Introduction to Proteomics
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Lecture - 13
DE Applications& Challenges

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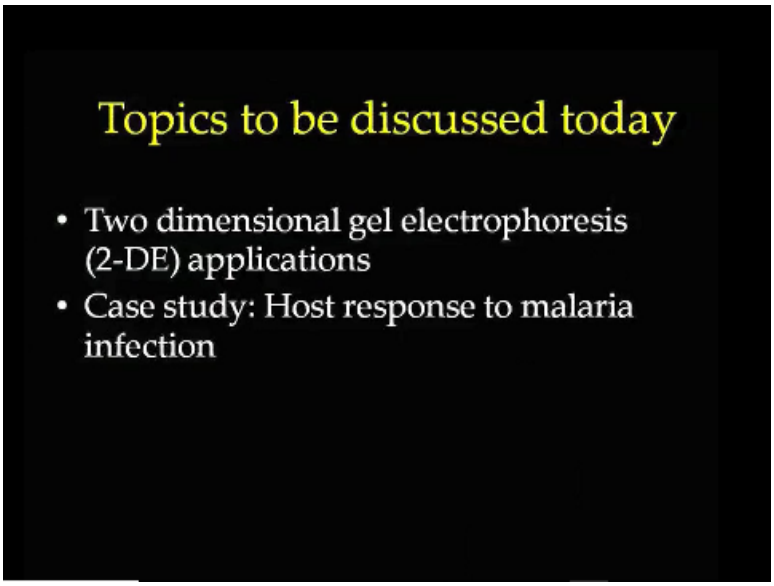

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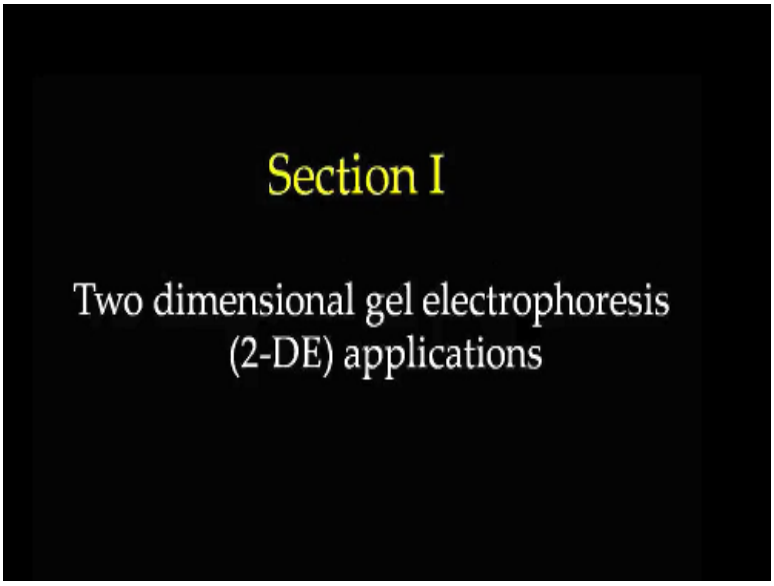

So, now let me give you a few applications one can use by applying this technique. So, people apply 2-DE for various objectives for example.

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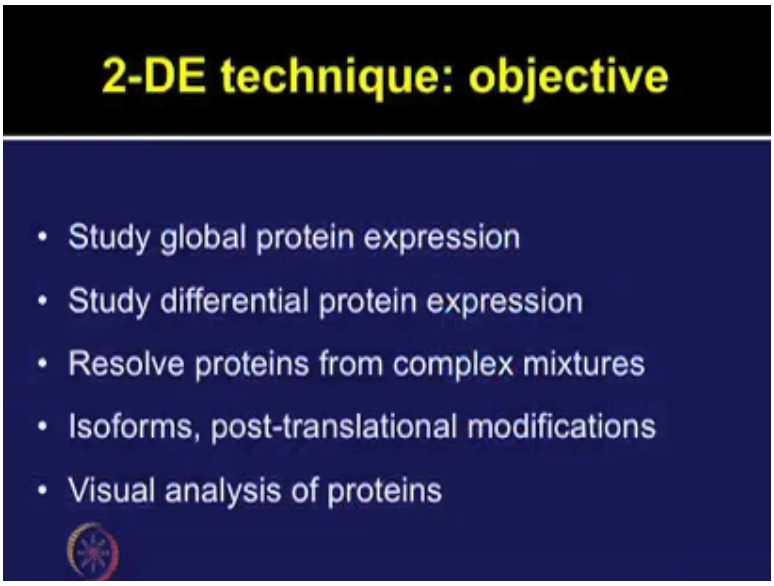

For studying the global protein expression when I say global protein expression it means you want to identify or separate all the proteins present in a given protein sample. So, if you do not have information about all of the protein which could be present in your sample mixture. Then first of all we need to create that fingerprint or blue print of all the proteins and use that by the 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 upregulated or downregulated in their expression because of the given treatment or because of a disease condition or because of your experimental condition.

So, in both of these cases the two-dimensional preferences can be used to resolve the proteins from very complex mixtures, people also used to study different type of Isoforms or the post translational modifications which occur in that process. One of the major advantage of this technique is the visual analysis of protein. When you stain a gel after the two-dimensional flor for assess process then you can see all the protein spot 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 or you have to rely on your analysis. In this case here your all the protein points are already present, and you can get visualize that and then you can use each of these spots to further analyze and compare the images. So, the protein profiling if you want to compare your different sample types.

