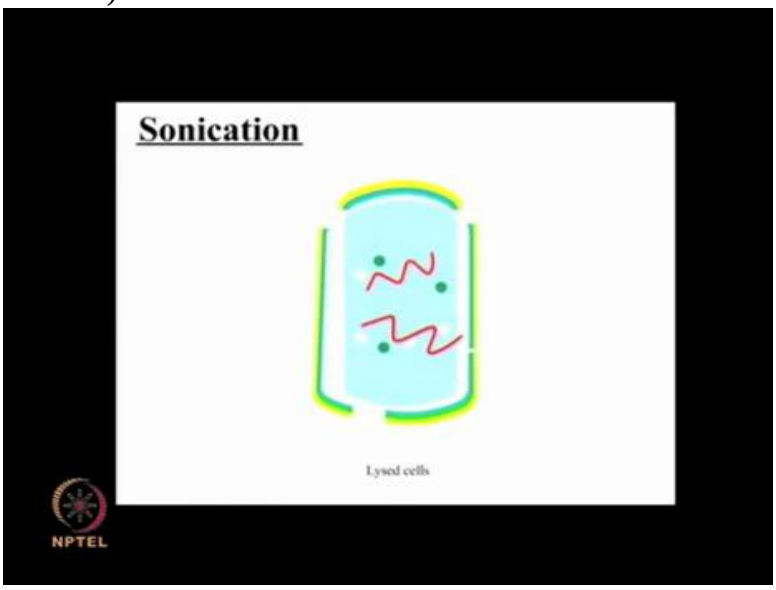

…so that its contents are released. Sonication involves the use of high energy sound waves that are capable of breaking open the outer membranes of cells. All cellular contents including proteins of interest leak out of this disrupted membrane.

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Carry out the sonication procedure for 30 seconds…

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…with a pulse of 1 second at 40% amplitude

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Once it is complete, centrifuge the contents…

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And collect the supernatant that is obtained.

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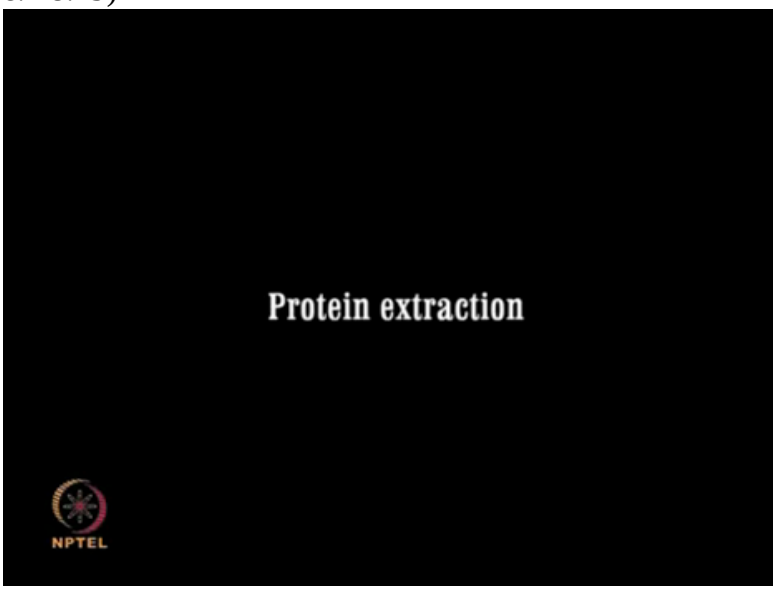

Protein Extraction

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The Trizol extraction protocol allows …

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…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.

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Mix the contents thoroughly by Vortexing.

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Next, add chloroform to this solution, mix the contents…

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… And place the tube on ice for few minutes.

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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 interface containing proteins and the bottom layer is organic and consists of DNA

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Discard the transparent top layer having the RNA.

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Then add…

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Absolute alcohol to the remaining layers…

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… And mix the solutions well.

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Centrifuge the contents at 2000 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.

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Collect the supernatant in the fresh tube.

