Proteins and Gel-Based Proteomics
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Mod 04 Lecture Number 15

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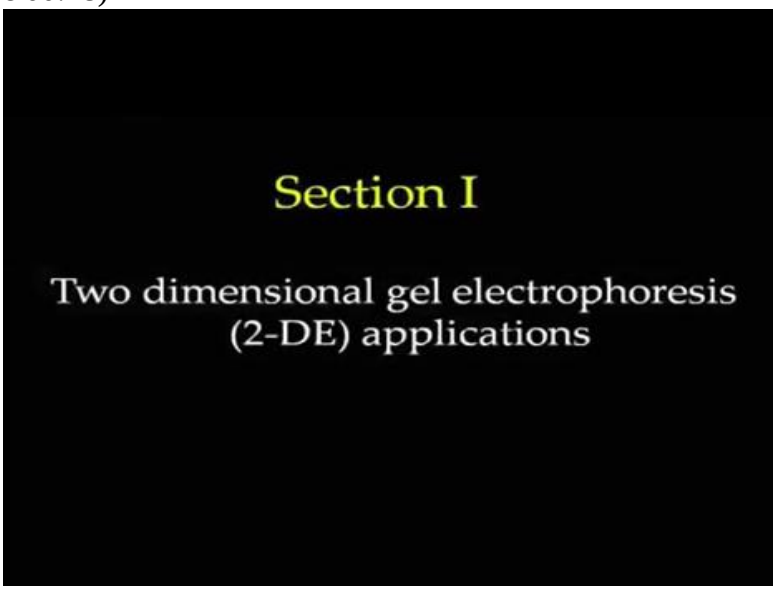

So now, let me give you few applications one can use by applying this technique. So people apply 2D for various objectives

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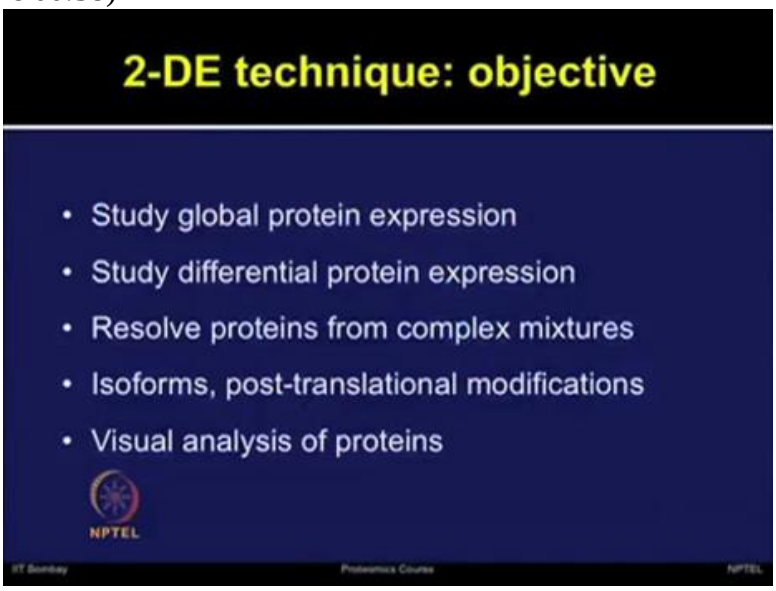

For example studying the global protein expression When I say global protein expression, it means you want to identify or separate all proteins present in a given protein sample. So if you do not have information about all of the proteins which could be present in your sample mixture, then first of all, one needs to create that fingerprints or blueprints of all the proteins and use that by process known as global protein expression.

Second thing which is most commonly being used is you want to compare the protein abundance from a given sample to a treatment sample, so that is known as differential protein expression or also known as abundance based proteomics.

You want to look at the proteins which are either going up-regulated or down-regulated in their expression because of a given treatment or because of a disease condition or because of your experimental conditions. So in both of these cases the two-dimensional electrophoresis can be used to resolve the proteins from very complex mixture.

People also use to study different types of isoforms or post-translational modifications which occur in that process. One of the major advantage of this technique is the visual analysis of proteins.

When you stain the gel after the two-dimensional electrophoresis process, then you can see all the protein spots on the gel which is not possible from the gel-free approaches such as mass spectrometry. Over there most of your analysis depends on your spectra and you have to

rely on your analysis. In this case here, your all the protein spots are already present and you can visualize that and then you can use each of these spots to further analyze and compare the images.

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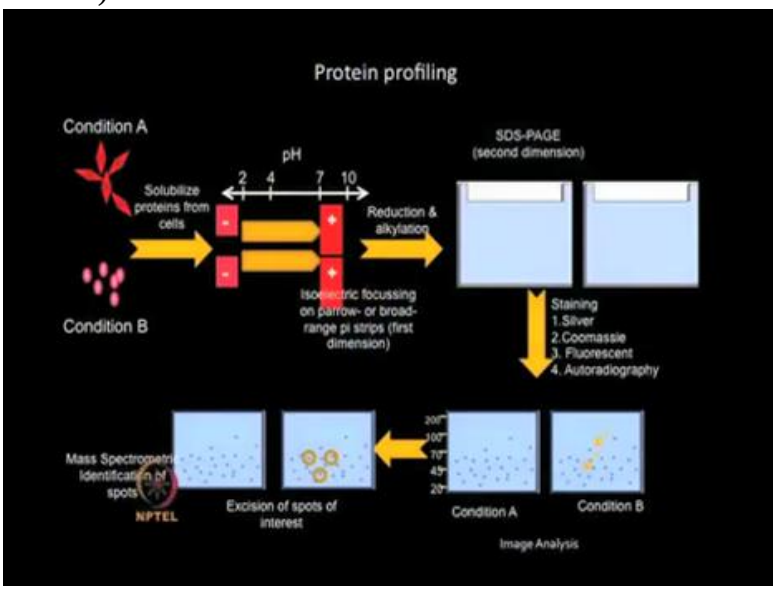

So the protein profiling, if you want to compare different sample types, from condition A to condition B, you need to solubilize the proteins from the cell and separate all the proteins by applying the pH which is shown here in the first dimension, after that you need to reduce and alkylate your samples and then separate that in the SDS PAGE based on the second dimension molecular weight and depending on your staining method being used, whether Coomassie brilliant blue or silver stain, different types of fluorescent stains or autoradiography, you can visualize the protein spots

Now, one can compare these images from Condition a to Condition b and if there are some proteins which are different in their expression, those can be the proteins of your interest.

One needs to analyze that from different types of gels, different replicates both technical and biological and then obtain the statistical information for these spots and if these are significant, then this is the protein of your interest which you would like to identify by using mass spectrometry.

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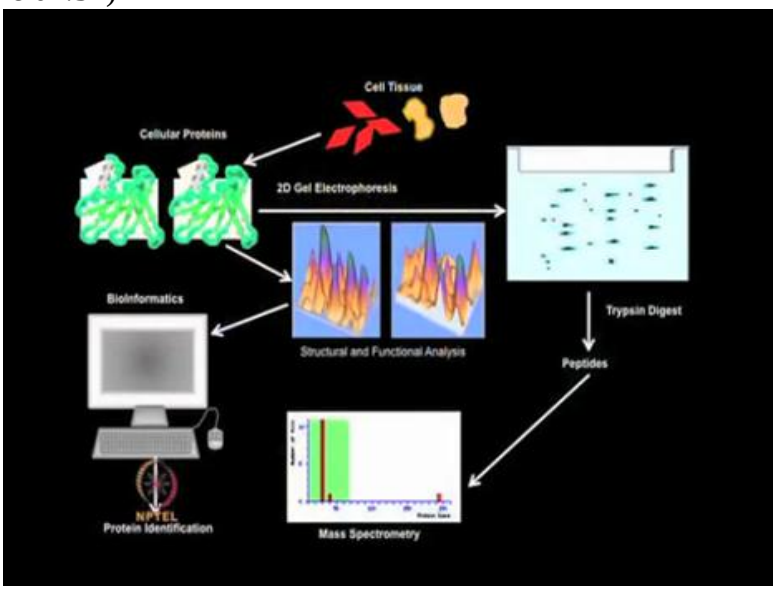

So I am giving you the view of abundance based proteomics or differential expression profiling where you can separate the proteins and you can compare the abundance of each spot as shown in the 3D View, and then a spot of interest could be trypsinized and it can be identified, the peptide spectra can be generated from mass spec.

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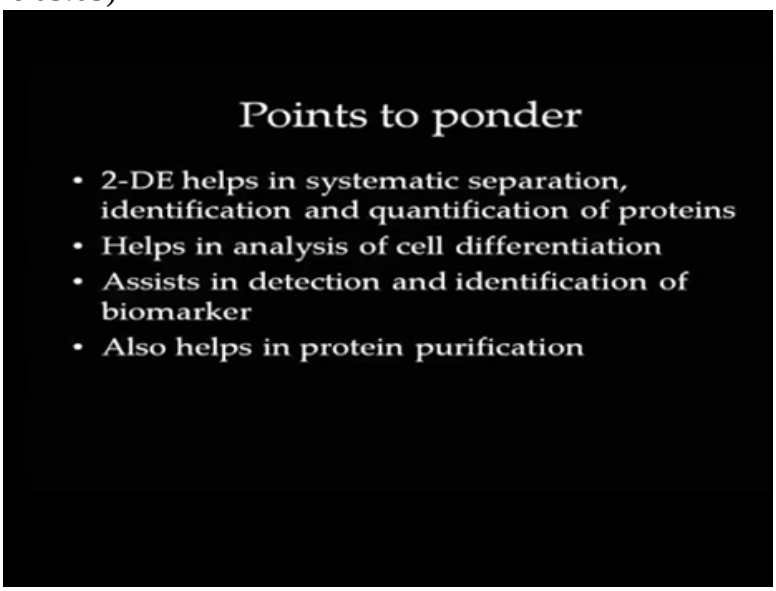

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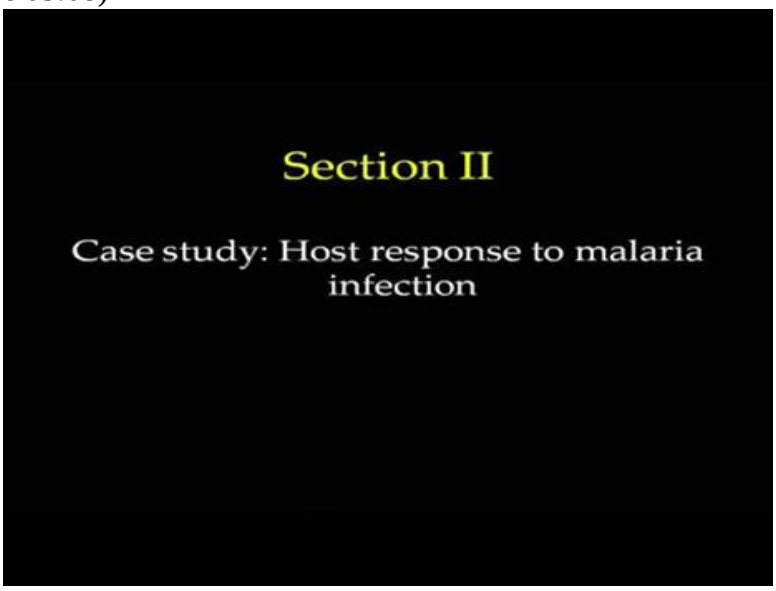

Now let's move on to the case study

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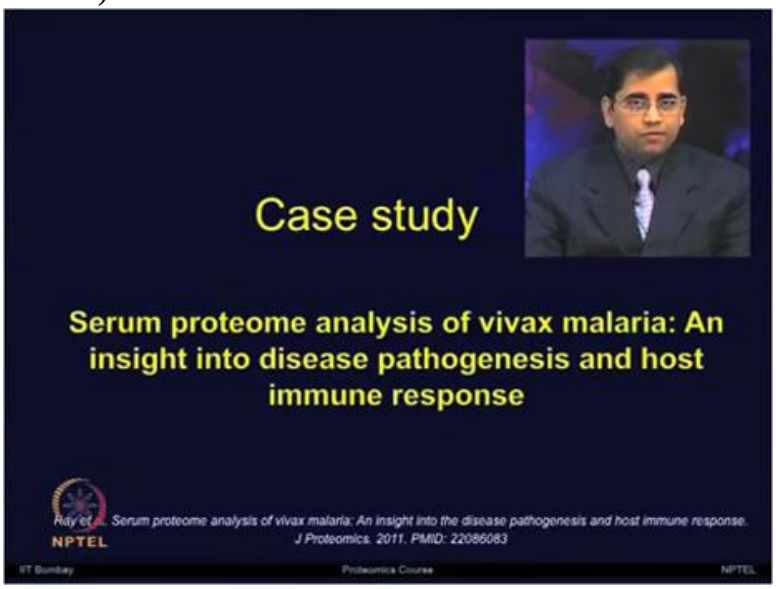

. In first study we will talk about serum proteome analysis of Vivax malaria and insight into disease pathogenesis and host response, study by Ray et al. So the study was performed by my group and we have tried to investigate the host response against malarial pathogen Vivax.

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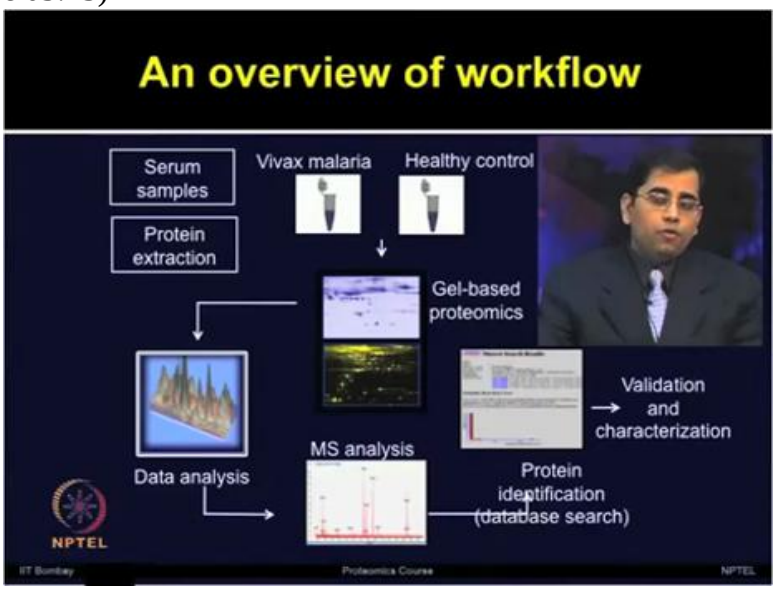

So this slide gives an overview of the workflow which we followed to investigate the host response against Plasmodium vivax in human serum.

Serum samples were obtained, proteins were extracted and the protein samples were compared from the healthy and vivax patients by applying gel-based proteomics including two-dimensional electrophoresis and difference in gel electrophoresis which separated large number of proteins on the gels, compared the data, looked for those proteins which are significantly differentially expressed in Vivax as compared to the healthy controls.

And then those proteins were subjected to mass spectrometry analysis. Further we validated few targets and analyzed the data for various type of pathways which could be involved in such disease.

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So before we move to details of various experiments performed in this study, let me show you this animation to give you an overview of the workflow followed in this experiment.

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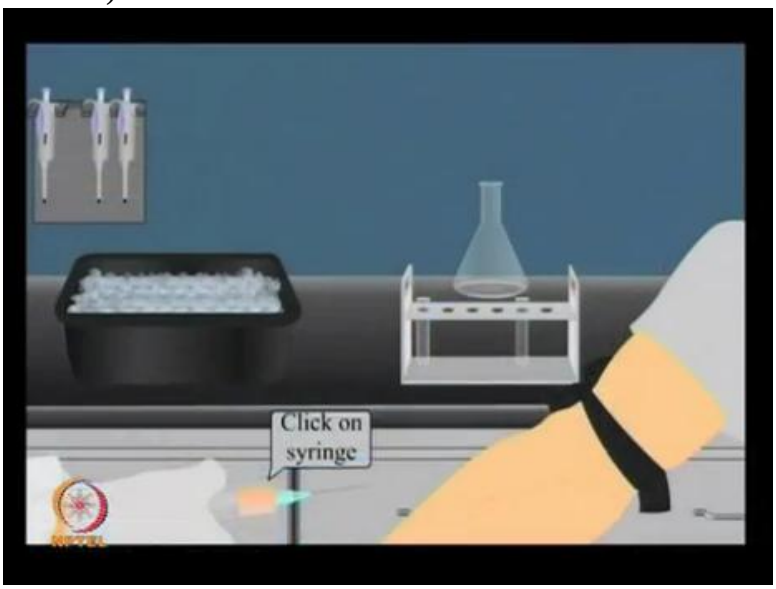

So let me give you an overview of all the steps involved …

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… in performing such an experiment So first of all, carefully withdraw the intravenous blood

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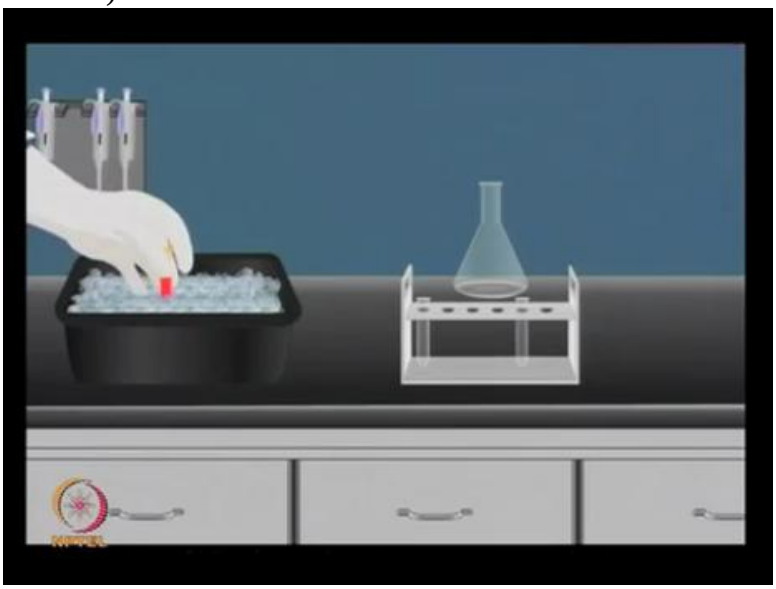

… into a vacutainer tube and store the tube on ice to allow the blood to coagulate.