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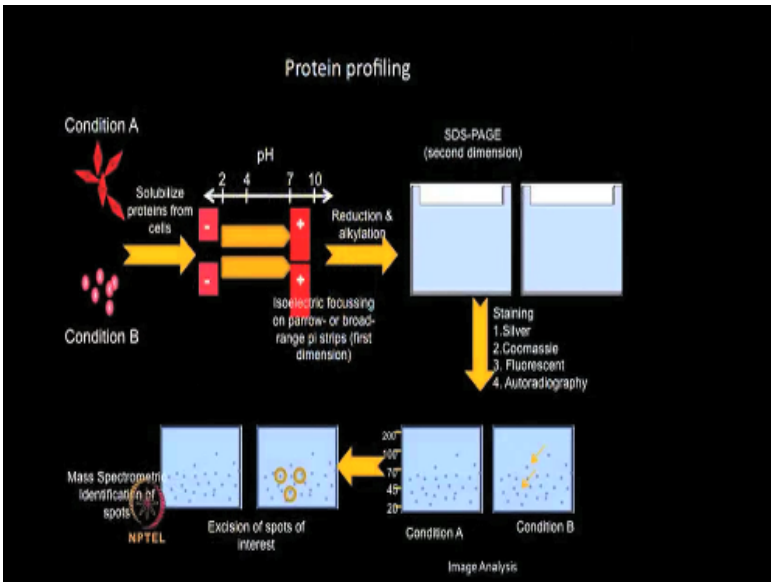

From condition A to condition B you need to solubilize the proteins from the cells and separate all the proteins by applying the pH which is shown here in the first dimension. After that you need to do reduce and alkylate your samples and then separate that in the SDS page based on the second-dimension molecular weight depending on your staining method being used. Whether Coomassie brilliant blue or silver stain different type 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 the expression those can be the proteins of your interest. One need to analyze that from different type of journals different replicates. Both technical and biological and then obtain the it is a statistical information for the spot.

And if these are significant then this is the protein of your interest which you like to identify by using mass spectrometry.

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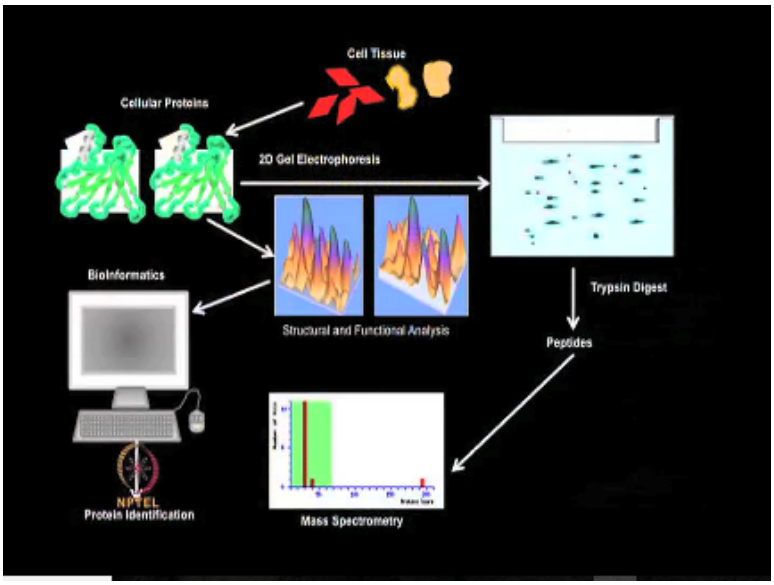

So, I am giving you a view of abundance-based proteomics or differential expression profiling where you can separate the proteins and you can compare the abundance of each spot as shown in the 3D views. And then the spot of interest could be trypsin iced and it can be identified, the peptide spectra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

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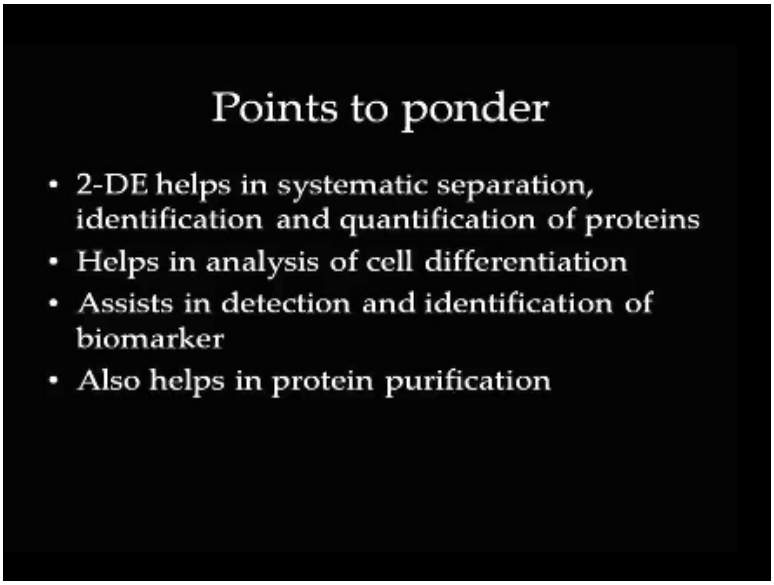

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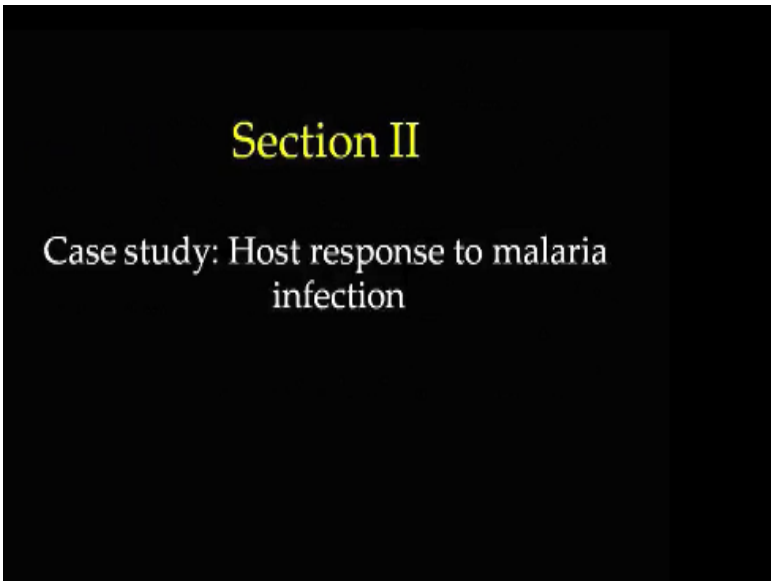

Now let us move on to the case study.