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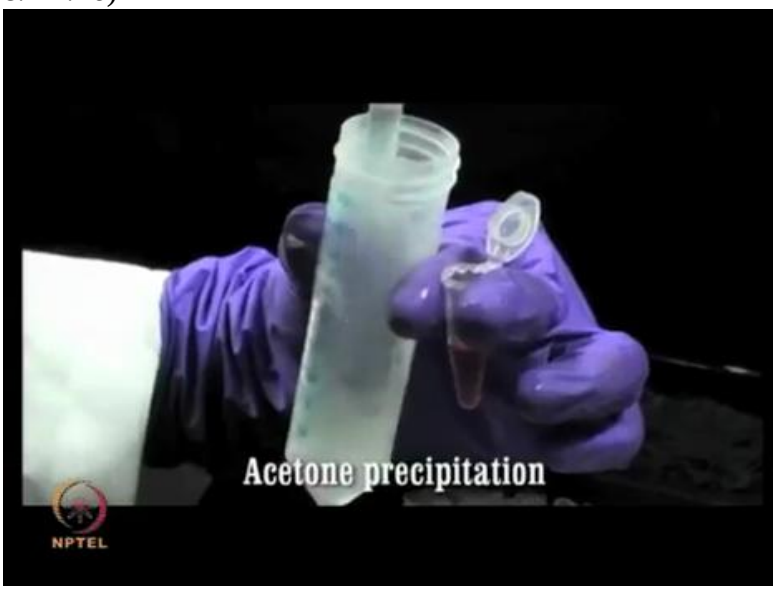

Then add chilled acetone to this tube…

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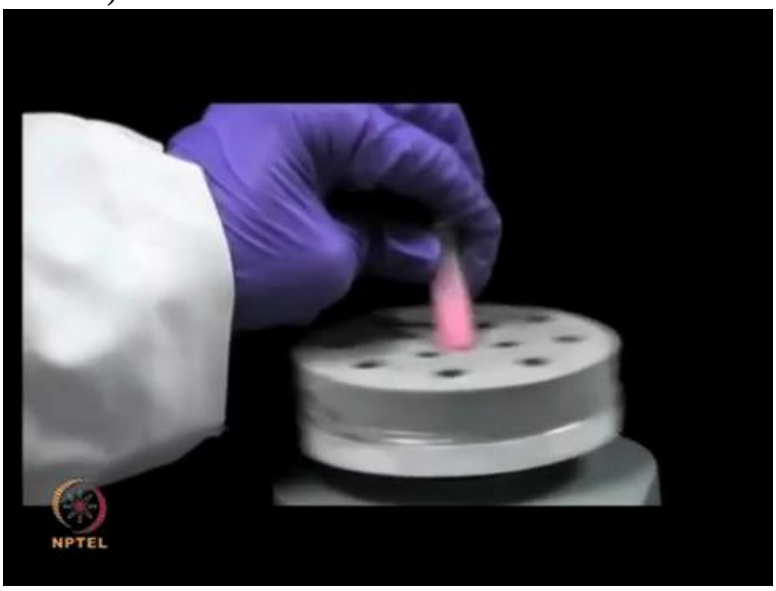

… And mix well by Vortexing.

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Store the solution at -20 degrees Celsius for at least an hour…

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Before centrifuging it, to obtain the protein pellet…

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Discard the supernatant and….

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…dry the pellet at room temperature

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Reconstitute the dried pellet with rehydrated buffer and store overnight

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… at -20 degrees Celsius before carrying out protein quantification.

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Ok, so I hope the 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 types 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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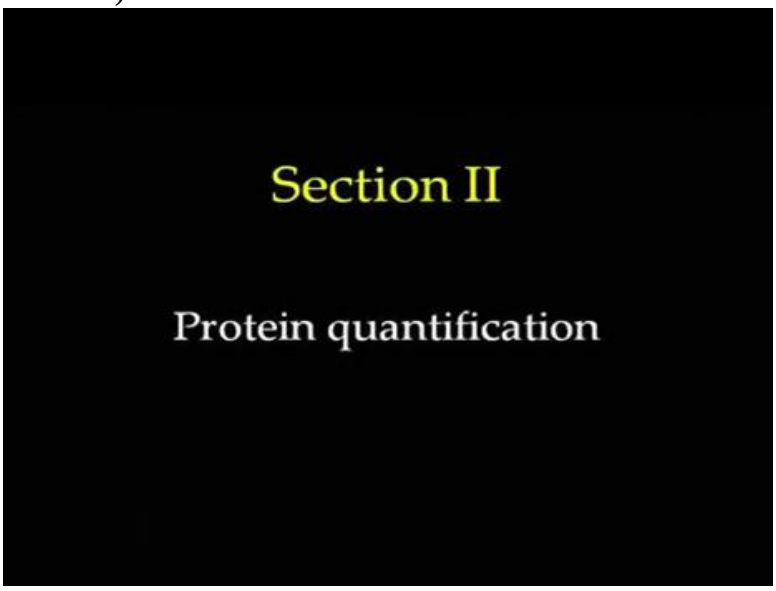