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Centrifuge the contents to separate coagulated blood cells and clotting factors

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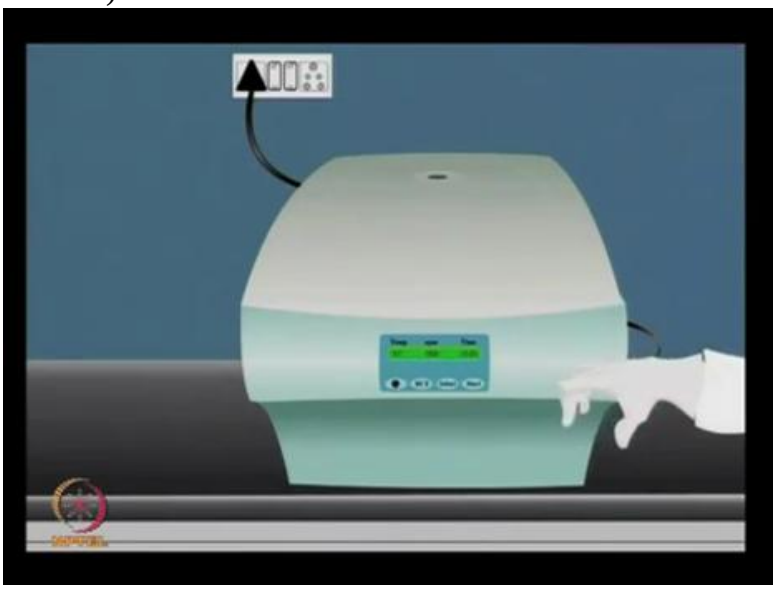

… from the serum which forms the clear supernatant After centrifugation is done, then collect the obtained serum in the fresh tube.

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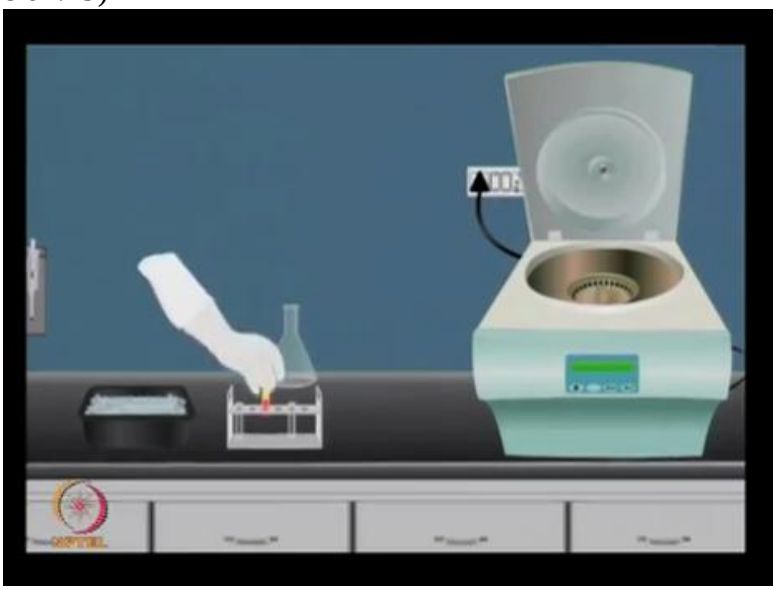

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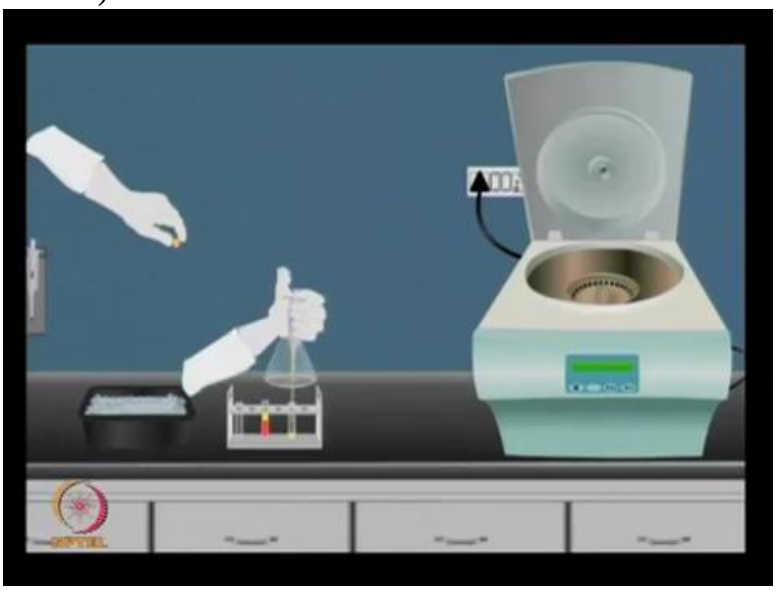

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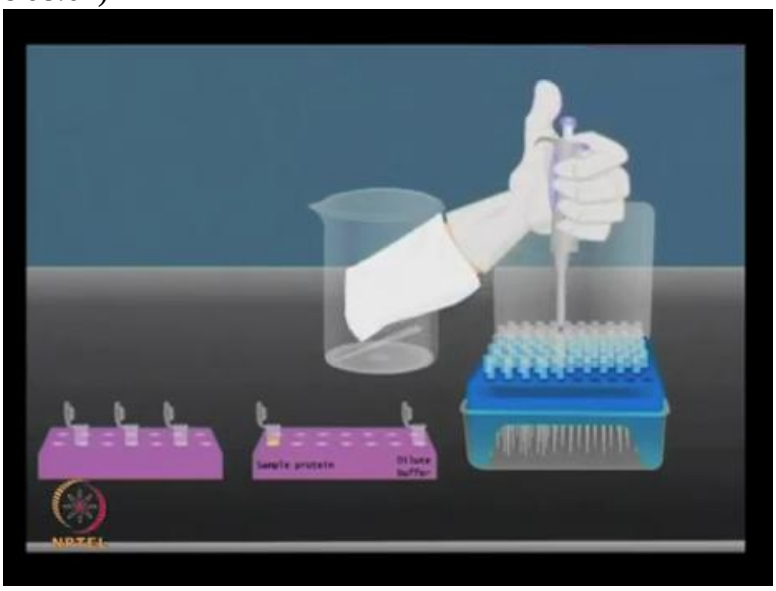

Sonicate the serum to break down any large protein complexes.

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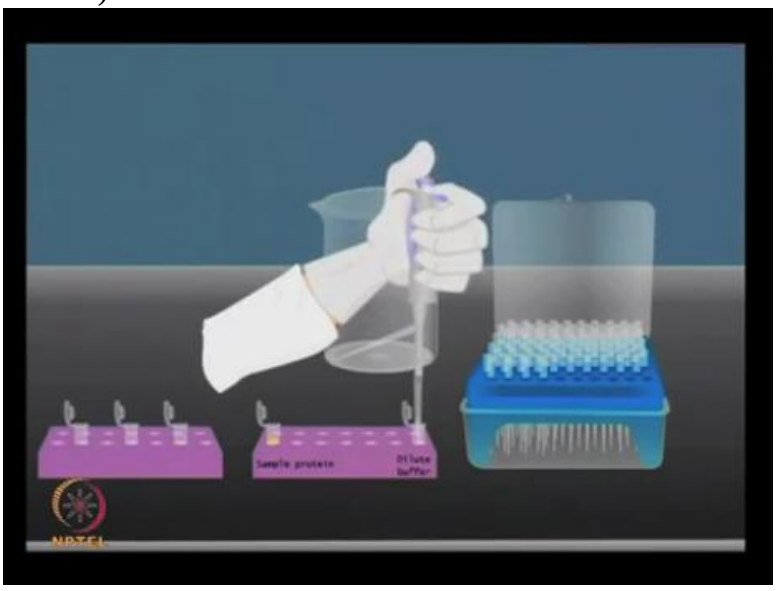

Serum contains several proteins in wide range of concentrations of which

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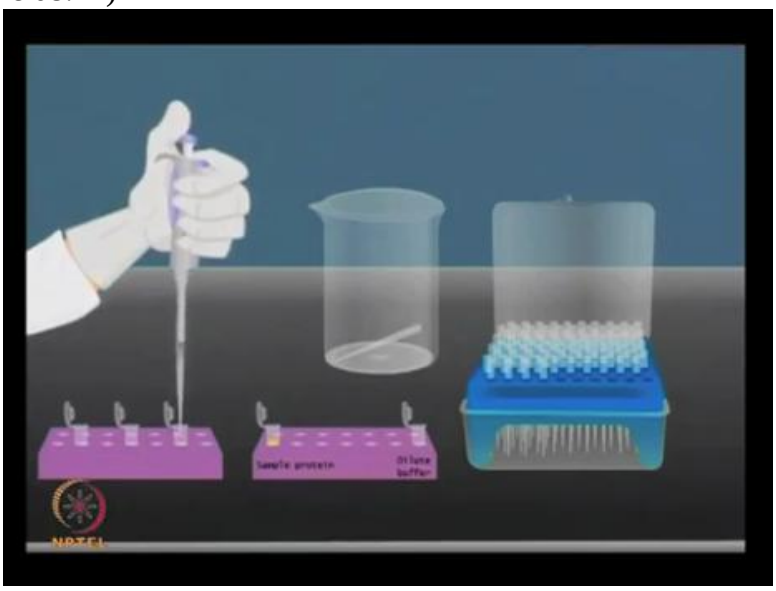

… albumen and immunoglobulin G are the most abundant ones.

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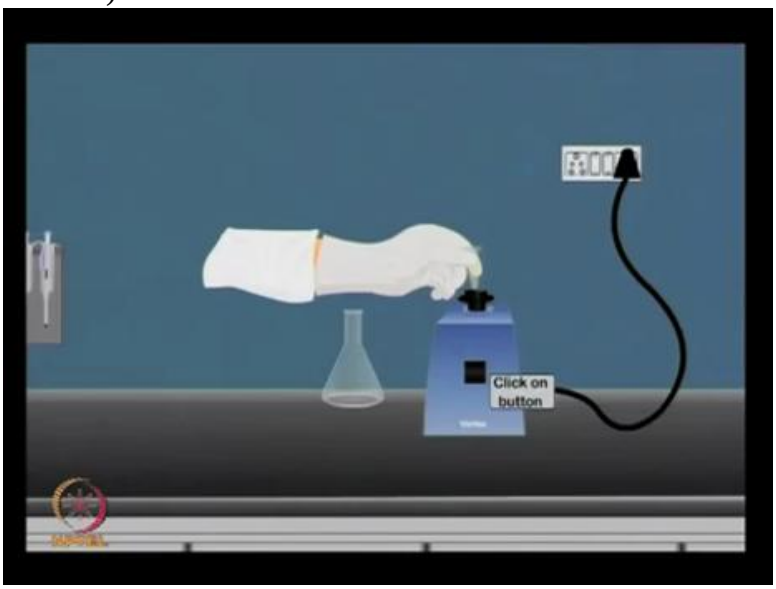

So presence of such high abundance proteins can interfere with the experiment, therefore we remove...we need to remove these high abundant proteins.

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So this experiment we tried both sonication and depletion of the high abundant proteins.

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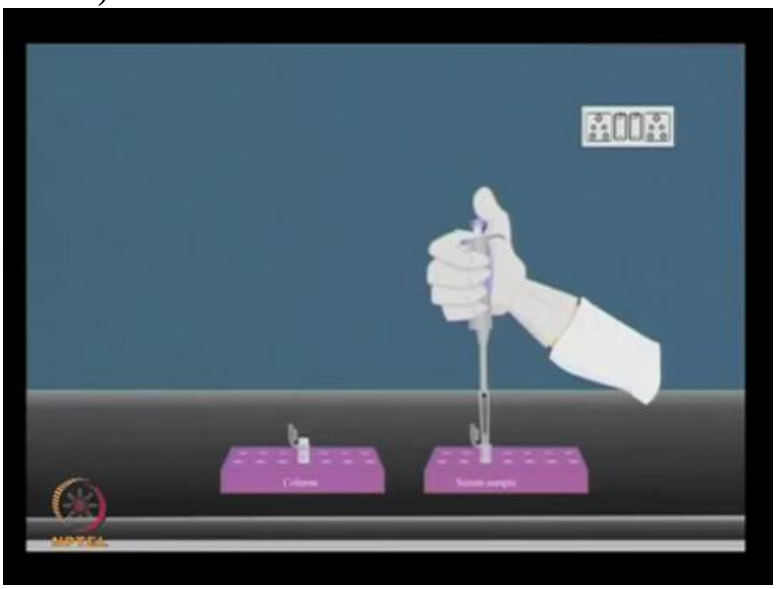

After sonication is done, then we can add

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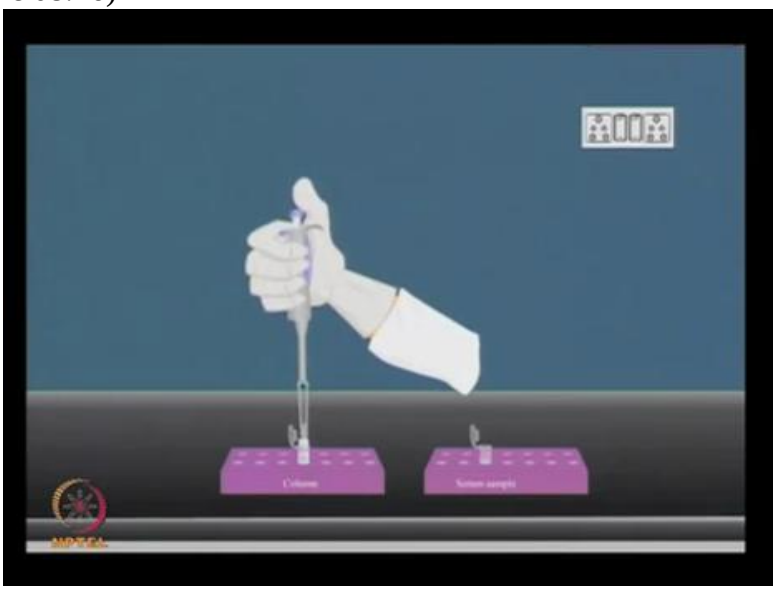

… the serum sample on the columns. Usually these are commercially available …

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…which can deplete various high abundant proteins

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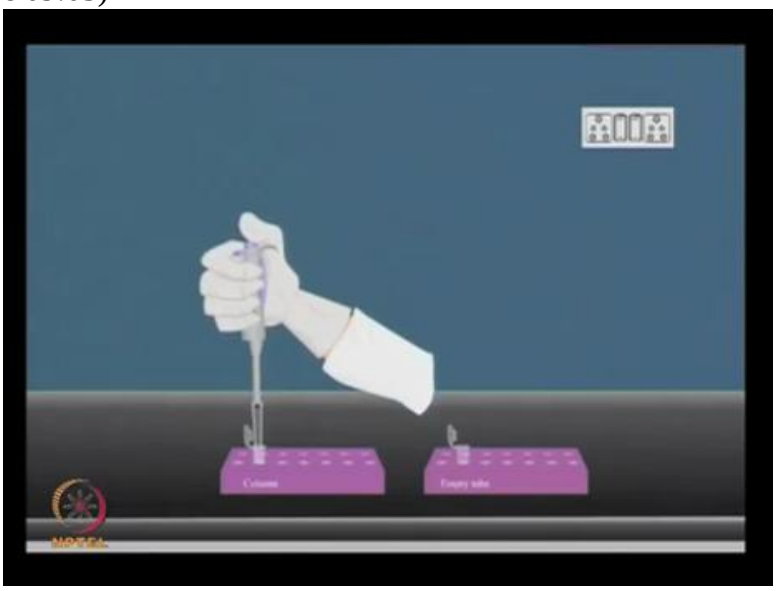

Now these depletion columns bind only the high abundance proteins on to their matrix

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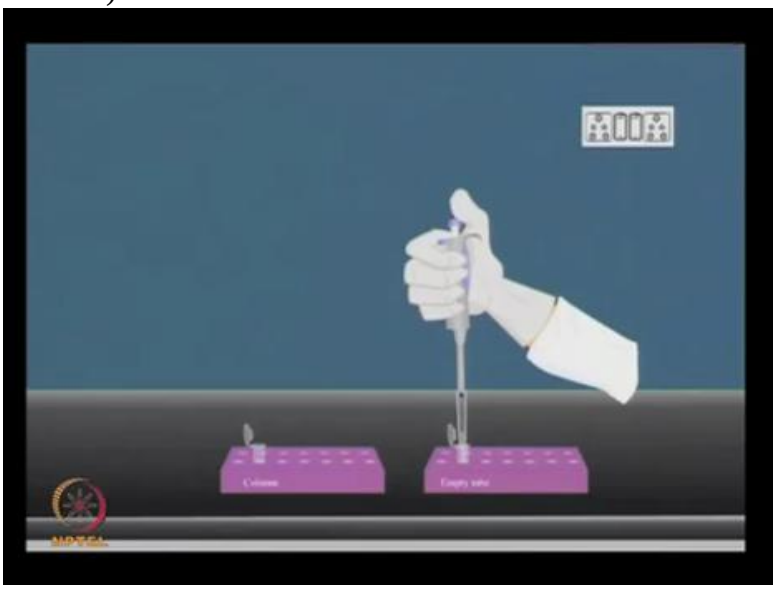

…through the affinity interactions Once the serum has been processed using a depletion column

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… precipitate out the remaining proteins by using Trichloro acetic acid and acetone.