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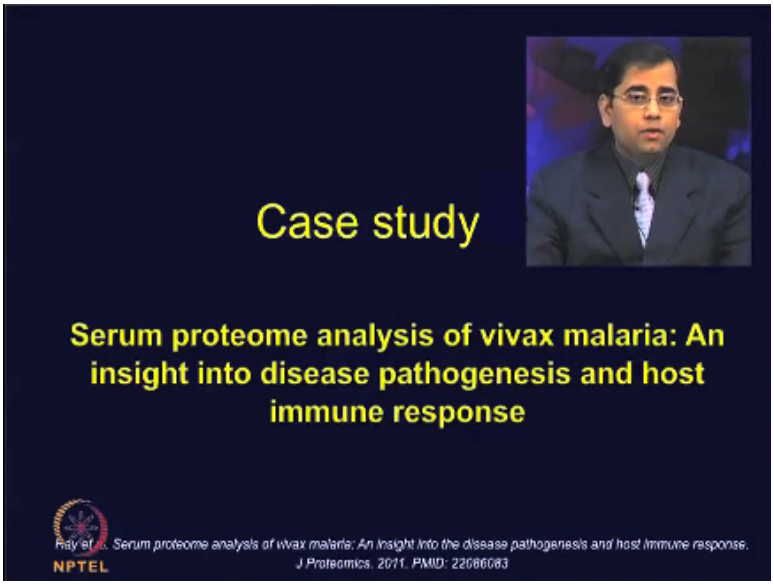

In first study we will talk about serum proteome analysis of vivax malaria and insight into disease pathogenesis and host response a study by Ray et al. So, the study was performed by my group and we have tried to investigate the host response against malaria pathogen vivax.

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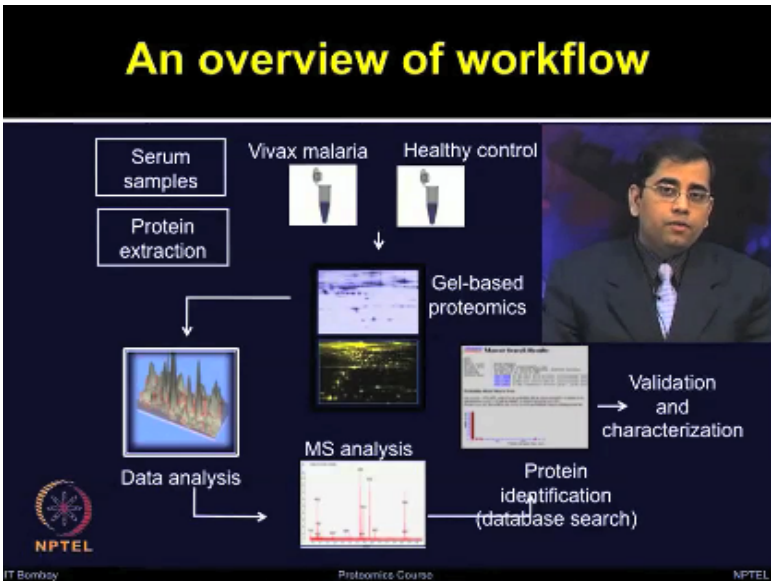

So, this slide is an overview of the work flow which we followed to investigate the host response against plasmodium vivax in human serum. So, serum samples were obtained proteins were extracted and the protein samples were compared from the healthy and vivax patients by applying gel-based proteomics including two-dimensional electrophoresis and difference in the electrophoresis which separated large number of proteins on the gels.

Compared the data to look 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 a disease.

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So, before we move to details of this experiment performed in the study. Let me show you this animation to give you an overview of the work flow followed in this experiment

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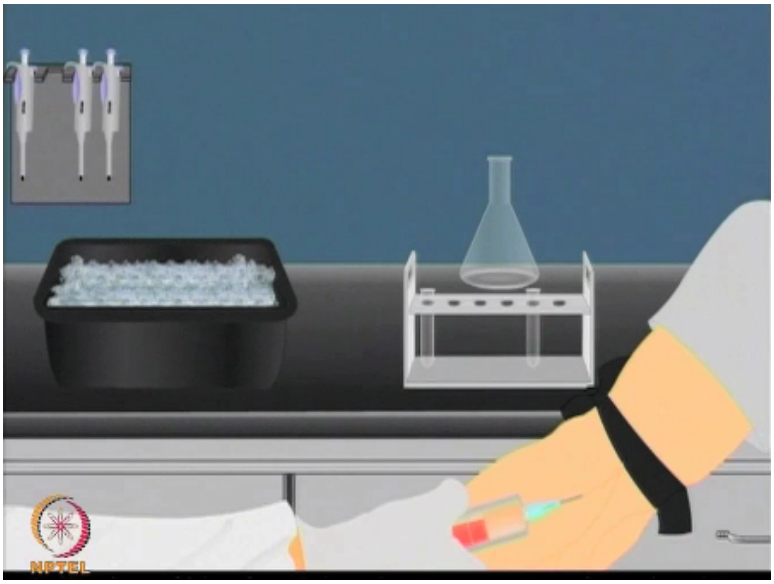

So, let me give you an overview of all the steps involved in performing such an experiment. So, first of all carefully we draw the intravenous blood in to a vacutainer tube.

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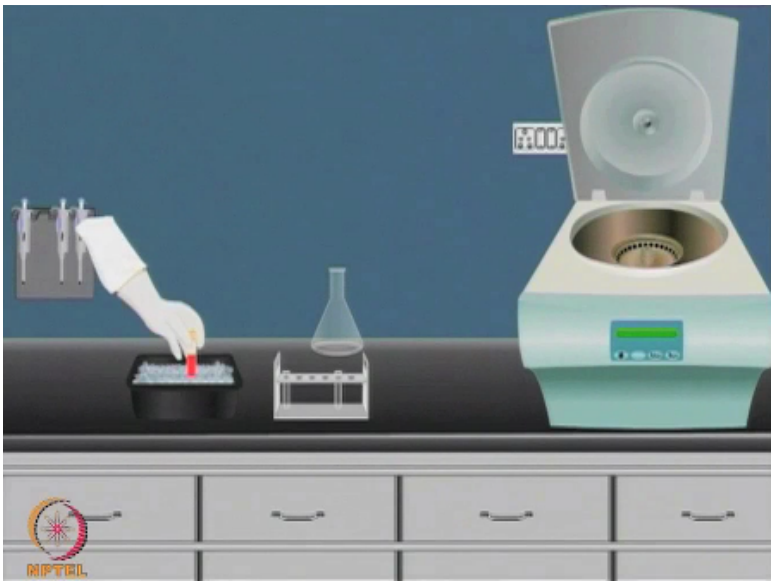

And store the tube on ice to allow the blood to coagulate, centrifuge the content to separate coagulated blood cells and clotting factors from the serum which from a clear supernatant.