So now, let's move on to the next topic which is protein quantification. So by now, you have prepared your protein sample by using different types of methods, by keeping a very careful eye on different types of interfering substances and contaminants and you tried to eliminate all of those components.

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.

So the protein concentration determination by the UV absorption; that is one of the very commonly used methods, in fact the oldest method used to determine the protein concentration by the absorbance at 280 nanometers.

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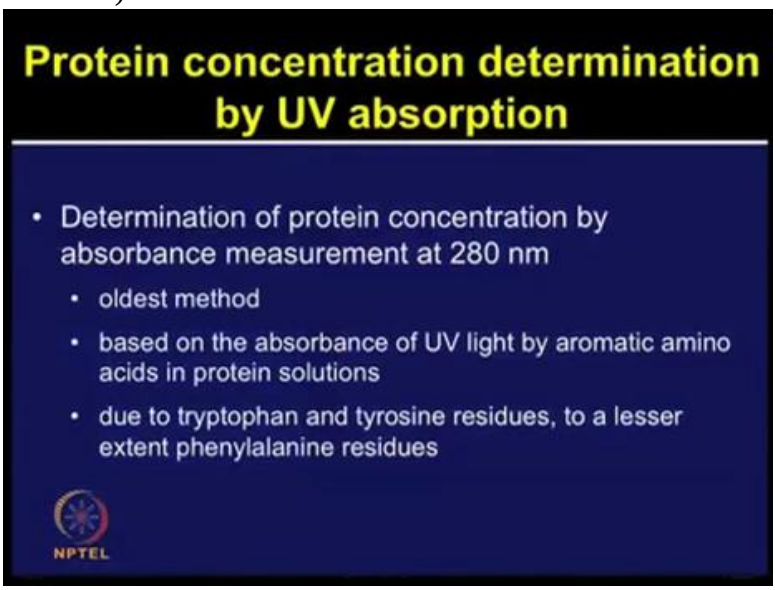

This method is based on the absorbance of Ultra Violet by aromatic amino acids such as Tryptophan and Tyrosine residues which are present in the protein solutions. Phenylalanine, it is also aromatic amino acid but up to a lesser extent it is responsible for this absorbance. So to determine the protein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

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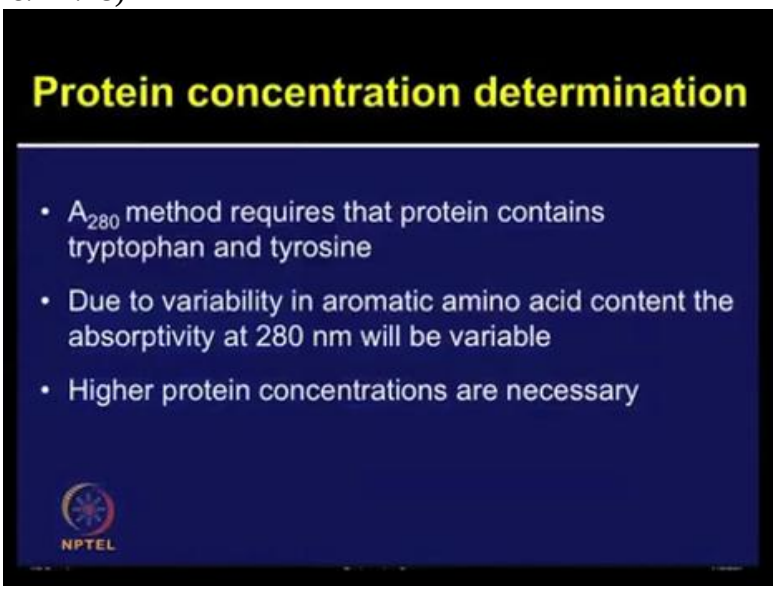

Since the protein samples will have variability in overall amino acid contents, so the absorptivity at 280 nanometers will be a variable factor for this type of method. Also if you need very high protein concentration for your protein preparation, for your proteomic applications, then this method is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

So, high 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 limitations. One, the aromatic residues have to be there to obtain the absorption at 280 nanometers and the protein concentration should be in very high amount.