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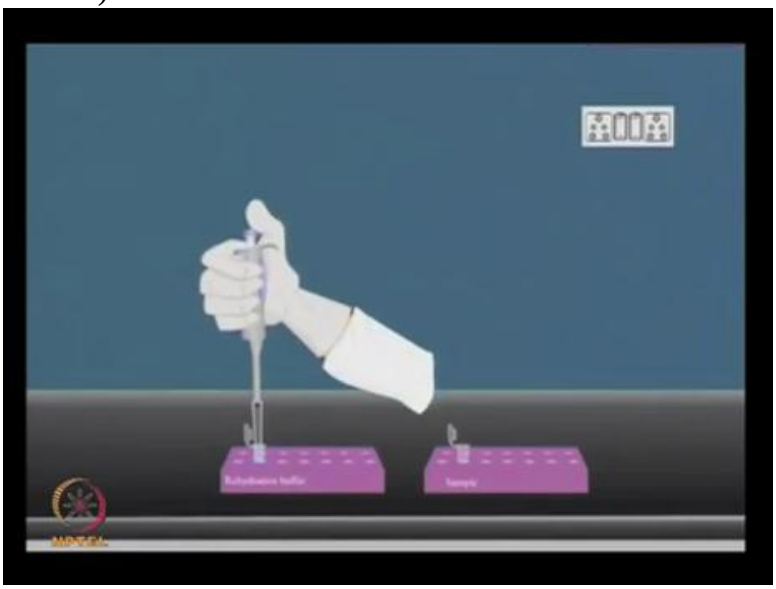

Once the protein extraction is done, then we need to add …

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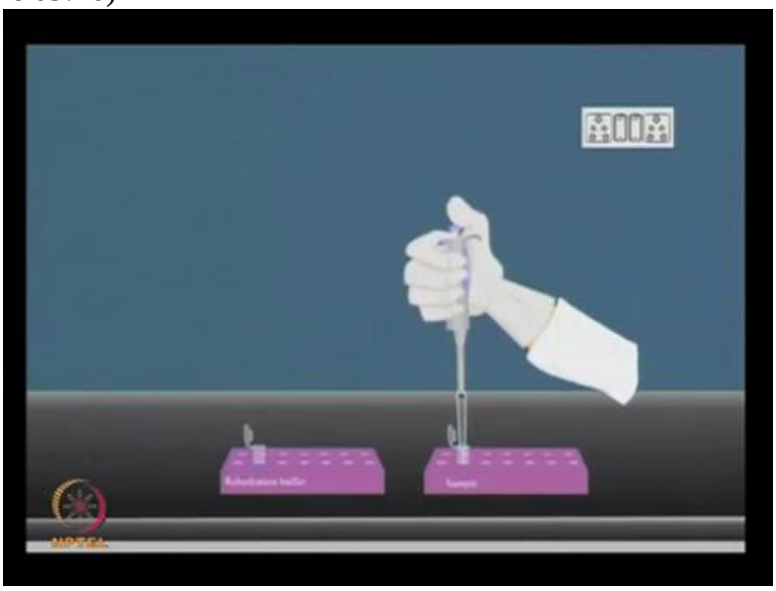

…the dehydration buffer which contains urea, thiourea….

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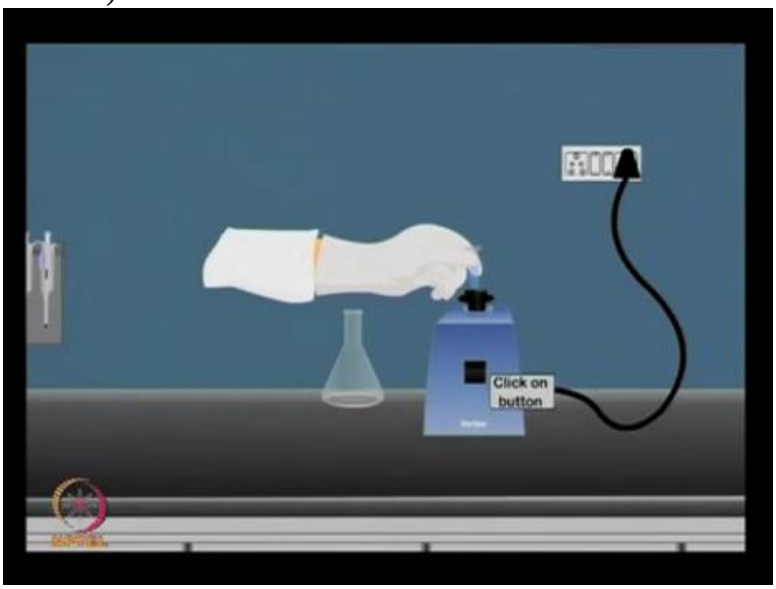

… CHAPS, DTT and BPB

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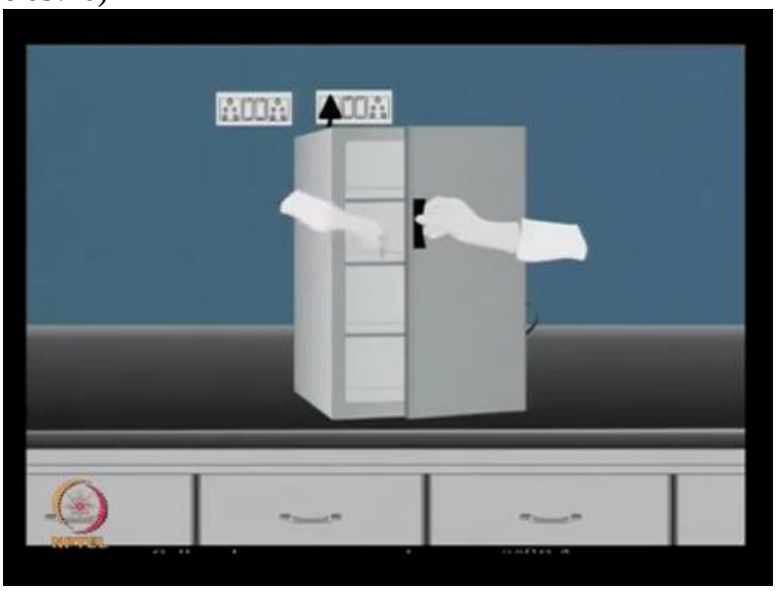

After overnight incubation, then we can centrifuge and collect the supernatant.

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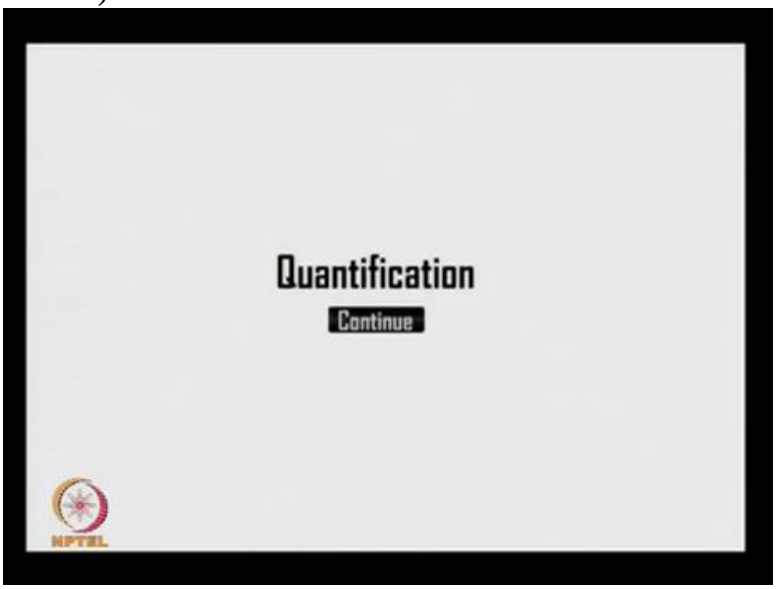

Now we watch the quantification of the proteins.

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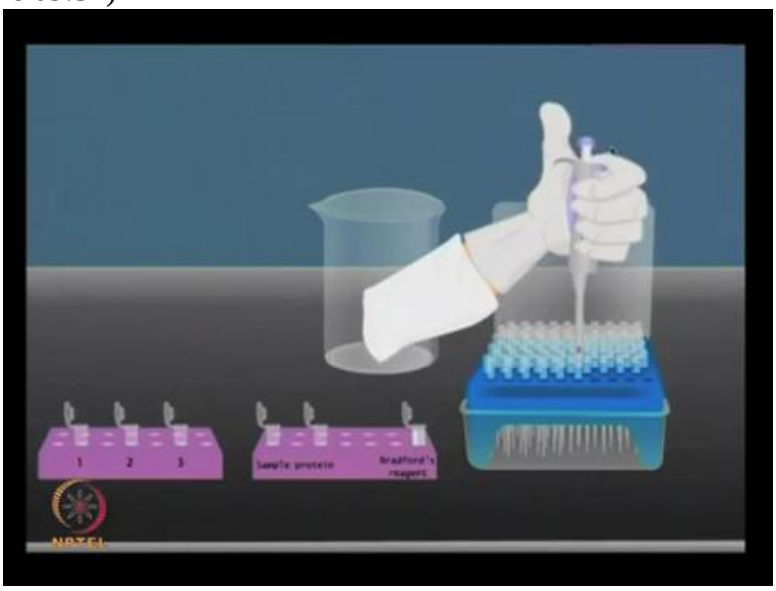

Prepare the Bradford Dye Reagent and label the tubes suitably for the standard and …

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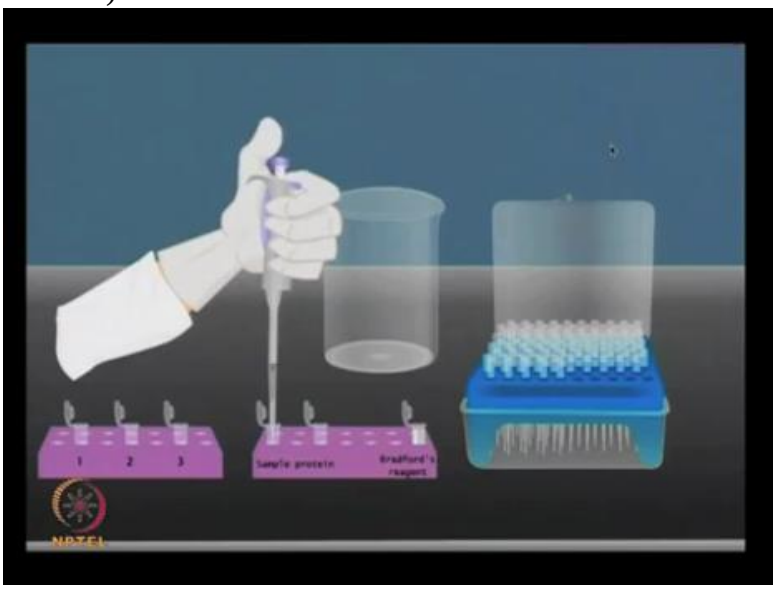

.. the test samples

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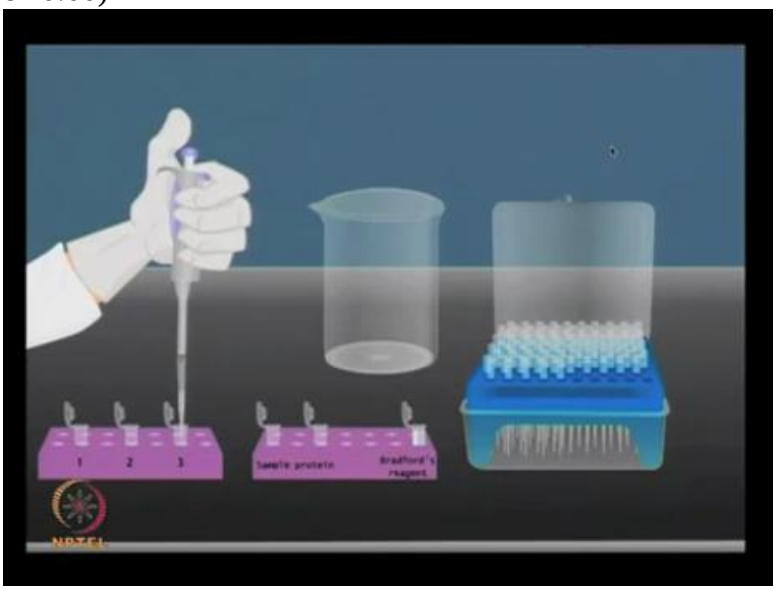

Add the standard and sample solutions to their respective tubes.

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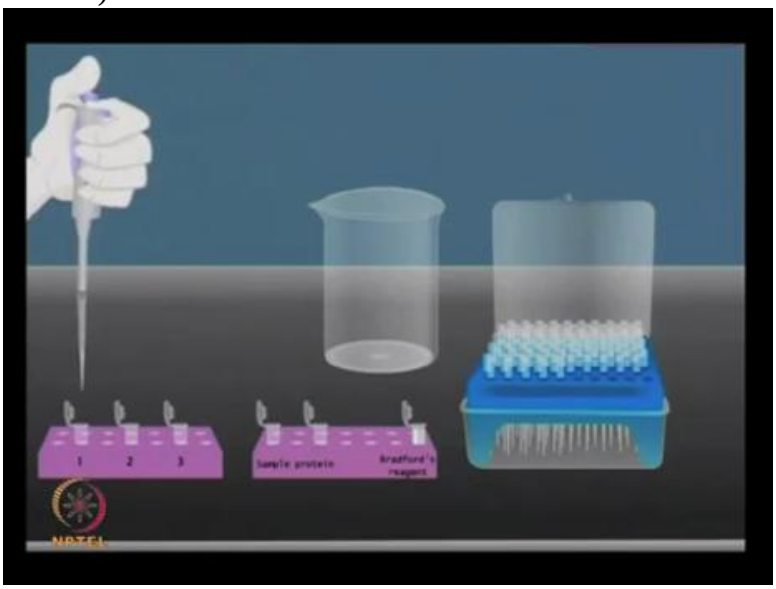

Now add the Bradford color Reagent to the tubes.

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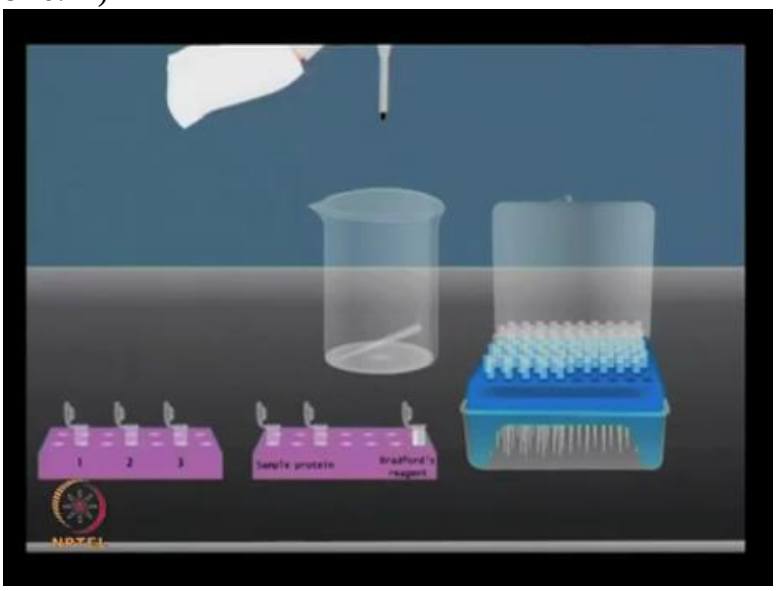

and mix them thoroughly

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Once the color reaction has occurred, then…

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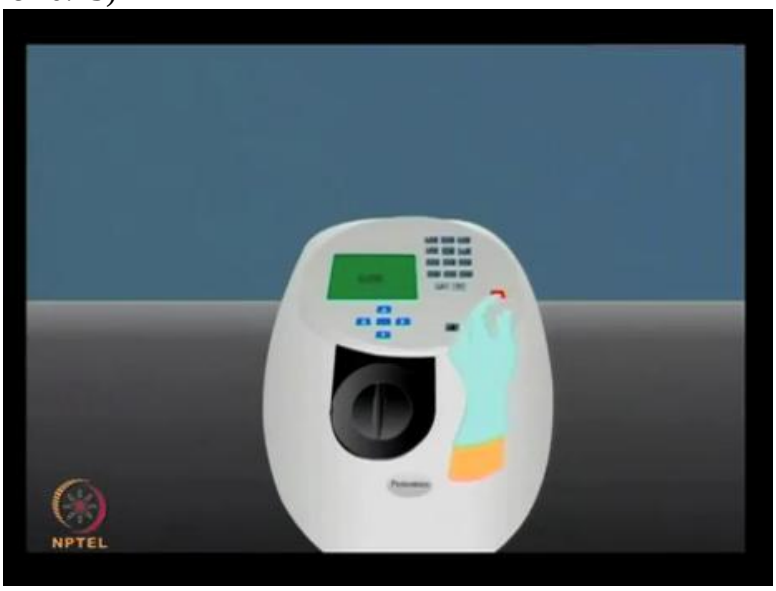

…perform the absorbance measurement at 595 nanometers.