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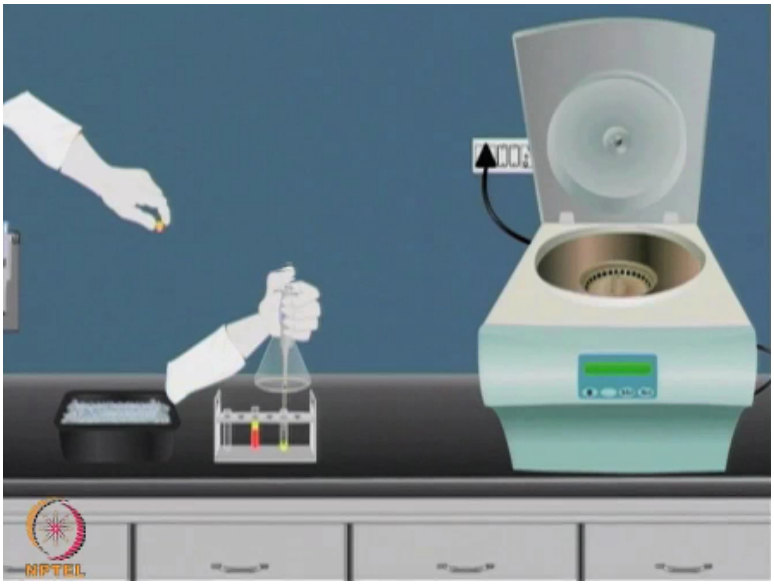

After configuration is done then collect the obtained serum in a fresh tube.

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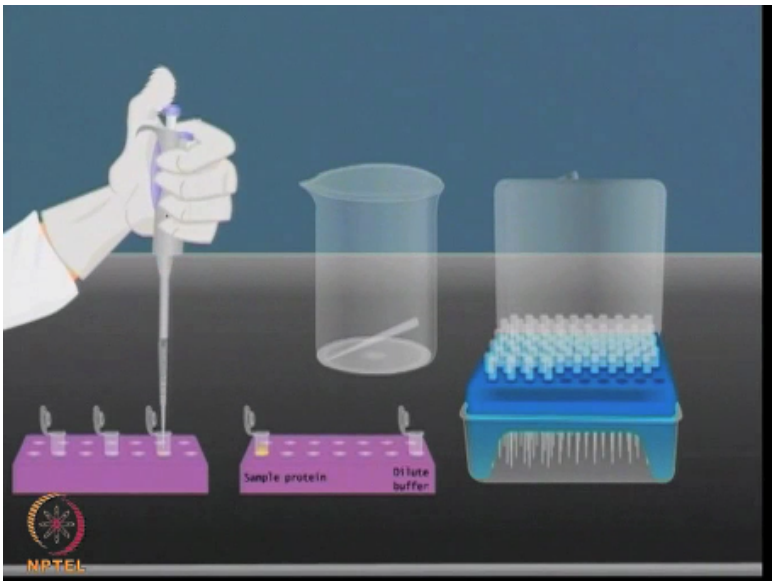

Solicit the serum to break down any large proteins complexes serum contains several proteins in a wide range of concentrations of which albumin and immunoglobulin G are the most abundant ones.

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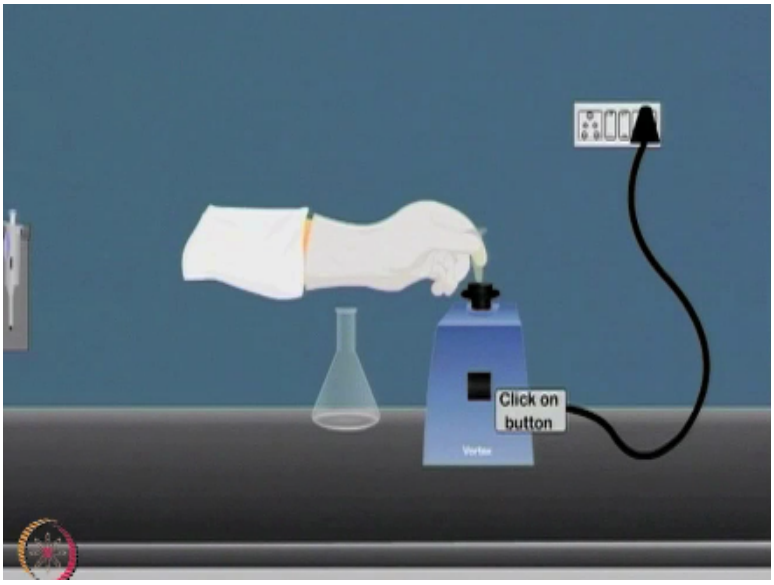

The presence of such high abundance proteins can interfere with the experiment therefore we remove we need to remove these high abundant proteins.

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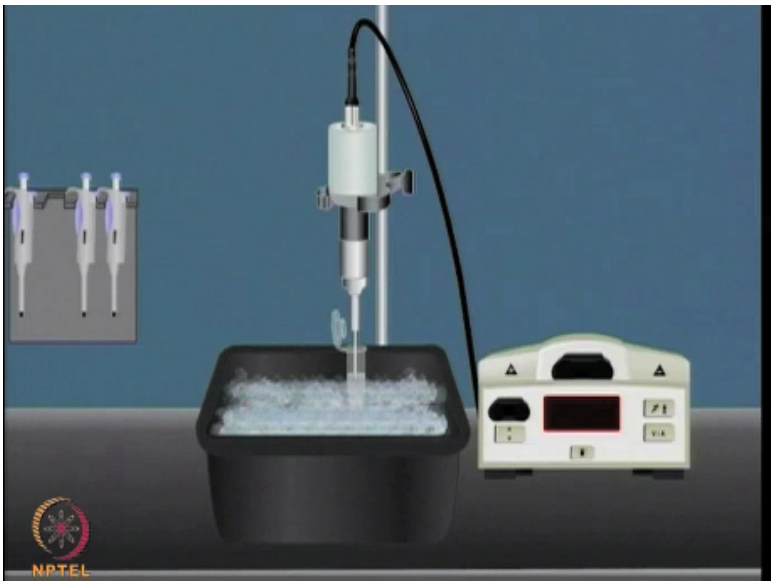

So, this ferment we try both sonication and depletion of the high abundant proteins.