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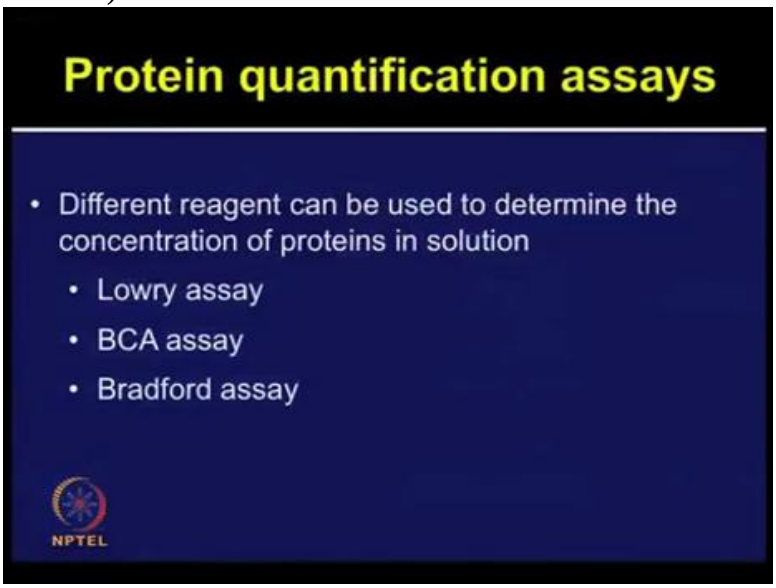

To overcome these limitations, there are different reagents, different quantification assays have emerged and these reagents can be used to determine the concentration of proteins in a given solution. These methods include Lowry assay, BCA assay and Bradford assay. There are several other assays as well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

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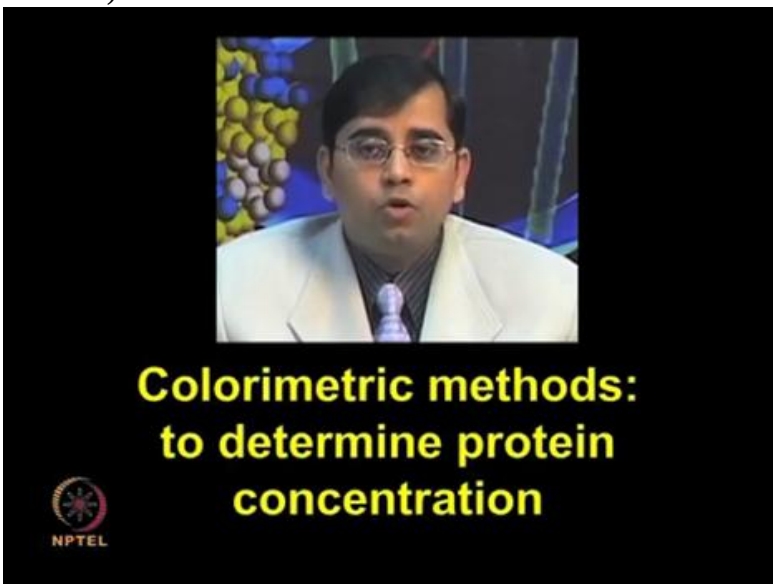

So let's talk about different types of colorimetric methods to determine the protein concentration. So how to perform the quantification…?