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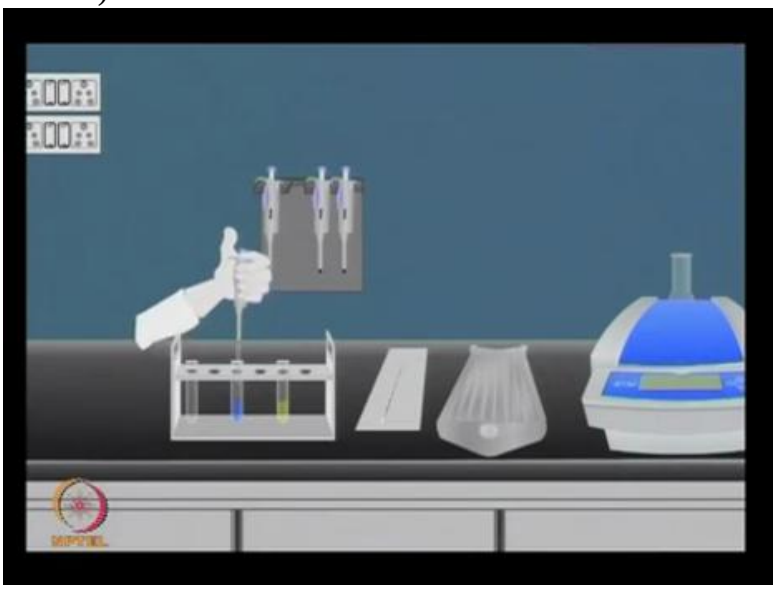

Once you have obtained good amount of proteins which was determined by the protein quantification, now proceed for the rehydration of immobilized pH gradient strip and focusing of these rehydrated strips.

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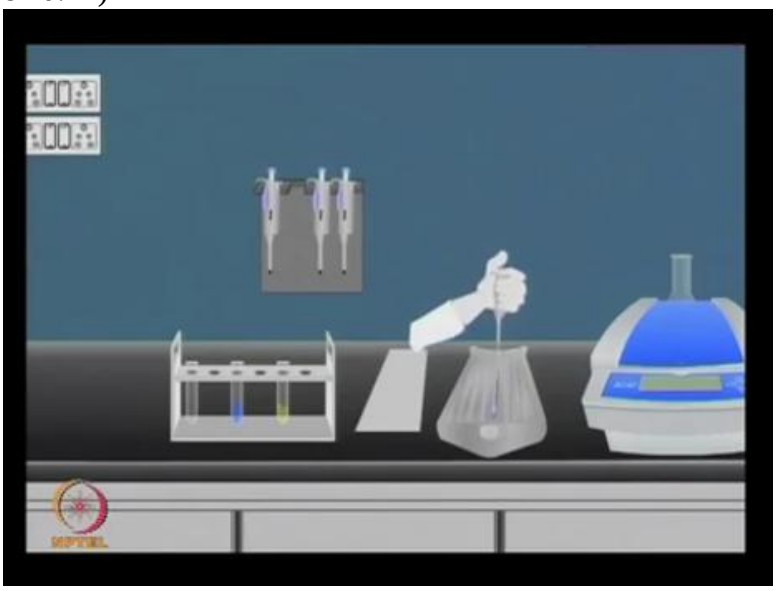

So remove the IPG strip from the cover

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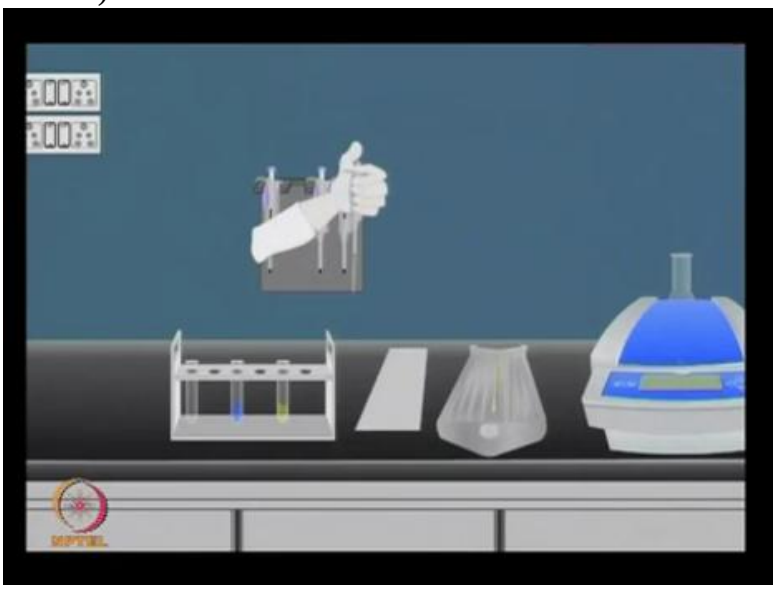

, place it in the rehydration tray

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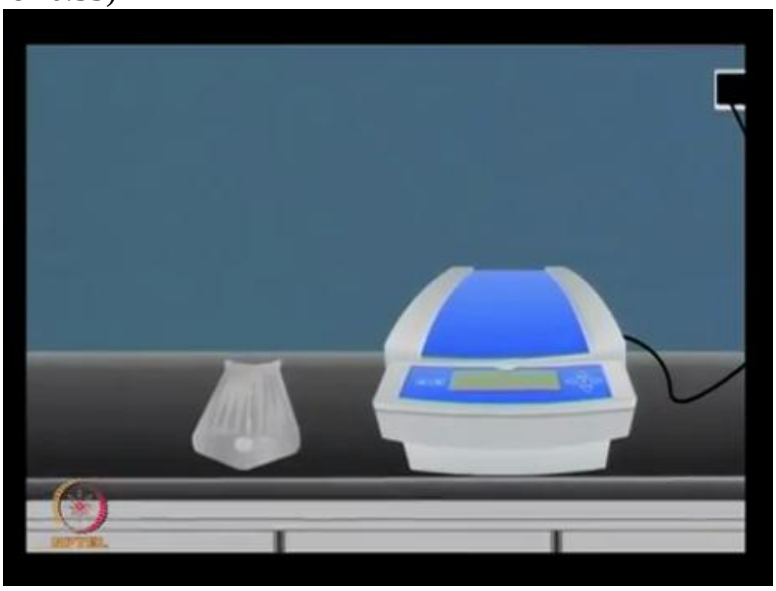

add the reconstituted protein sample on the strip,

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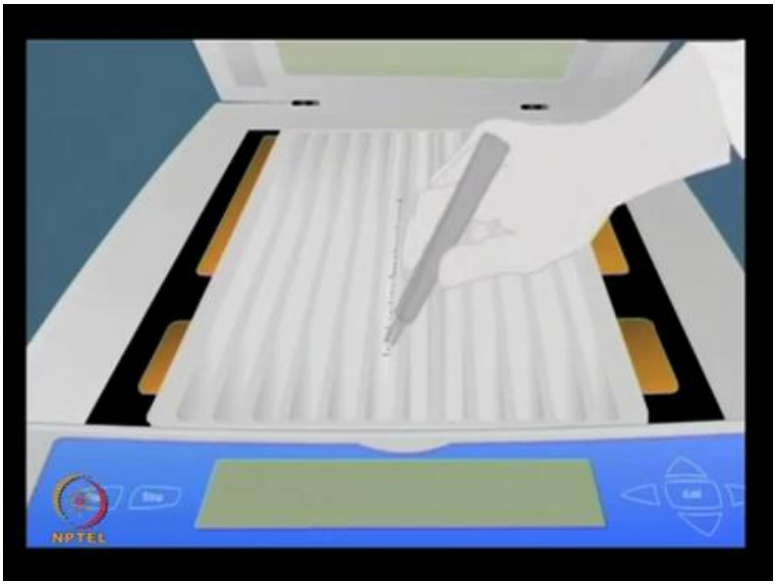

Pour mineral oil to prevent it from drying and then move forward for the isoelectric focusing.

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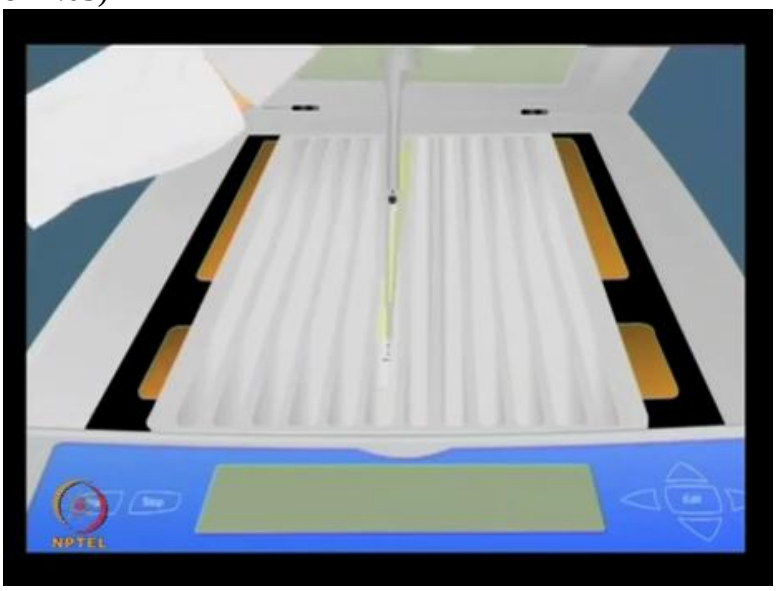

So place the wick at the either end of the IPG strip followed by

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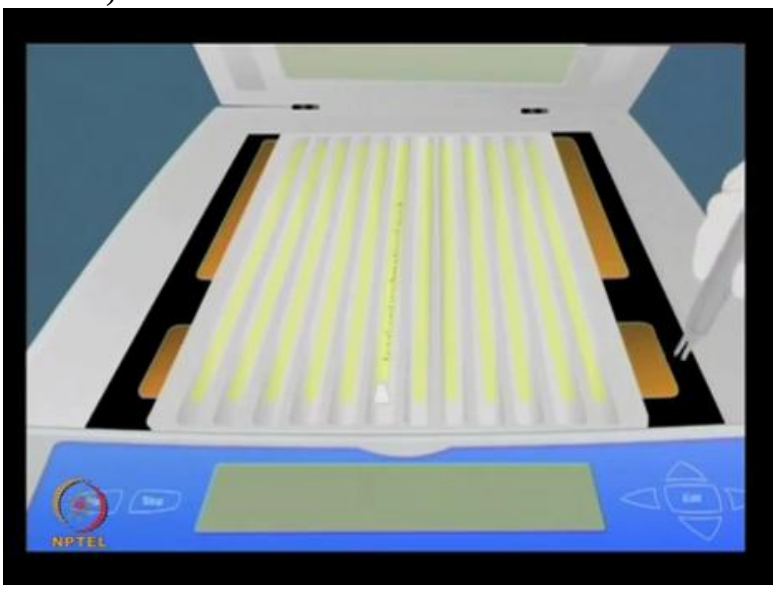

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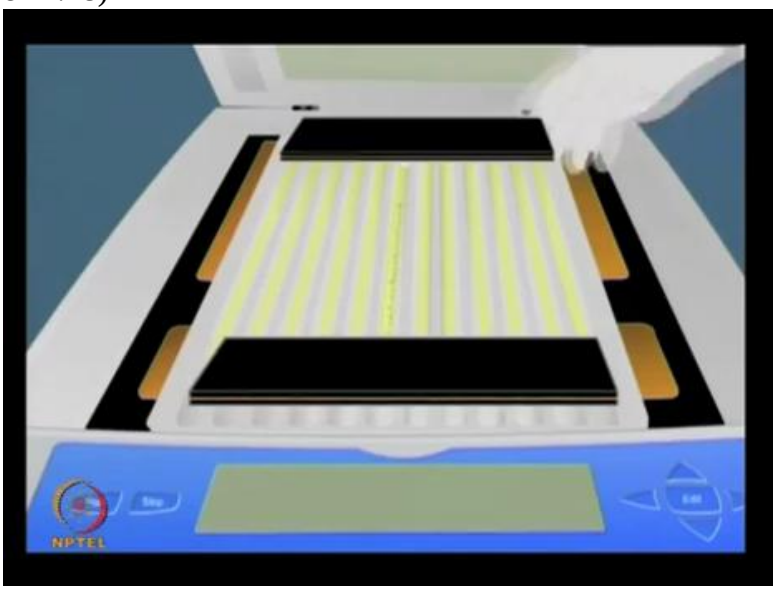

… an electrode at each end Fill all the adjacent wells with the mineral oil to ensure that uniform current flow

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You can input the desired protocol on the instrument software along with the details of strip length, pH Range, number of strips etc. and start the focusing process.

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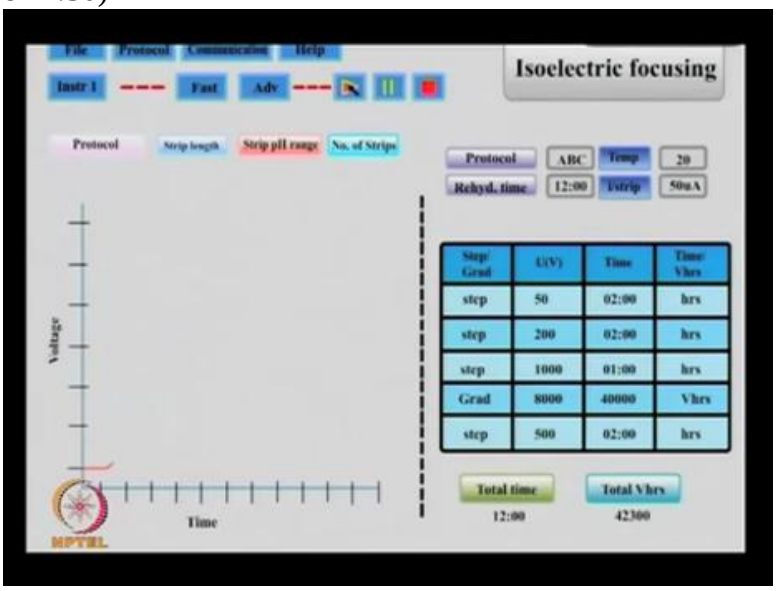

You can monitor the focusing progress on the software and you need to ensure that focusing is proper.

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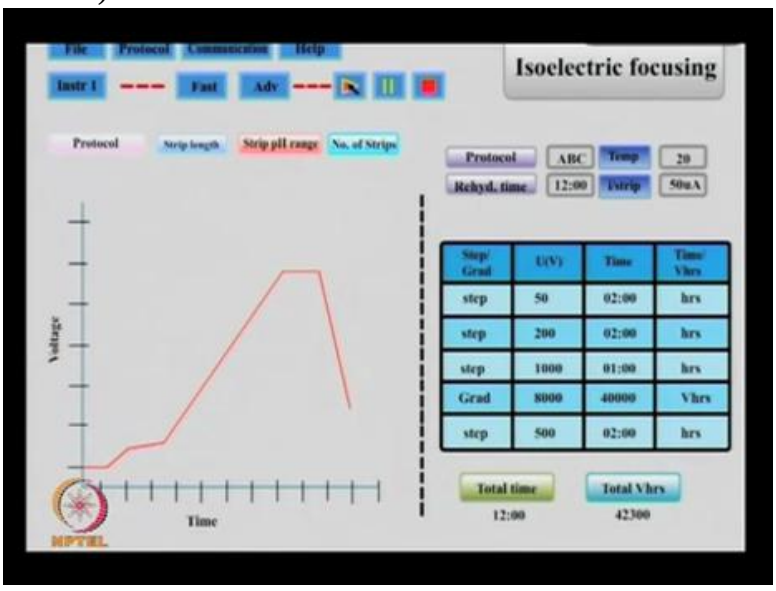

During the IEF, proteins will migrate along the strip and come to rest at a point when

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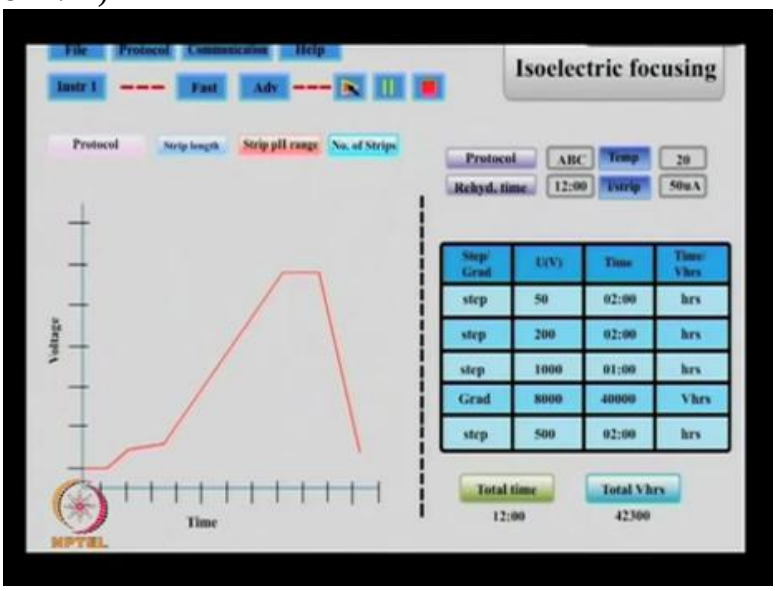

their net charge becomes zero which is known as their isoelectric point.