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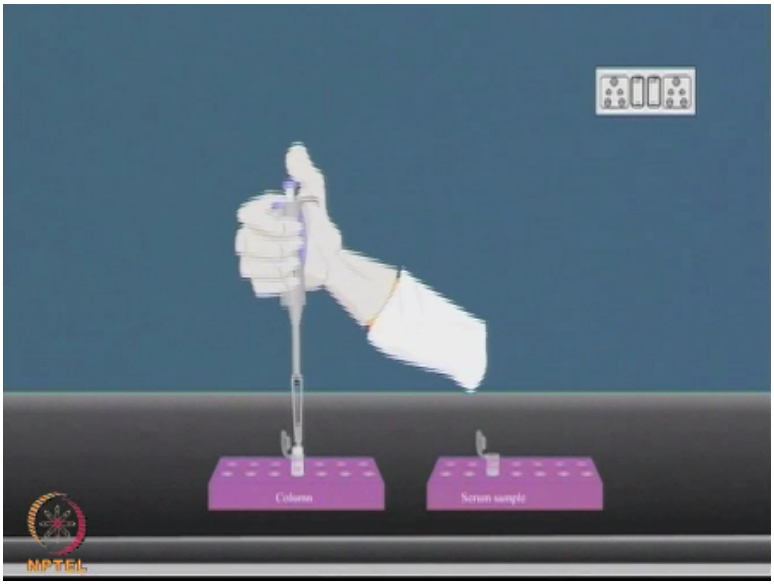

After sonication is done then we can add the serum sample on the columns.

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Usually these are commercially available which can deplete various high abundant proteins.

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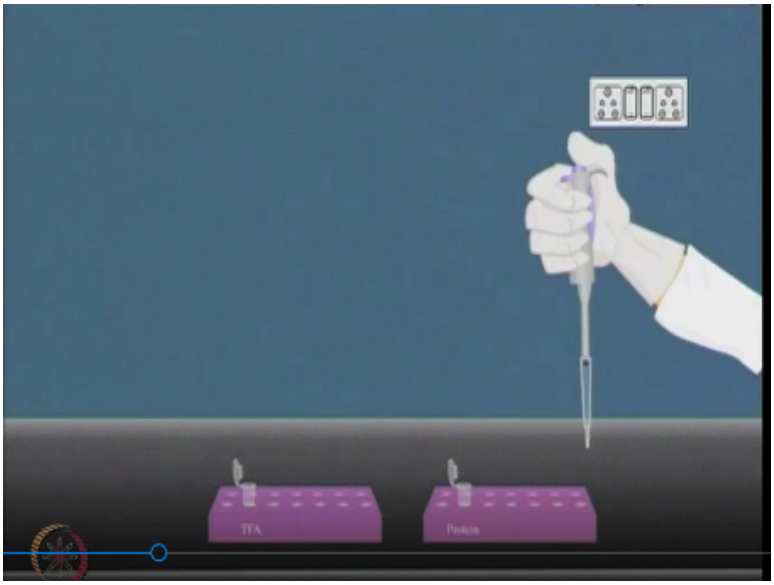

Now the depression column buying only the high abundance proteins onto their matrix through the affinity interactions, once the serum has been processed using a depletion column precipitate out the remaining proteins by using trichloroacetic acid and acetone. Once the protein extraction is done then we need to add the rehydration buffer.

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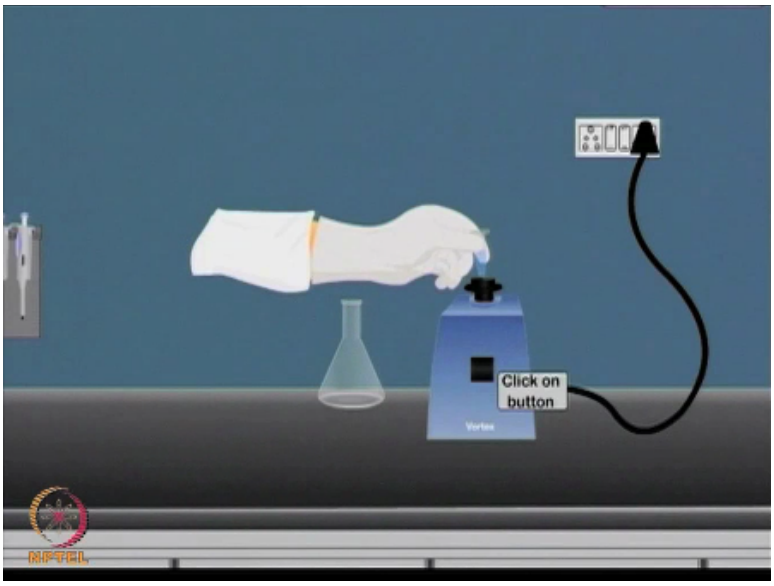

Which contains Urea, thiourea, chaps, DTT and BPV.

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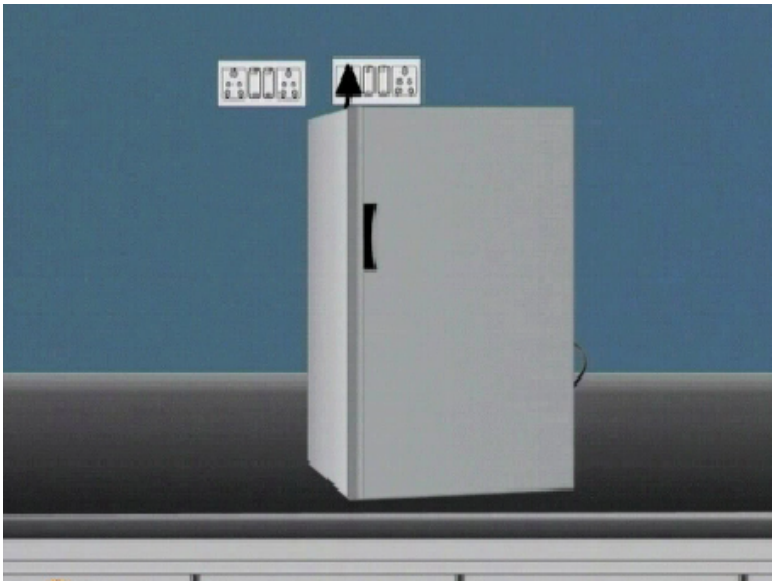

After overnight inhibition then we can centrifuge and collect the supernatant.