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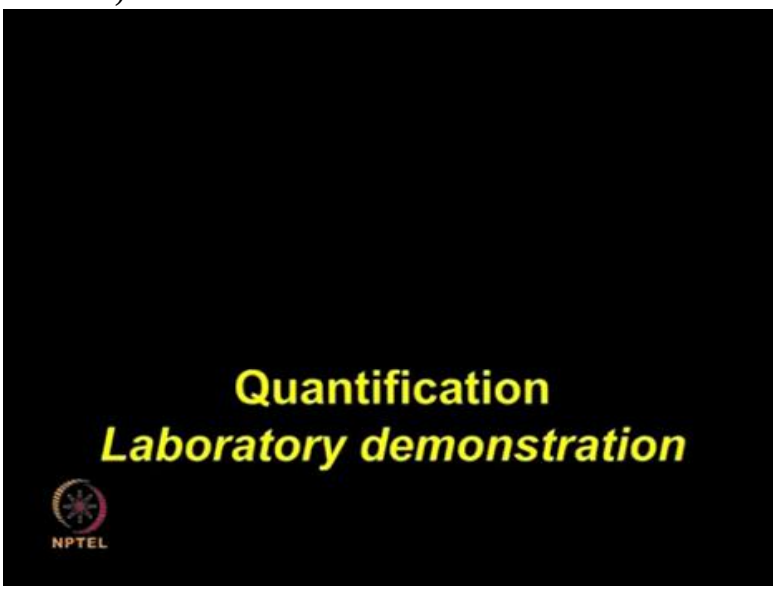

Let me show you one video, a laboratory demonstration of how to perform protein quantification.

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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.

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Label the tubes suitably for standard and test samples.

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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 albumen is often used as a standard.

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Then add the unknown protein sample whose concentration is to be determined to the appropriately labeled tube.

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Dilute all the samples uniformly using the sample buffer.

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Then add the Bradford color reagent to each tube…

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… And mix well.

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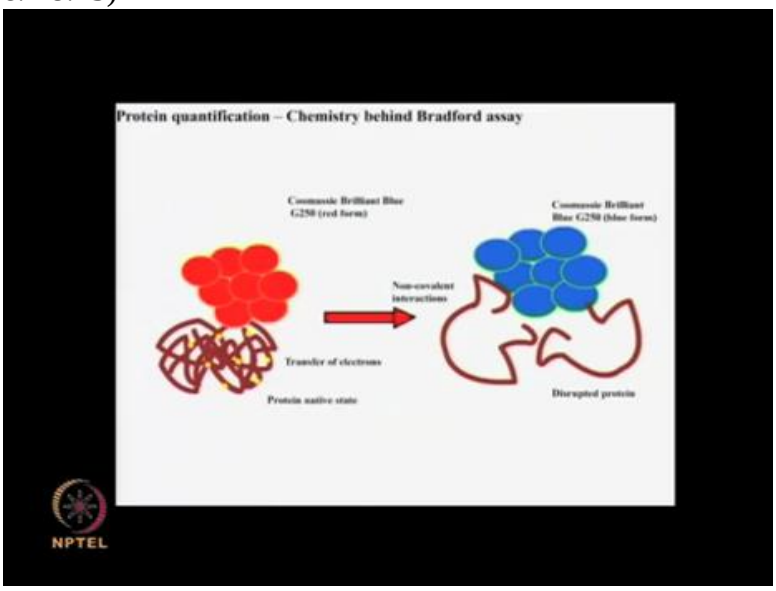

An electron transfer reaction takes place between the red form of Coomassie dye of the reagent and the native protein. This disrupts the protein structure and establishes several non-covalent interactions between the dye and protein. The transfer of electrons converts the dye into its blue form thereby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

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Set the wavelength of the UV spectrophotometer to 595 nanometers.

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Adjust the reading to zero using a blank solution containing only the dye solution and no protein.

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Then measure the absorbance of all the standard protein samples of known concentrations followed by the unknown samples.

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Protein concentration of the unknown can be determined…

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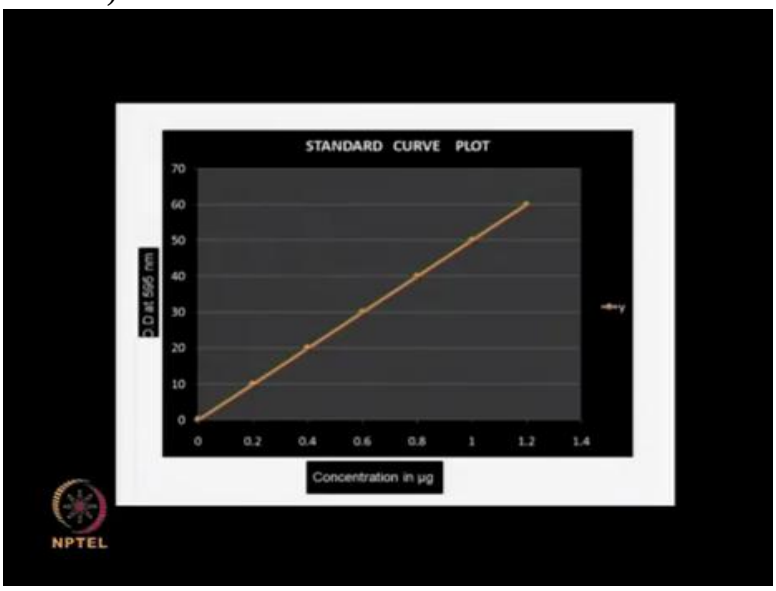