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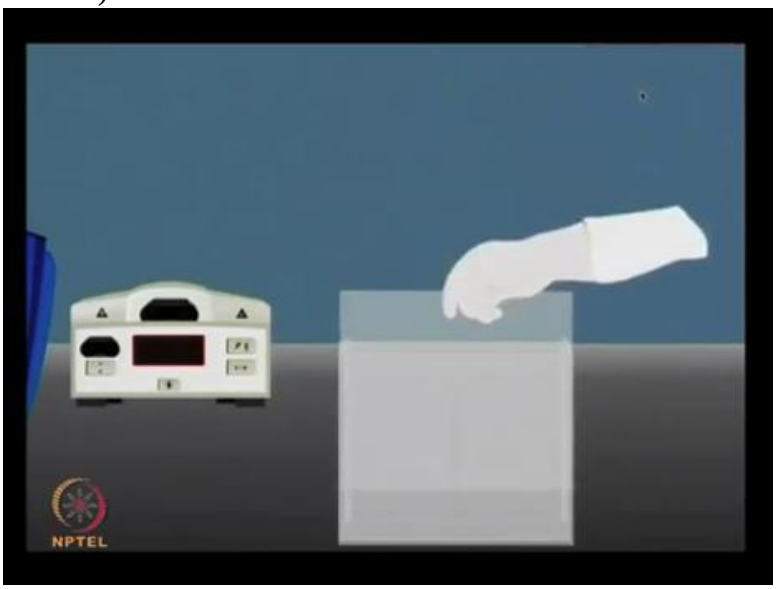

After the IEF is done, then we need to separate the proteins in the second dimension on SDS PAGE

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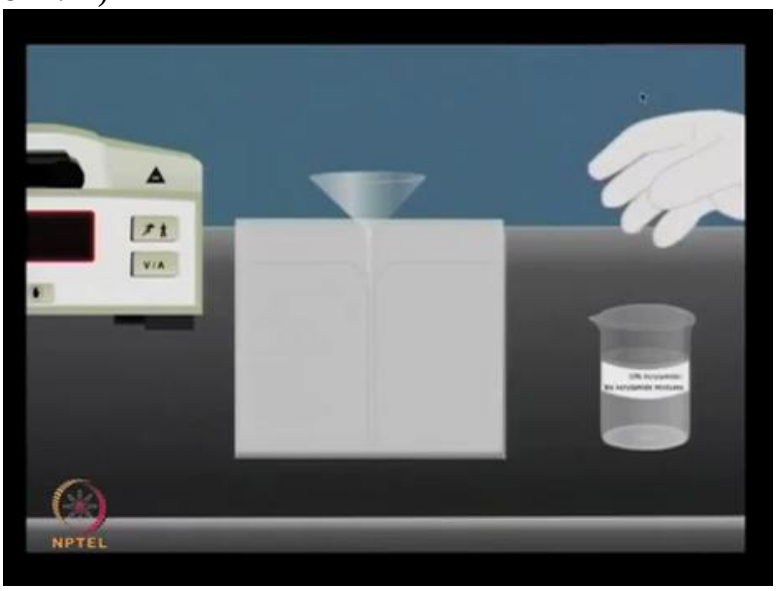

Remember in between, you have to do the equilibration step as well.

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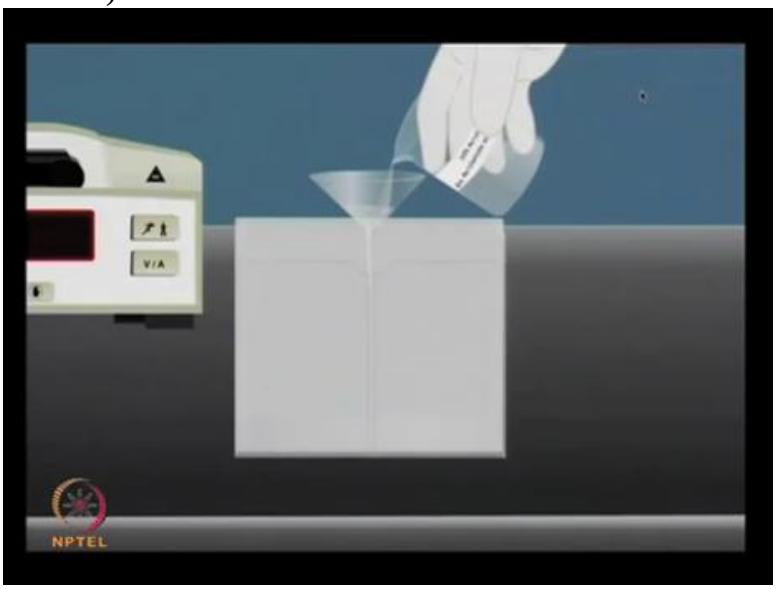

SDS PAGE which constitutes the second dimension of two-dimensional electrophoresis involves assembly of the gel apparatus, gel casting, equilibration of IPG strip

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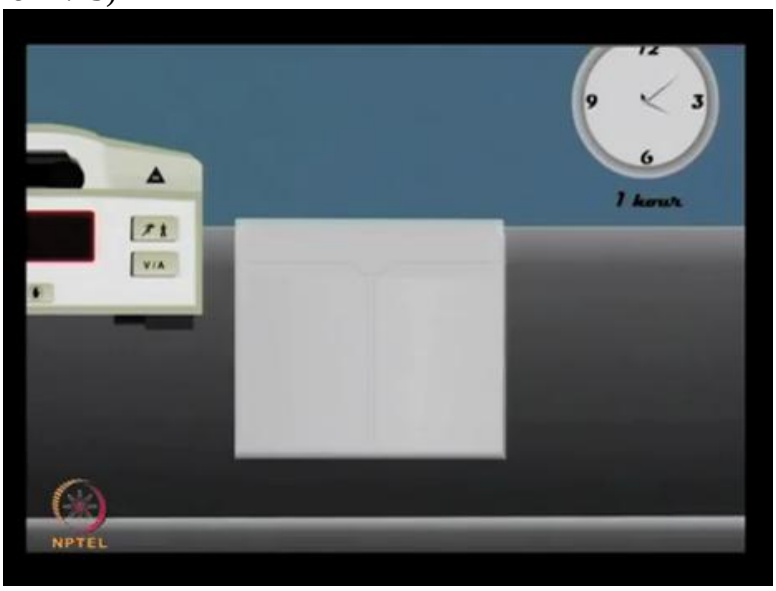

… followed by placement of the IPG strip on the gel and protein separation

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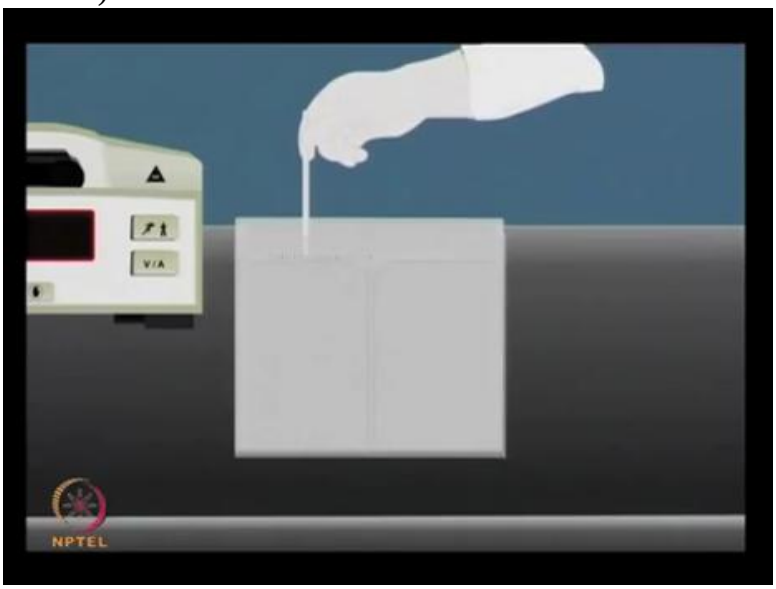

So prepare the gel casting solution consisting of Acrylamide Bis-acrylamide, Tris chloride, SDS, APS and TEMED. Once the gel is polymerized then you can

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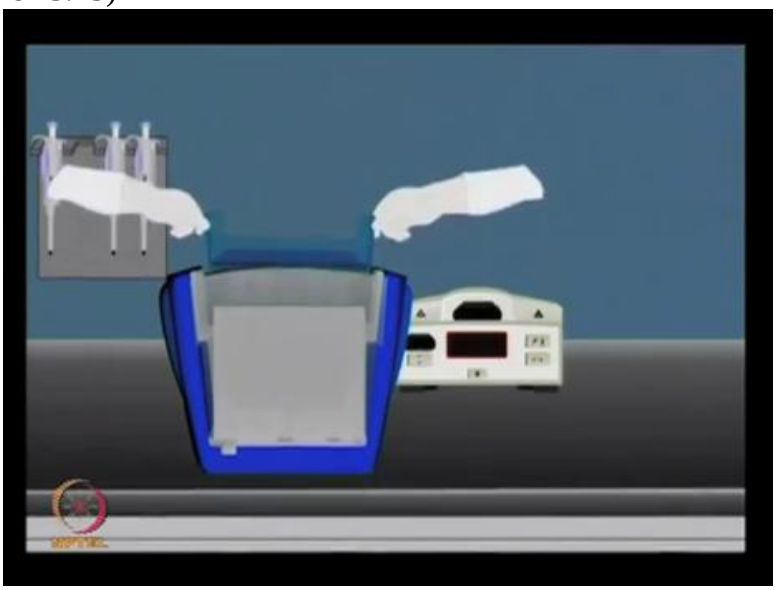

add the IPG strop and now ensure that

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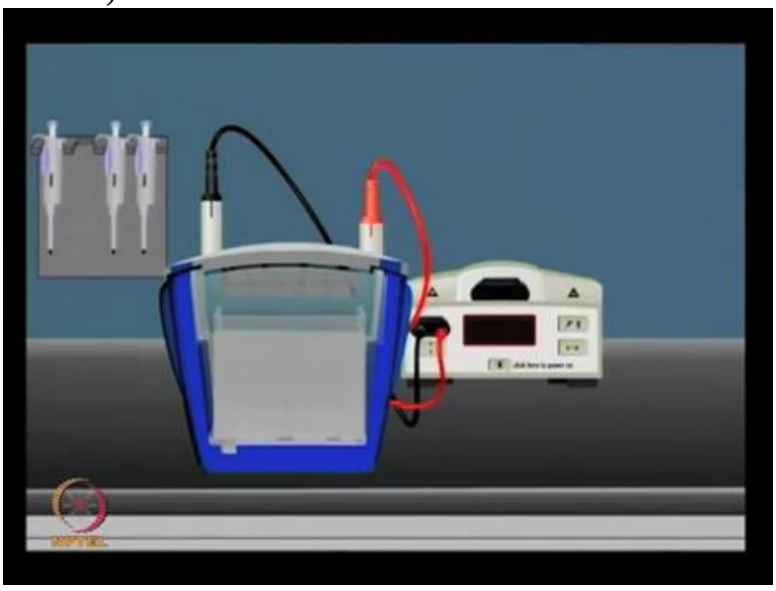

assembly is properly sealed and buffers are properly placed so that proteins can be separated based on their molecular weight. Depending on the gel length, you can perform the second dimension separation for few hours. After second dimension separation is done,

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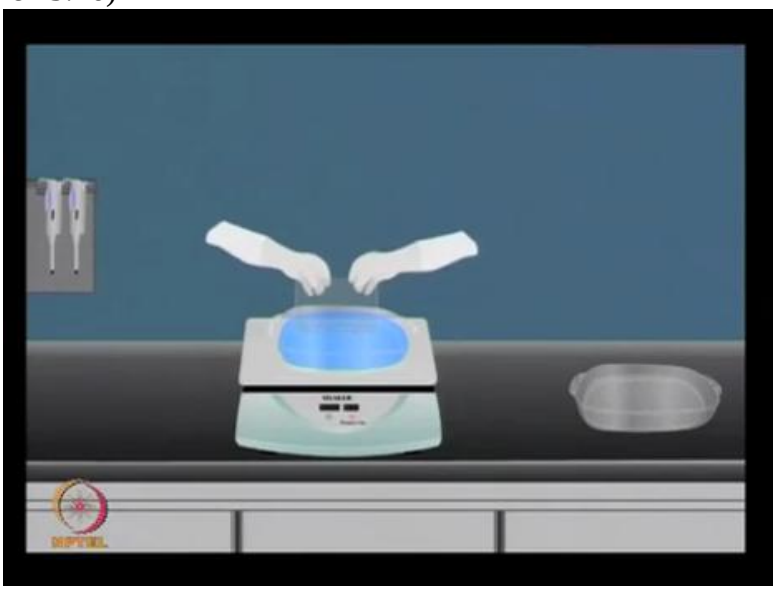

then we can perform the staining and de-staining. So this process involves removal of the gel from the electrophoretic assembly followed by …

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…treatment with a fixing solution,

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staining solution and.

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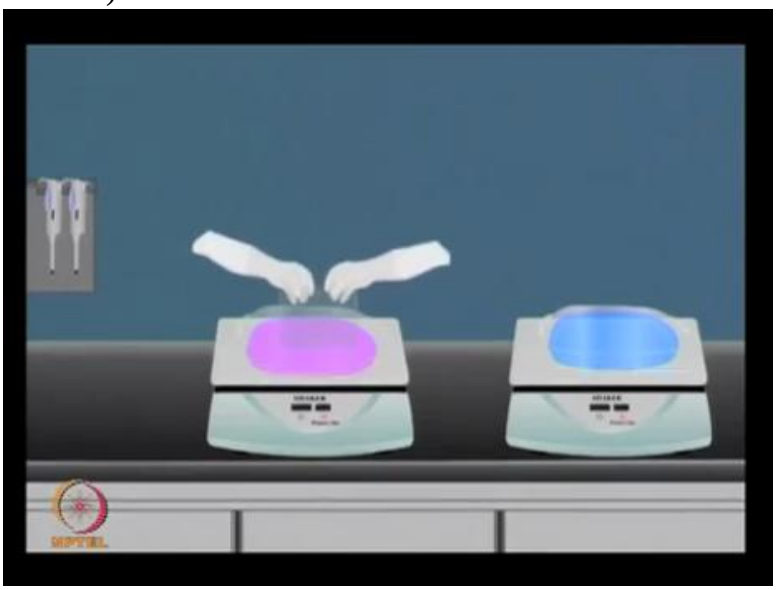

finally the de-staining solution You need to ensure that

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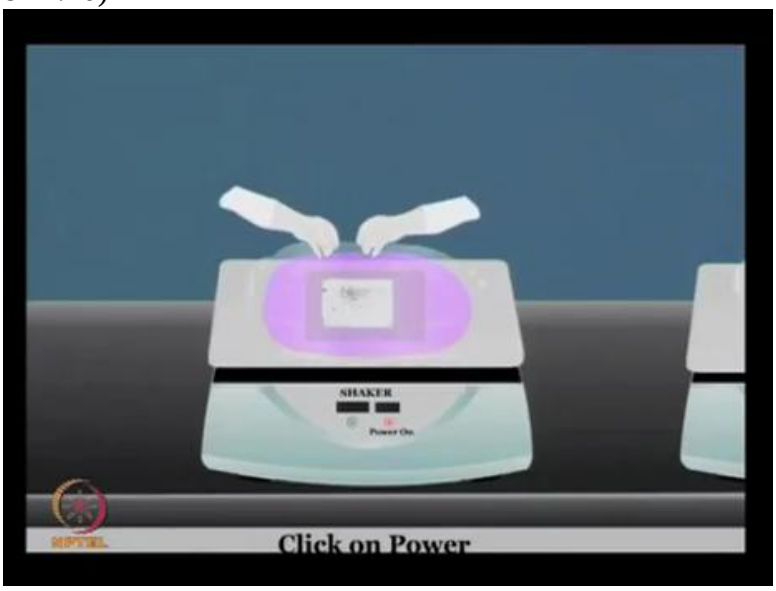

proper shaking conditions are maintained and you need to allow 10 to 14 hours, usually overnight step

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for the staining

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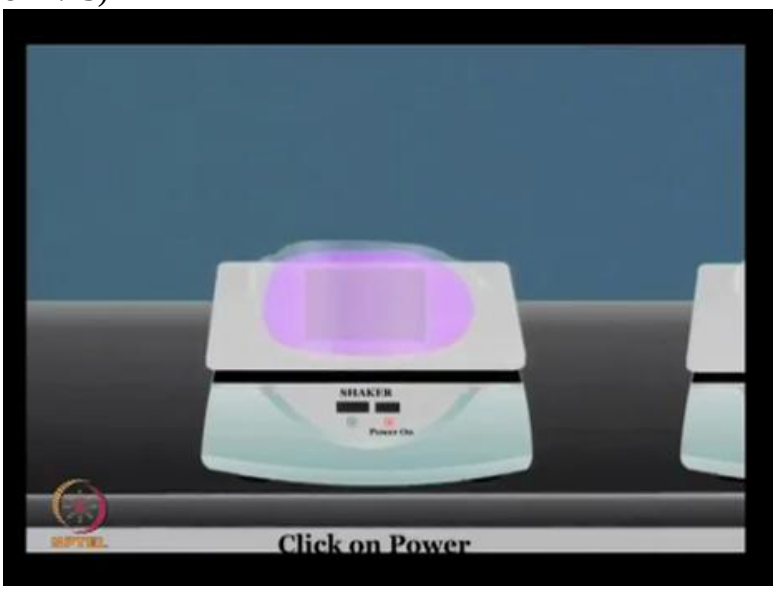

as well as de-staining steps

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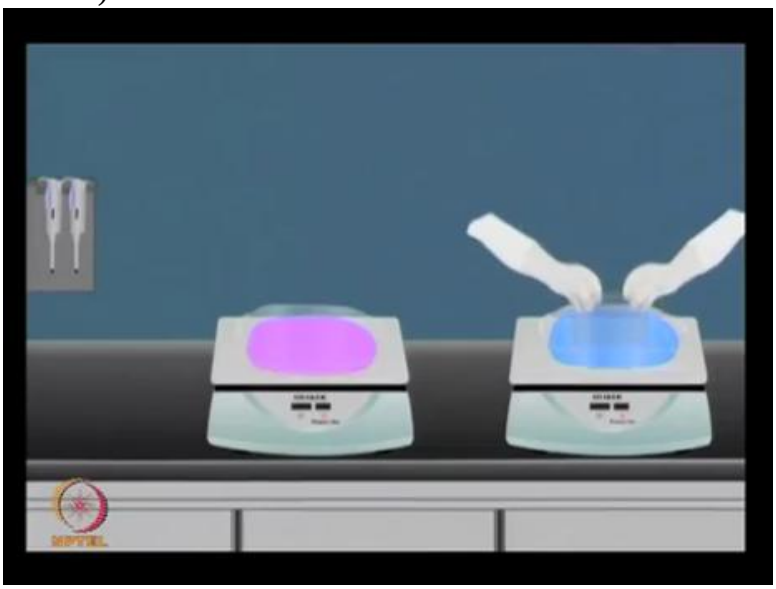