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Now we will watch the quantification of the proteins.

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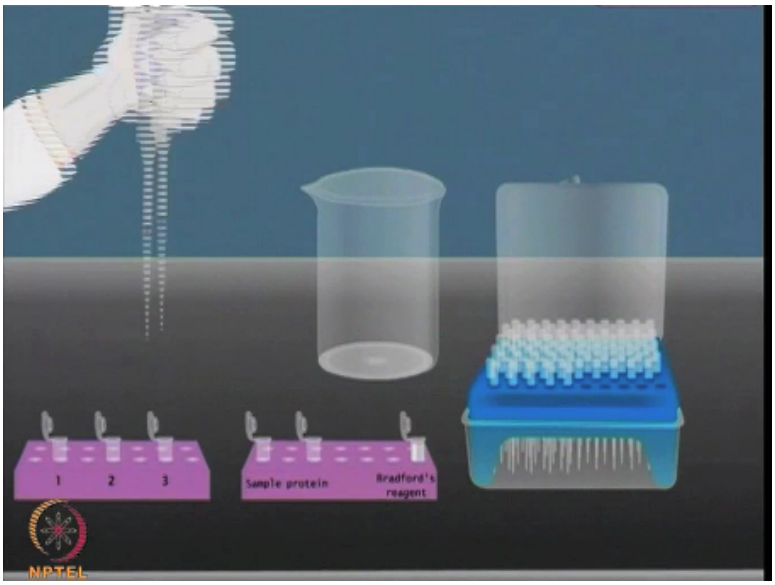

So, prepare the Bradford reagent and label the tube suitably for the standard and test samples add the standard and sample solutions to their respective tubes. Now add the Bradford color reagent to the tubes and mix them thoroughly.

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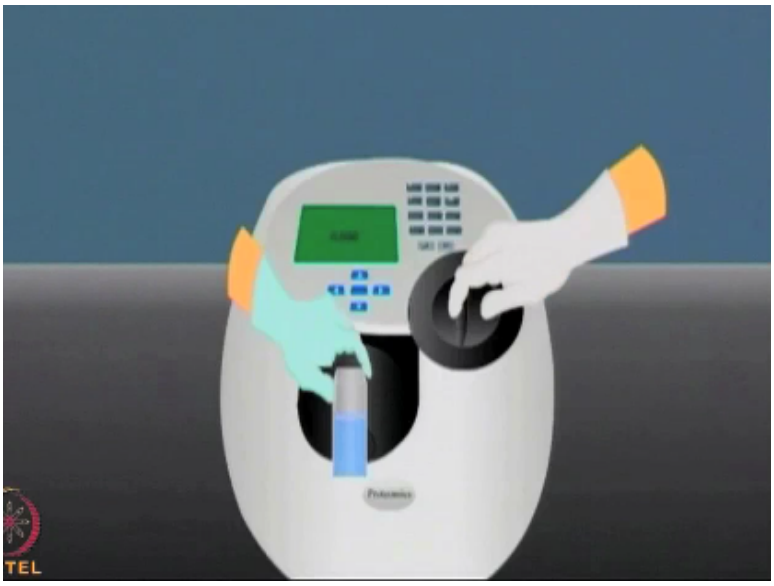

Once the color reaction has occurred then perform the absorbance measurement at 595 nanometers.

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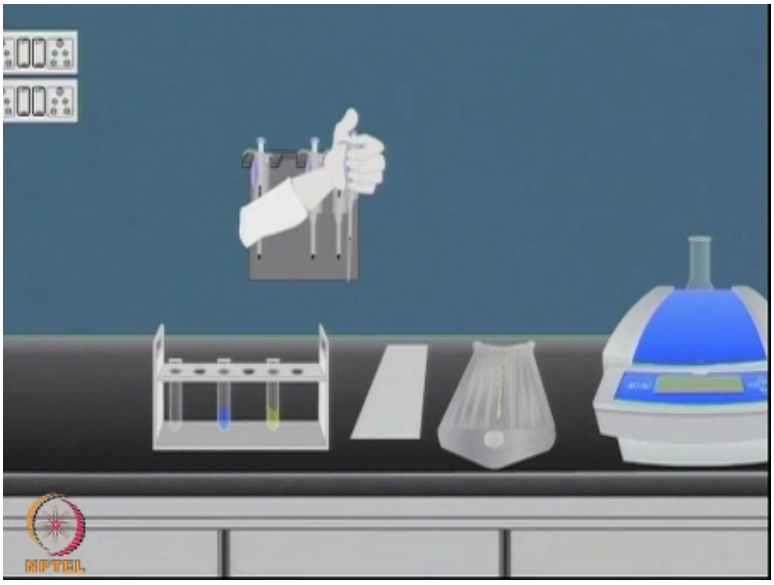

Once you obtain good amount of proteins which will determine by the protein quantification. Now proceed for the rehydration of immobilized pH there in the strips and focusing of these rehydrated strips.

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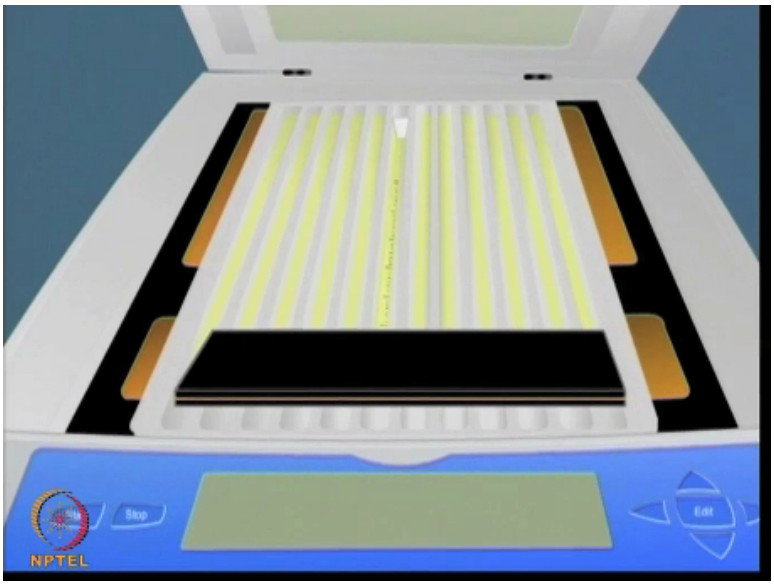

So, remove the IPG strip from the cover place it in the rehydration tray. Add the reconsidered protein sample on this strip. Pour mineral oil to prevent it from the dry and then move forward for the isoelectric focusing. So, place the width and ideal length of the IPG strip followed by an electrode at each end fill all the adjacent dwells with the mineral oil to ensure the uniform current flow.