…from the standard curve based on the absorbance value obtained Ok, so I hope it was useful for you to watch the video for protein quantification and you got a feel how to perform the experiment in the laboratory.

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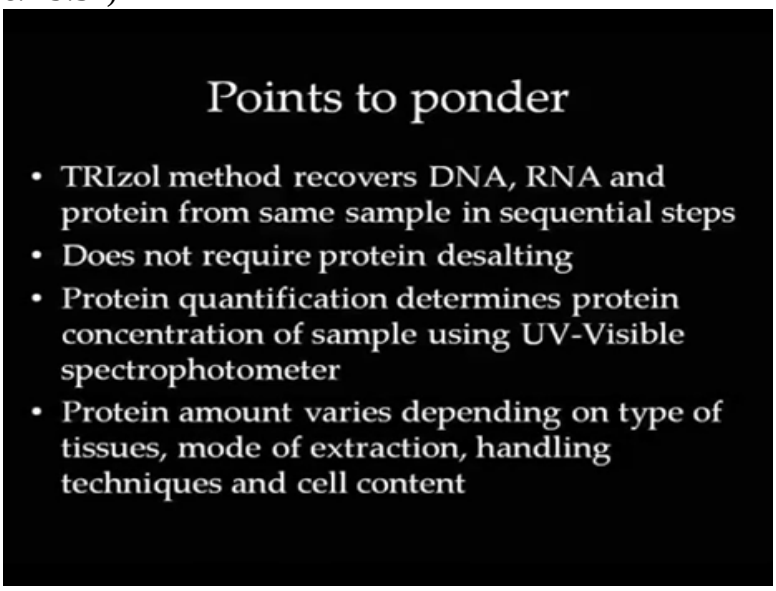

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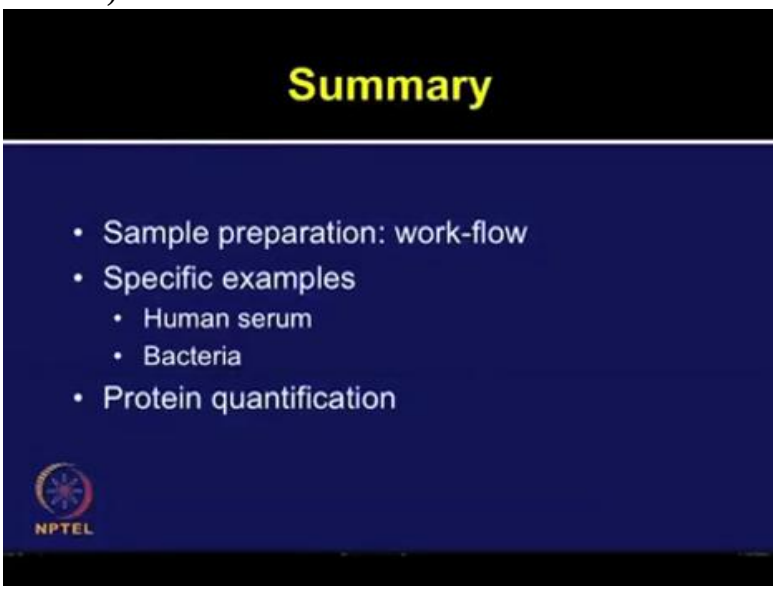

So in summary, in the last 3 lectures, we talked about strategies for sample preparation. I gave you a workflow where we talked about different types of lysis methods. We talked about how to prefactionate the samples, then how to precipitate the samples, how to remove the interfering substances and then we moved on to the specific examples.

We discussed in more details, different types of criteria being used for analysis of clinical samples and then we talked about how to analyze human serum samples. 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 proteomics, and now since you have prepared a good sample, it can be applied either for using the applications on gel-based proteomic approaches such as two-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.