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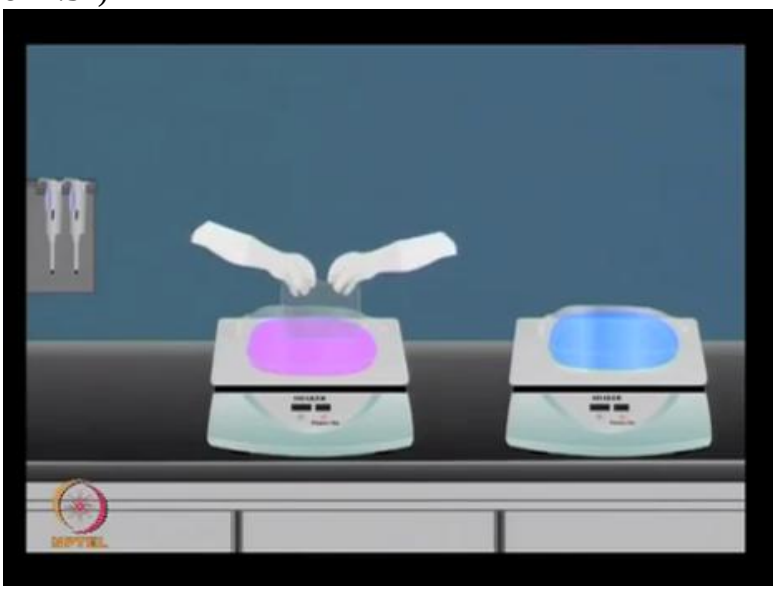

. After de-staining is done, then

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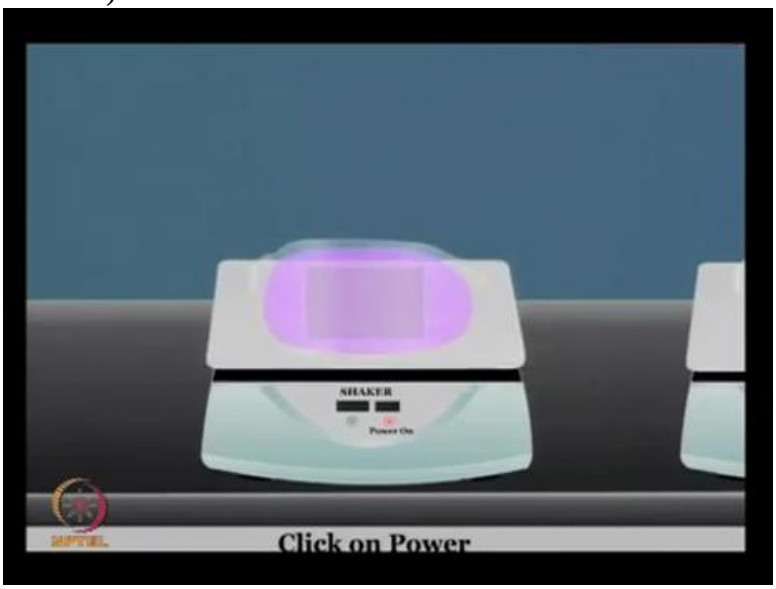

… you can see …

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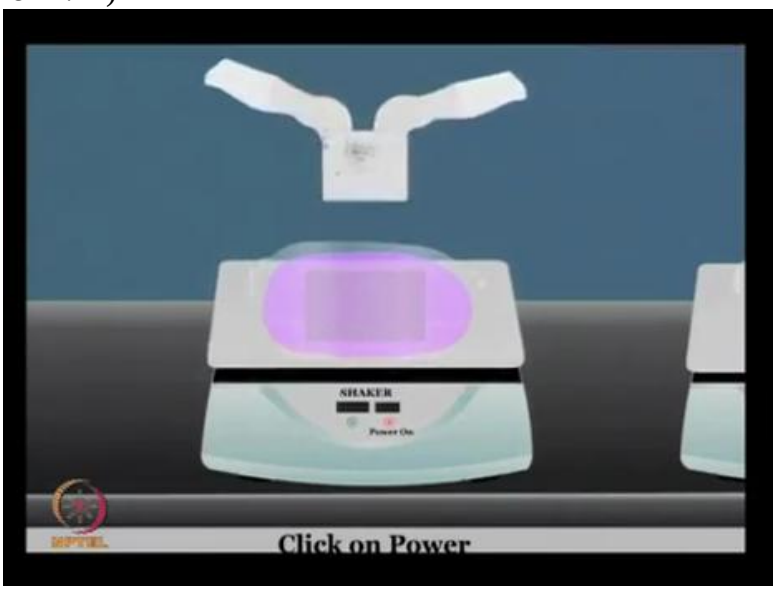

…protein spots on the gel with a very clear background.

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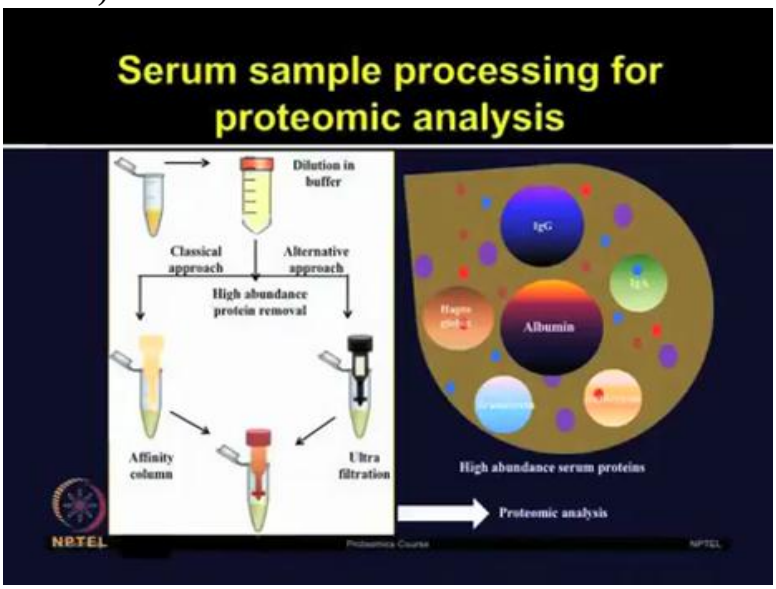

Now serum poses challenge of abundant proteins. So first of all, we had to remove the abundant proteins from the serum and there are standard columns available from various commercial manufacturers from which one can remove selectively very highly abundant proteins such as serum albumen IGG and various other abundant proteins.

In fact there are columns which can deplete almost 14 abundant proteins present in the serum. Now what should be ideal sample which one can use for such proteome experiment? So, first of all to address this question we tried separating the proteins from various types of samples.

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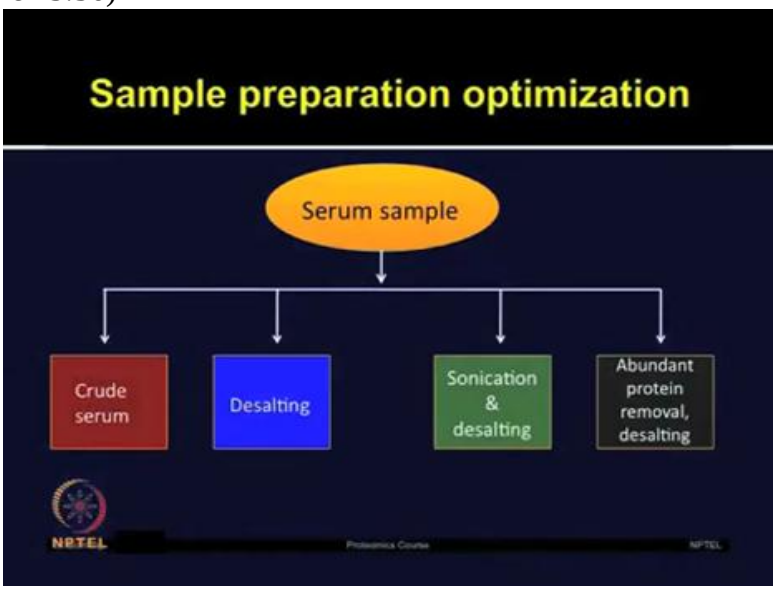

We took crude serum. Because serum will have mostly the protein content, so we thought, can we directly apply the serum on the IPG strip, immobilized pH Gel strip? Separate the proteins and see how many proteins we can separate on these 2D gels. But serum contains lot of salt.

So isoelctric focusing itself becomes challenging if we have crude serum. So we also tried, whether we remove salt component of it and after desalting, if we do the IEF?

Since serum contains highly abundant proteins and these commercial columns can deplete these proteins selectively, those are quite costly and considering that large number of patients' samples that you have to process, so we thought, can we disrupt these abundant proteins and remove these low abundant proteins from these abundant proteins by using sonication.

So effect of sonication as well as desalting was also investigated and then we also used commercial columns to remove the abundant proteins followed by the desalting step.

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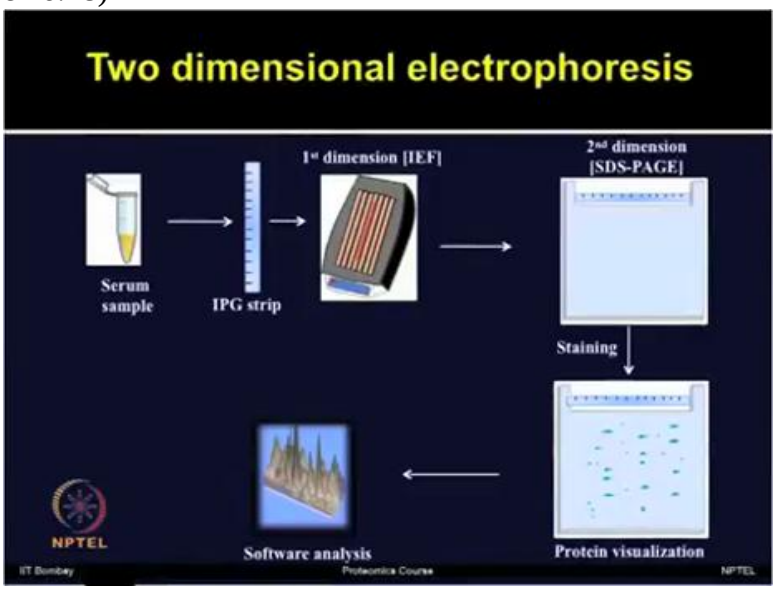

Now sample was corrected and processed with all these 4 variables which I talked to you and after that standard procedure of two-dimensional electrophoresis was followed.

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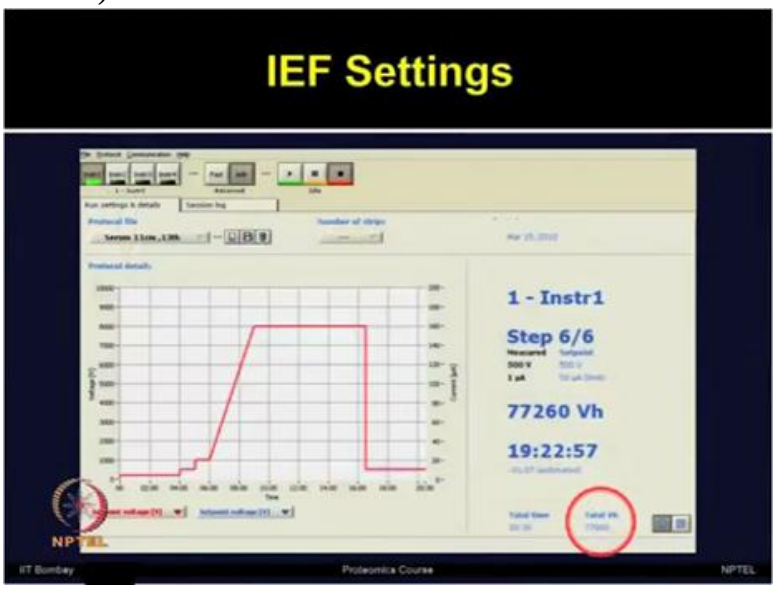

Now when you are doing this serum protein separation, the IEF becomes very critical because you have lot of salt component in it, and if your salt removal is not effective, your isoelectric focusing run may not be very smooth. So through the softwares you can monitor how smoothly your isoelectric focusing is progressing.

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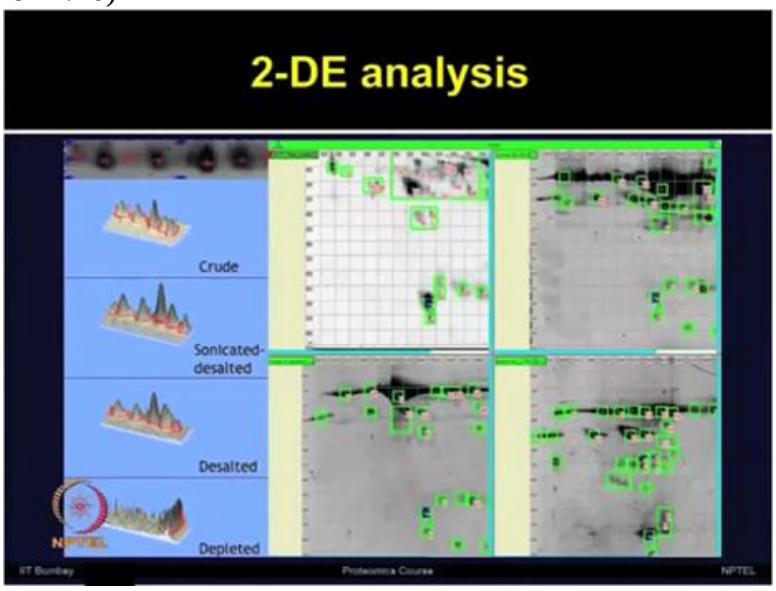

So we tried various types of effects; crude serum alone, or sonicated and desalted, desalted alone and depleted and desalted; and then tried to compare the effect of these types of processing on overall proteome coverage. We also tried to see that how many proteins we can separate on the small gels and then…

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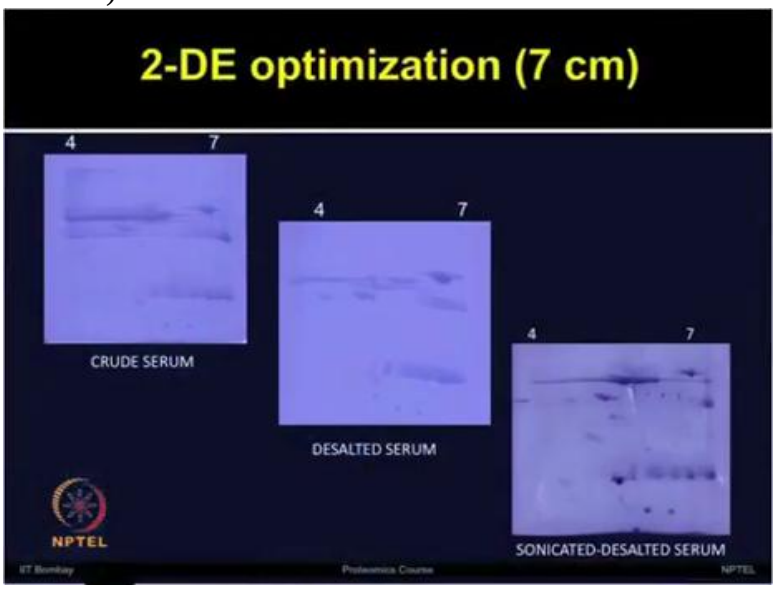

… as expected, the small gel of 7 centimeter cannot provide us very large number of proteins but even these gels can be used for standardization process. As you can see, starting from the crude serum to the desalted and then finally sonicated and desalted gets us better coverage of the proteome.

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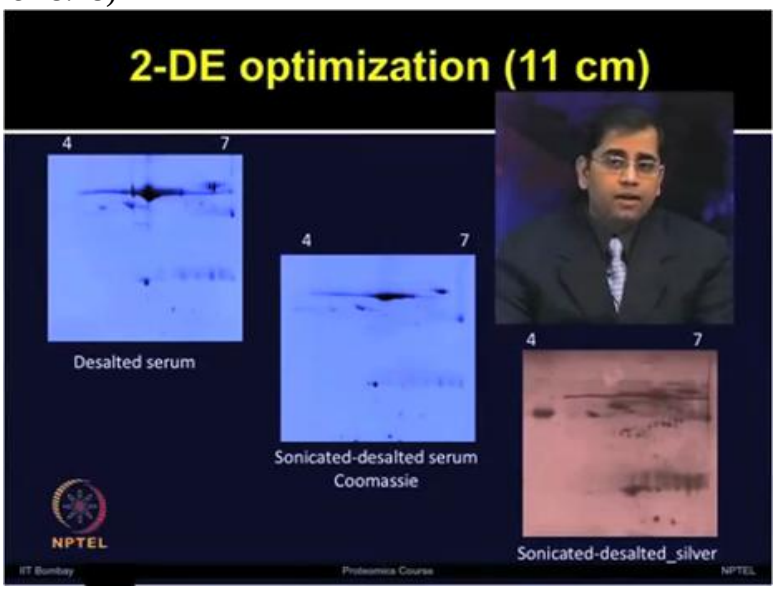

We tried the same even with the larger strip, 11 centimeter and it was again quite convincing. Then we moved on to the very large strips, 24 centimeters for the clinical studies.