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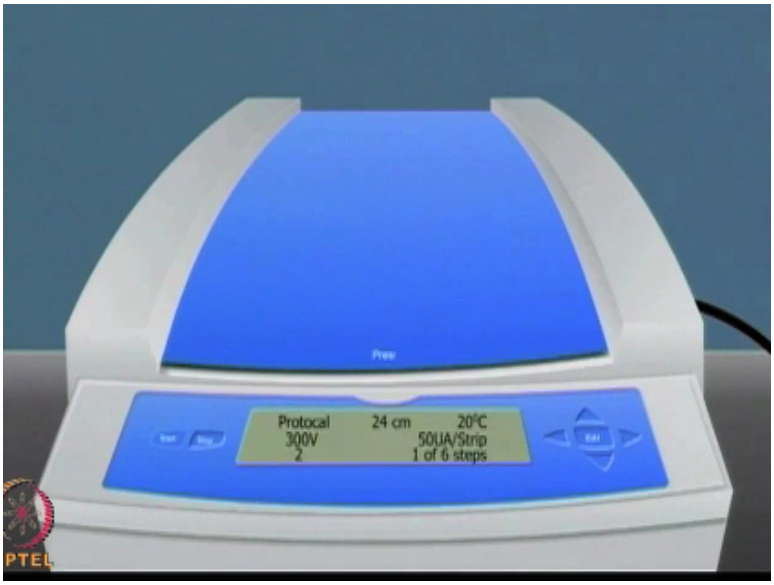

You can input the desired protocol on the instrument software along with the details of its strip length pH range number of its strip etc. and to start the focusing process.

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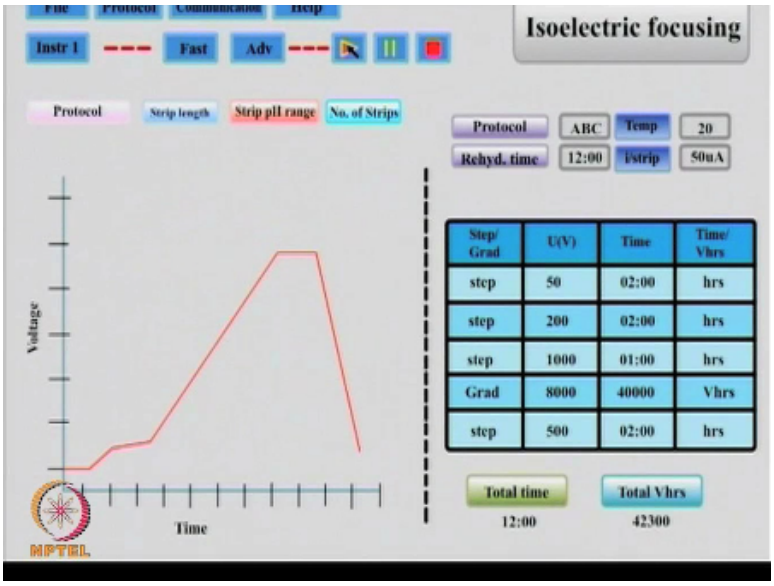

You can monitor the focusing progress on the software and you need to ensure that focusing is proper. During the IEF proteins will migrate along the strip and come to rest at a point when their net charge become 0 which is known as their Isoelectric point.

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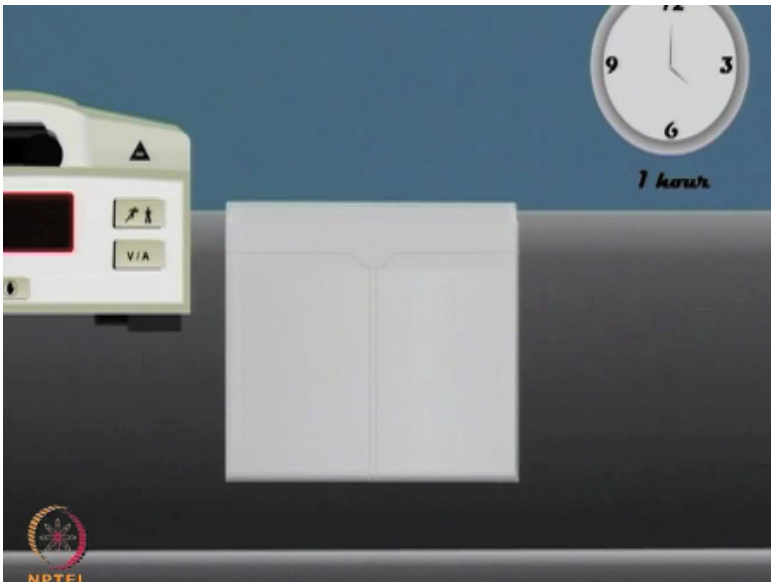

After the IEF is done then we need to separate the proteins in the second dimension on SDS page. Remember in between you have to do the equilibration strip as well. SDS page which constitute the second dimension of two-dimensional electrophoresis involves assembly of the gel apparatus gel casting equilibration of IPG strip followed by placement of the IPG strip on the gel and protein separation.

So, prepare the gel casting solution consisting of acrylamide, bisacrylamide, Trichlorides, APS and TEMED.