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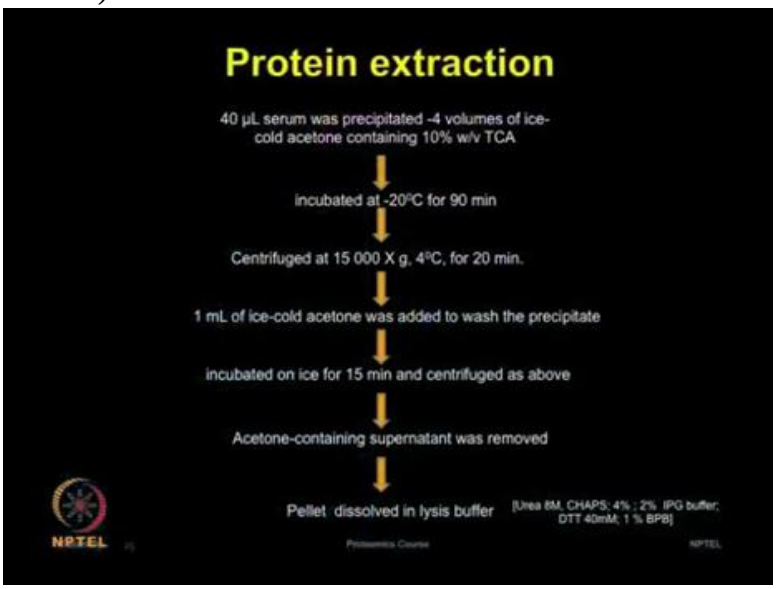

So in general, we took 40 micro liter of serum which was precipitated with the 4 volume of ice cold acetone containing 10% of TCA incubated at -20 degrees, centrifuged followed by added 1 ml of ice cold acetone to wash the precipitate. Then incubated it on the ice for 15 minutes and again centrifugation was performed.

Acetone content supernatant was removed and then pellet was dried in the lysis buffer. The recipe for lysis buffer we have talked earlier when we discussed about sample preparation; this is also mentioned in the slide here, we can use urea, CHAPS, IPG Buffer, DTT and BPB.

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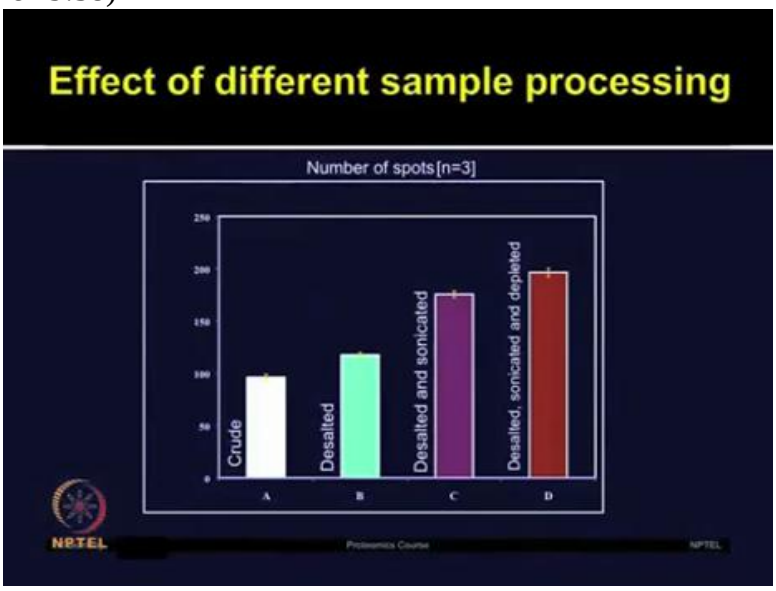

So from the previous study we looked at replicate gels, n is 3 here, and we found that desalted sonicated and depleted sample gave us largest number of spots on the gel as compared to crude alone or desalted alone, or desalted and sonicated.

So these studies we have performed on the small strip to standardize the procedure but we were able to draw the conclusion based on the reproducible pattern obtained in these gels and then we applied desalted, sonicated and depleted...these conditions for the processing all the samples.

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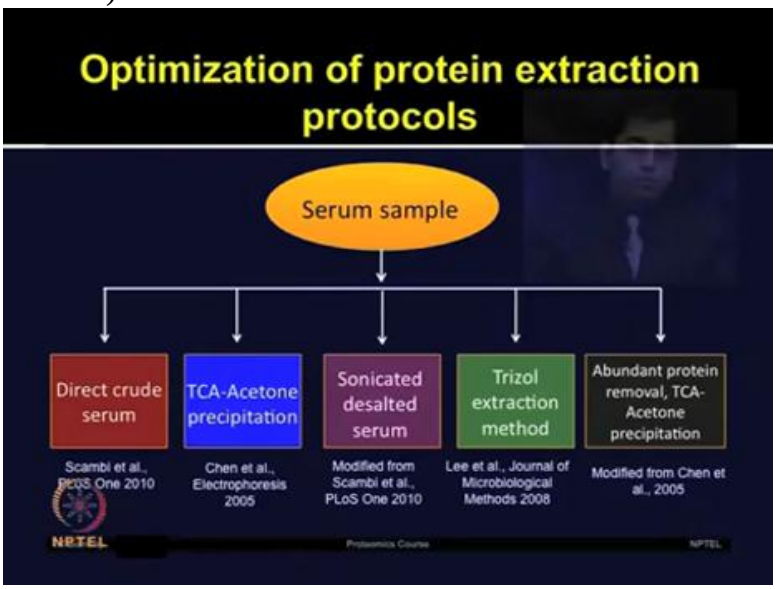

Now, after processing these samples, now how to extract the proteins…? So protein extraction protocol was also optimized and to optimize these protocols, first of all we looked

at what are different methods available in the literature. So, people have applied direct crude serum, TCA acetone precipitation, sonicated and desalted serum, TRIZOL extraction method as well as abundant protein removal and TC Acetone precipitation.

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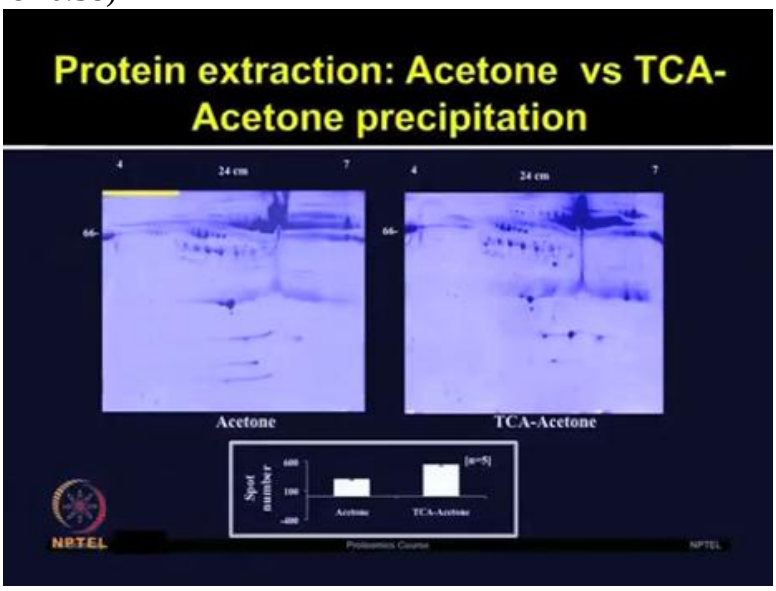

So we used this modified procedure of TC Acetone precipitation where we used depleted and desalted and sonicated serum. We also compared the effect of acetone alone or TC Acetone precipitation, as you can see in this slide, the left one is showing overall protein spot present in the acetone alone treatment and right side showing the TC Acetone precipitation. So, overall more numbers of spots were resolved on the TC Acetone precipitated gel.

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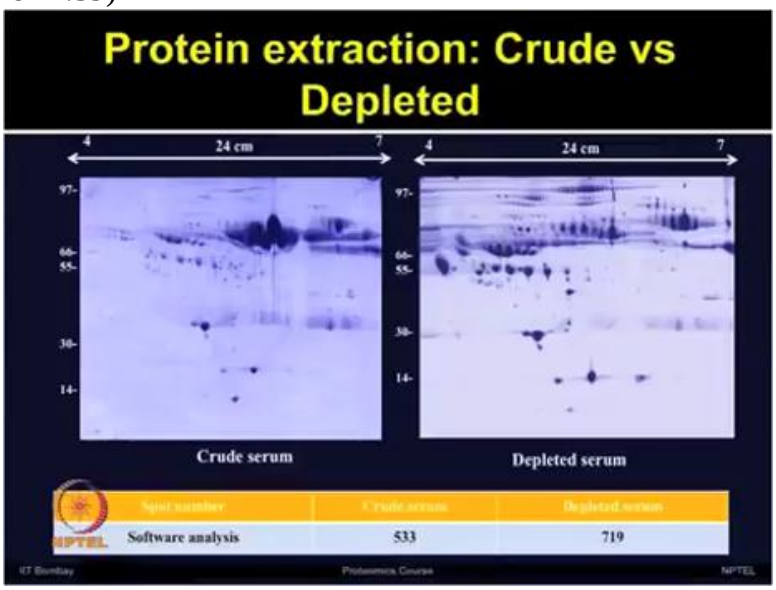

Since different dyes conditions on the small gel as we said earlier, we also tried to apply on the large gel of 24 centimeters because that is where we have to perform all our analysis. So

we again compared crude versus depleted protein response of the serum samples on the large gel and as expected, the depleted serum sample showed more number of spots as compared to the crude serum samples.

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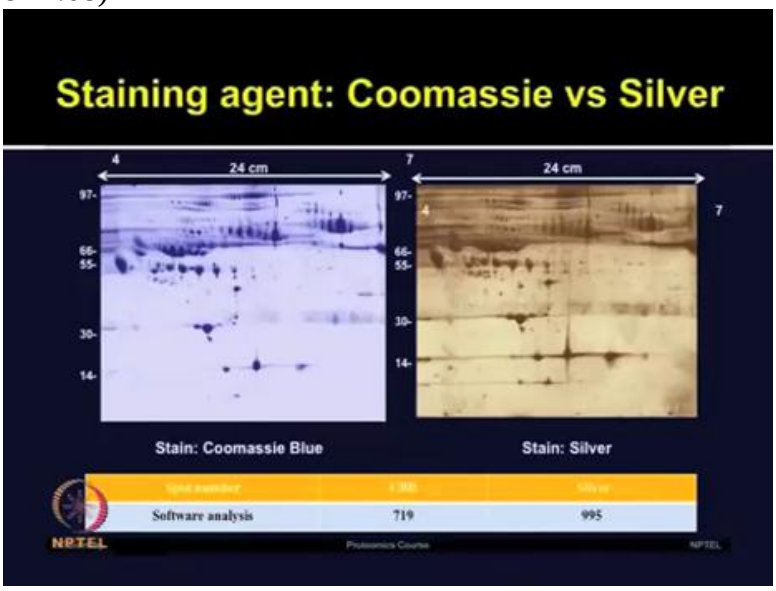

We also tried to look at effects of various types of staining, whether it is Coomassie Blue Staining or silver staining. We definitely identified more number of spots from the silver staining but just because we want to compare large number of clinical samples with the different healthy controls, it was very difficult to standardize the conditions, to keep it uniform staining for the silver.

So to overcome this limitation, we used the colloidal Coomassie and Biosafe Coomassie staining and we standardized the same conditions for all the gels.

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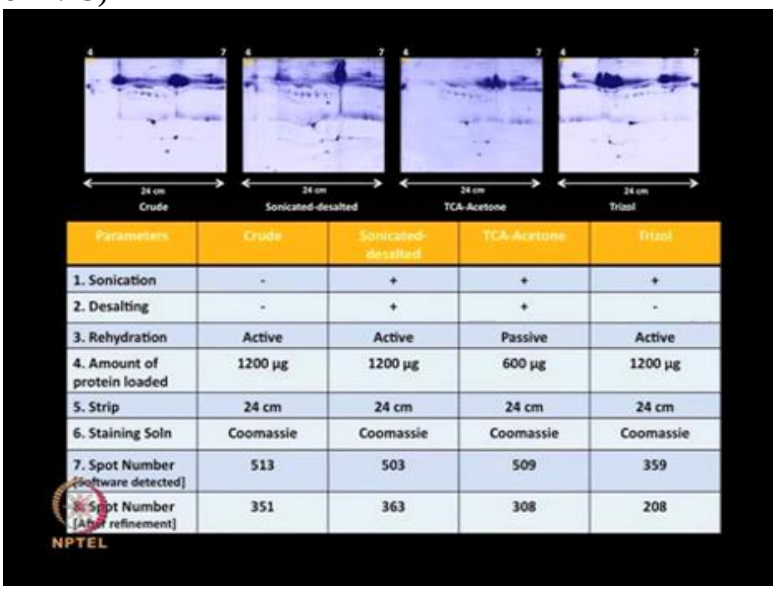

Parameters	Crude	Sonicated-desalted	TCA-Acetone	Trizol
1. Sonication	-	+	+	+
2. Desalting	-	+	+	-
3. Rehydration	Active	Active	Passive	Active
4. Amount of protein loaded	1200 µg	1200 µg	600 µg	1200 µg
5. Strip	24 cm	24 cm	24 cm	24 cm
6. Staining Soln	Coomassie	Coomassie	Coomassie	Coomassie
7. Spot Number [software detected]	513	503	509	359
8. Spot Number [After refinement]	351	363	308	208

So this slide gives you an overview of various types of parameters which we investigated from different types of treatments; crude serum, sonicated and desalted, TC Acetone precipitated and Trizol extraction methods.

So we looked at effect of sonication, desalting, rehydration, amount of protein loading, type of strip, staining solution, how many number of spots we can resolve on these gels and then, since software analysis automated gives lots of artifacts, how many real spots we can obtain after the manual refinement.

Sometimes, to give you all the details what one needs to actually perform before reaching to that stage where you can compare the healthy control with the clinical samples and this lecture is actually trying to cover various types of concepts which we have talked in the sample processing and in different lectures of two-dimensional electrophoresis.

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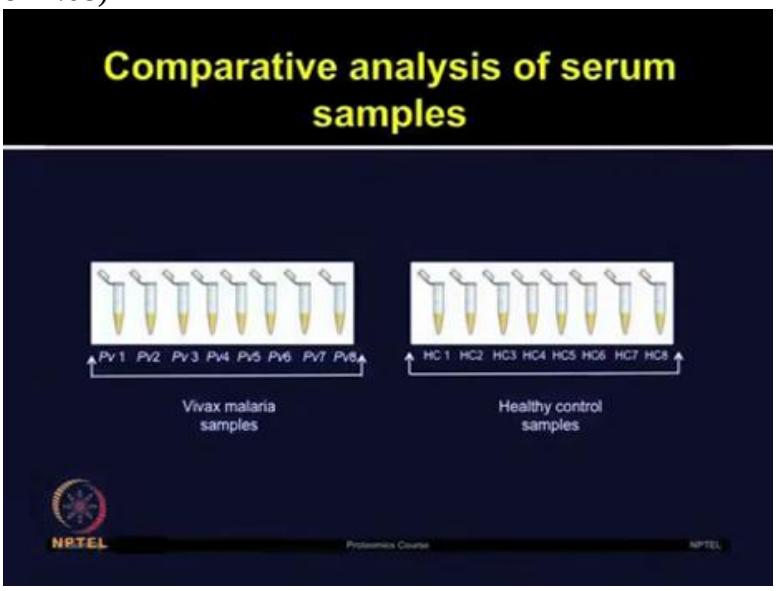

So now after doing lot of comparative analysis and standardizing the protocols for sample preparation and protein extraction, now we are ready to perform the comparative study of serum samples of Vivax samples with the healthy controls...

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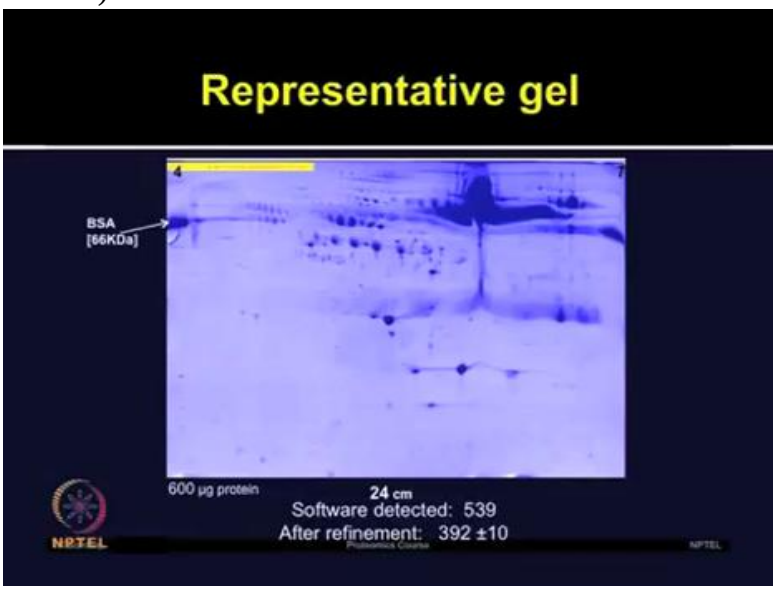

This is one of the gel image showing that that is what is expected on 24 centimeter large 2D gel and …

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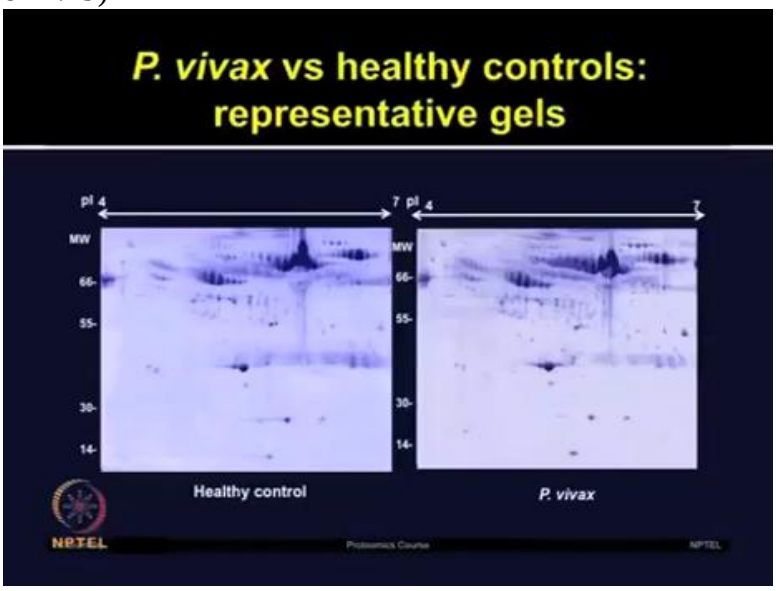

then a representative gel showing the healthy control and the Plasmodium vivax treated samples. So these samples, as you can see were showing number of protein spots resolved on these gels and then …

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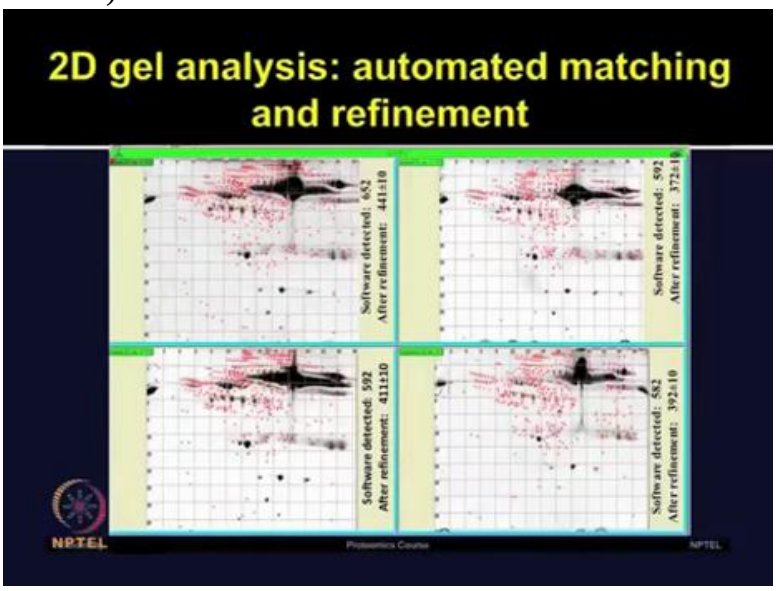

…after separating these protein samples on these 2D gels from various patients and controls, then most challenging job was the data analysis. So automated matching as manual refinements are performed to obtain the how many spots are significantly modulated due to Vivax infection

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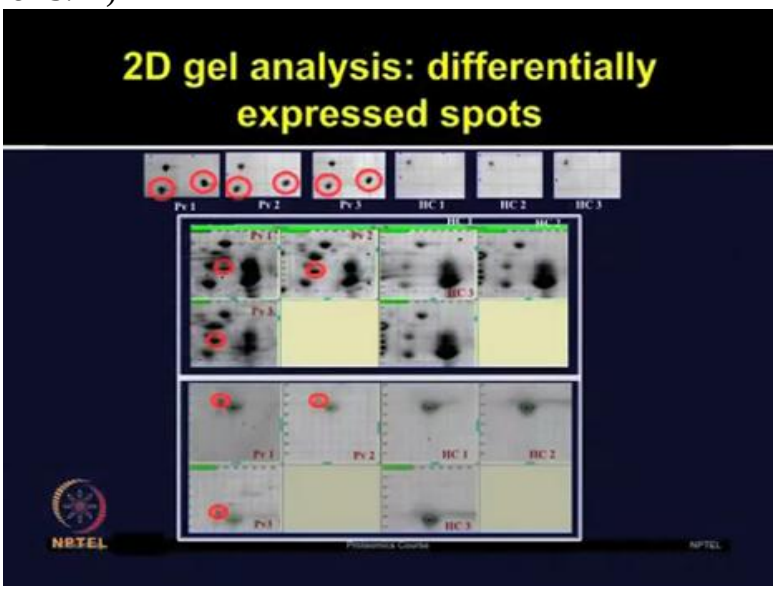

As you can see in this slide, we had looked at various controls and various Vivax patients. These are only few patient samples here but we analyzed large number of samples but those splits which were reproducible in all the patients.

For example one you can see in the top panel, two spots are showing significant alteration in the Vivax as compared to the healthy controls. So these types of spots were considered further for the mass spectrometry analysis

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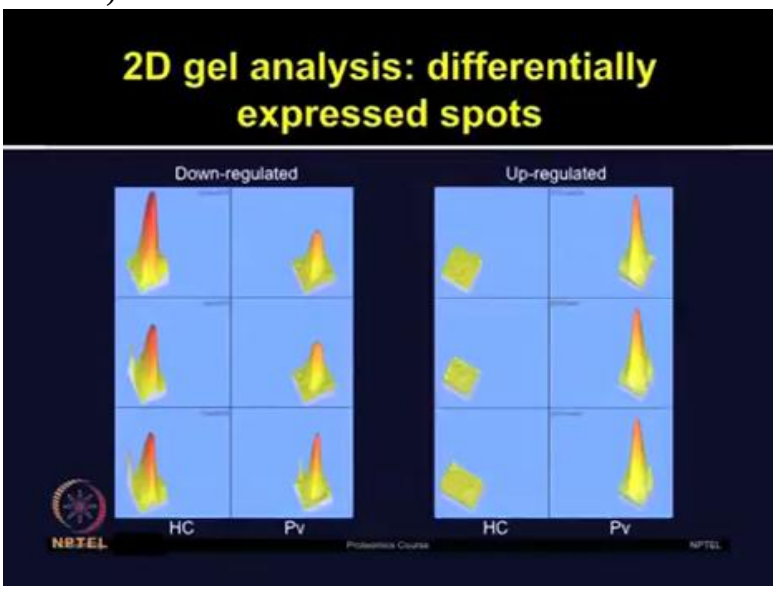

. Now those spots which we found were showing good differential expression, we looked at the 3-dimensional views of those spots to ensure that those are not artifacts and those are the real spots showing up or down regulation of these proteins in human cell.

Now after completing analysis we were able to obtain large number of proteins which were differentially expressed

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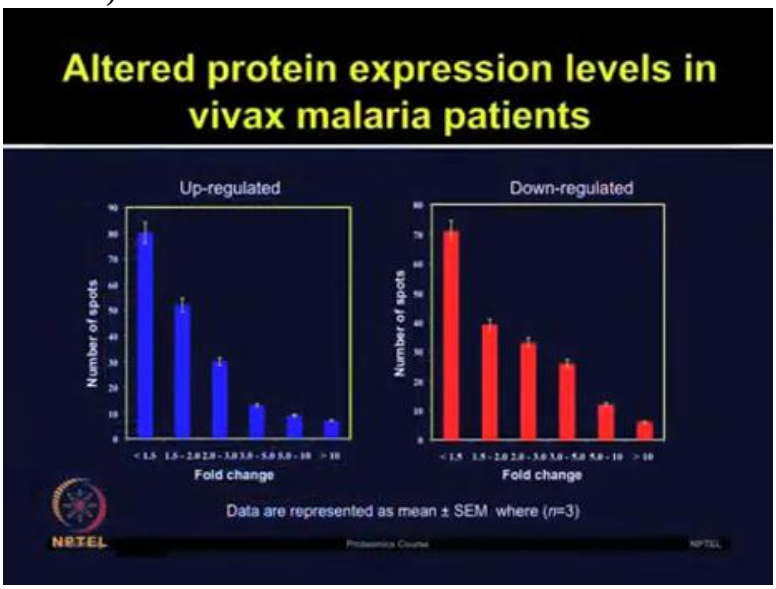

and as you can see in this graph, there are proteins which vary in different range of the fold change. Many proteins show very less fold change, between 1 to 1.54 and there are few proteins which show more than 10 fold up or down regulation. So these protein spots were considered interesting because those were statistically significant and then these spots were further analyzed using mass spectrometry techniques.

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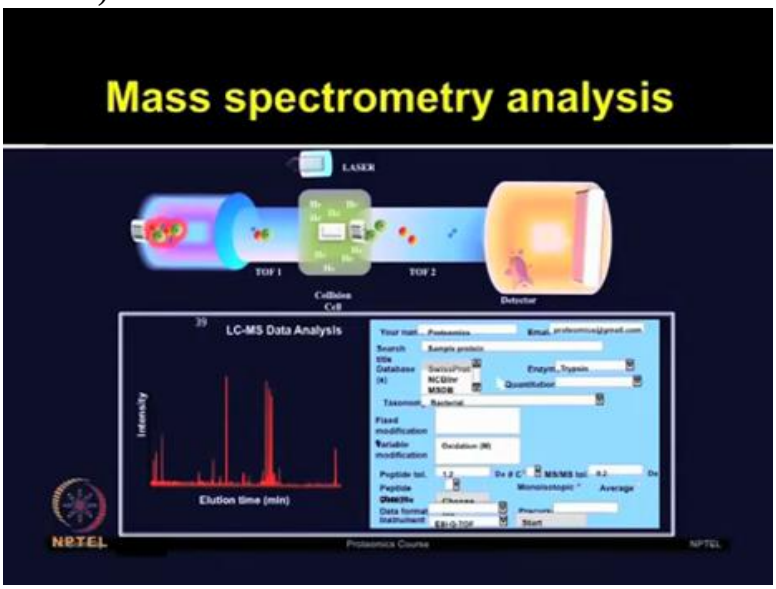

So this slide gives you an overview of Tandem mass spectrometry technique and followed by how well MS/MS data can be analyzed by using Mascot search engine. Different types of mass spectrometry techniques are available inducing MALDI-TOF/TOF and MS/MS based

approaches and we will talk about these techniques in much more detail in the next module when we talk about mass spectrometry

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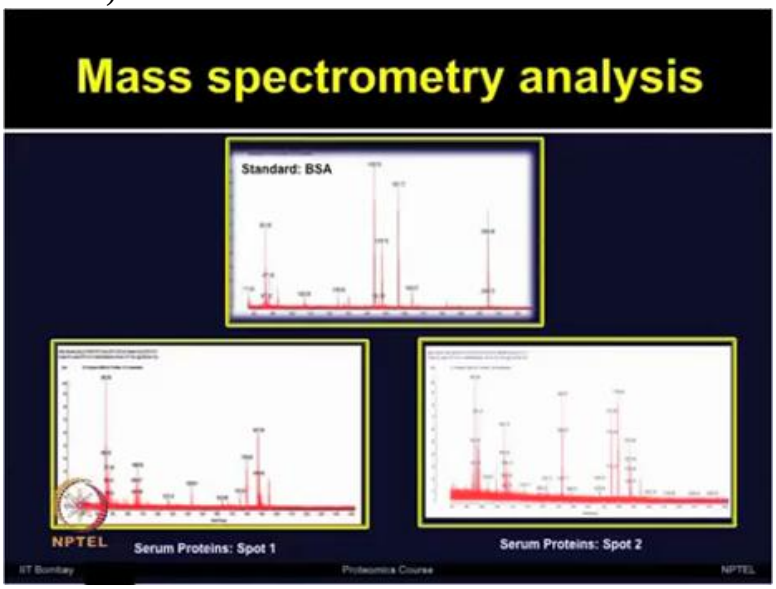

But just to give you an overview here that one needs to analyze the spectra and also one needs to have some standard proteins to ensure that correct spectra is obtained, so you have to do lot of quality control checks in mass spectrometry to ensure your data is of good quality. I will cover the details of mass spectrometry experiment when we talk about …

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… about mass spectrometry in more detail in the next module and again may be I will take this study at that time, that what are the different detailed procedures required to perform such analysis?

At the end, from these experiments of mass spectrometry, one can obtain the identity of these proteins

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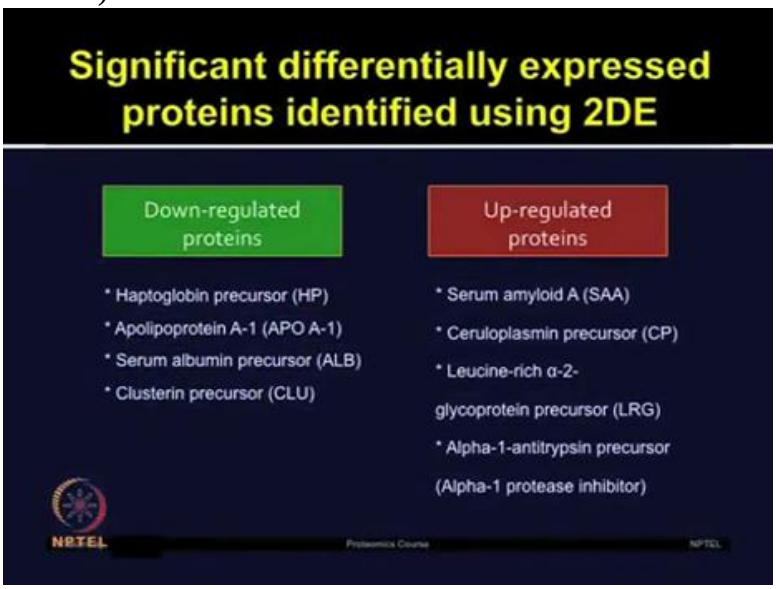

… as you can see in this slide, there are several proteins in the host human where differentially expressed due to the Plasmodium vivax infection and these are some of the proteins listed here such as haptoglobin, · Apolipoprotein A1, serum albumin precursor and clusterin precursor.

These were down-regulated and Serum amyloid A, Ceruloplasmin precursor, Leucine rich alpha 2 glycoprotein precursor, and alpha-1-antitrypsin precursor, these proteins were up-regulated among many other proteins.

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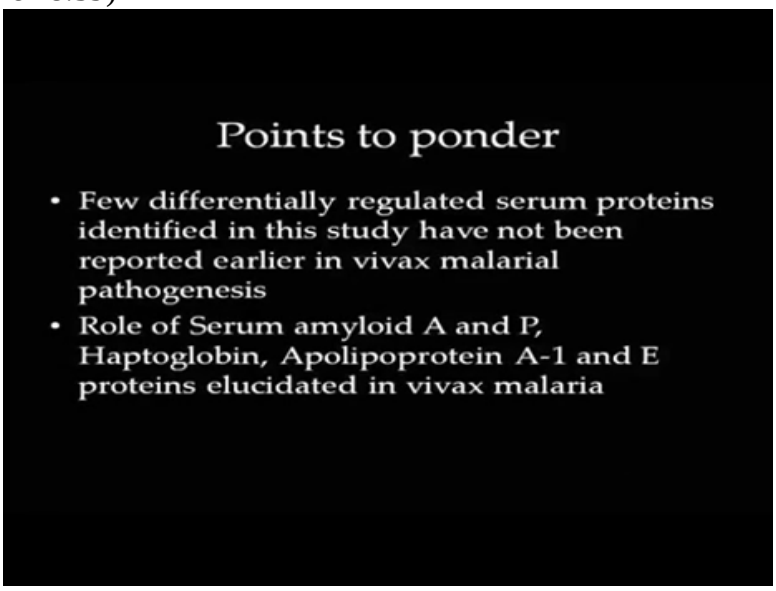

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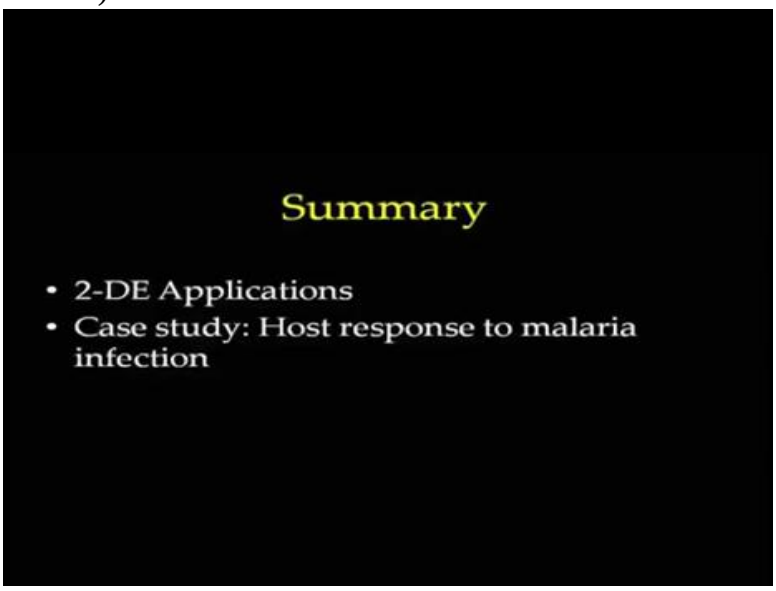

How two-dimensional electrophoresis technique can be applied for various biological applications.

By giving you a case study and details of various types of experiments one needs to perform, probably you got an idea that before performing that final experiment on your case and the controls again lot of optimization has to be performed and starting from your sample processing and how you can expand the coverage of the proteome, these types of quality control checks are very essential.

If you can increase the overall proteome coverage, you can separate over 1500 to 2000 proteins on the gel reproducibly then you have a good change of identifying various potential targets which could be novel drug target or potential bio-markers.

But if you have not done the proper quality control experiment, you have not optimized the protocols properly and your proteome coverage is very poor on the gel, then you are comparing only partial proteome and there is a good likelihood that you will miss out many important changes.

So not only today we discussed about one application how host serum protein changes due to one of the Plasmodium parasite, but also we discussed the various nitty gritty experiments one needs to perform to achieve such type of comparison.

We continue our discussion on some more applications of two-dimensional electrophoresis and also two-dimensional difference in gel electrophoresis because due to the variations in the 2DE, people also try two-dimensional DIGE experiments and also that is very sensitive, so that also expands the overall proteome coverage. We will continue our discussion in the next class. Thank you