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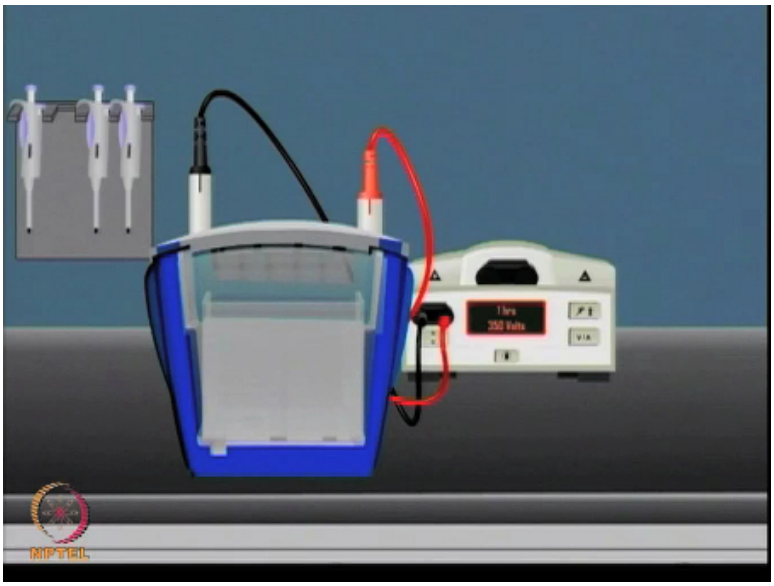

When the gel is polymerized then you can add the IPG strip and now ensure that assembly is properly sealed, and buffers are properly placed. So, that proteins can be separated based on their molecular weight depending on the gel length you can perform the second-dimension separation for a few hours.

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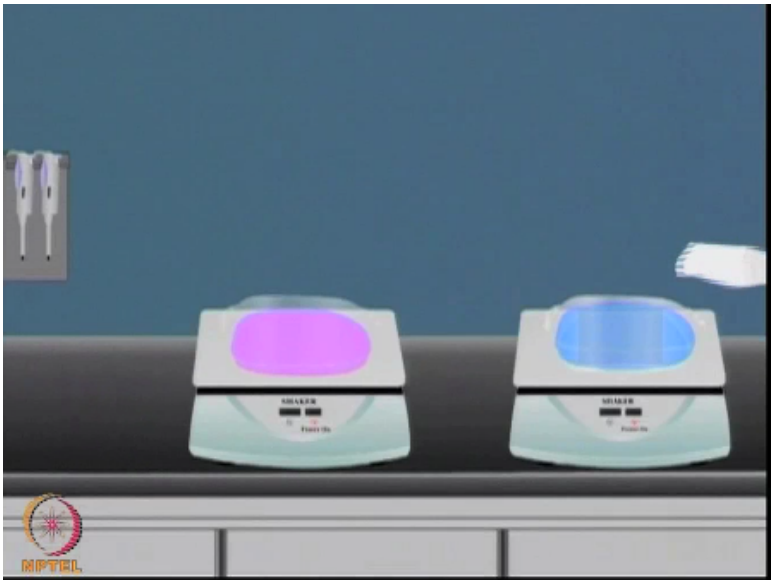

After second dimension separation is done then we can perform the staining and D staining. So, this process involves the removal of the gel from the electrophoretic assembly followed by

treatment with a fixing, staining solution and finally the D staining solution. You need to ensure that proper shaking conditions are maintained, and you need to allow 10 to 14 hours usually overnighted step further for staining as well as D staining steps. After D staining is done then you can see protein spots on the gel with very clear background.

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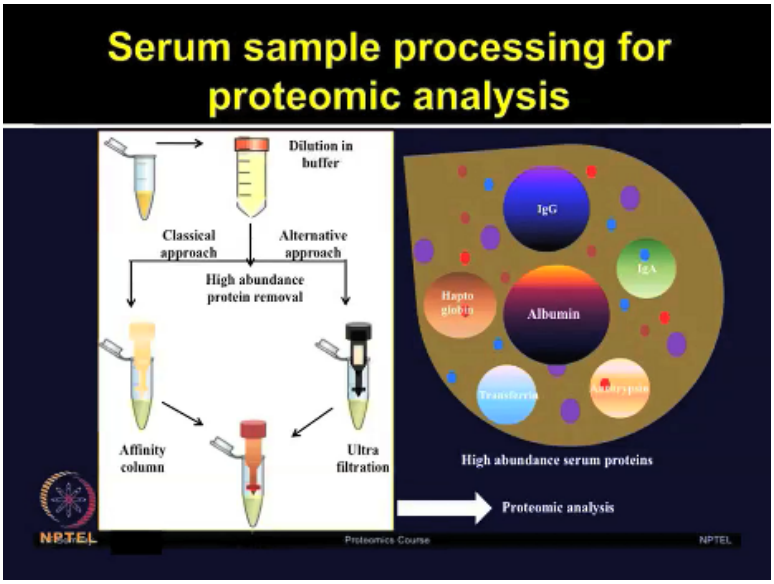

A serum poses challenge of abundant proteins. So, first of all we had to remove the abundant proteins from the serum there are standard columns available from various commercial manufacturing from which one can remove selectively the very highly abundant proteins. Such as serum albumin IGG and various 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 type of samples. we took crude serum.

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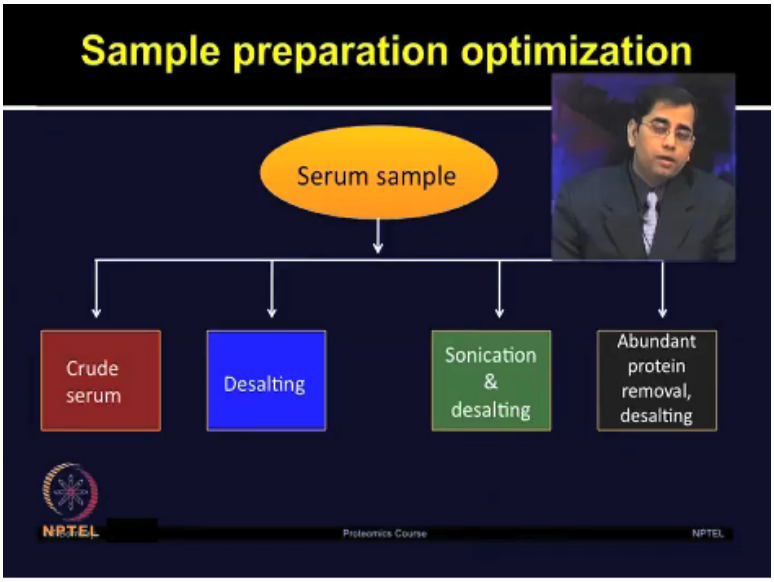

Because serum will have mostly the protein content. so, we thought can we directly apply the serum on the IPG strip immobile pH in a strip separate the protein and see how many proteins we can separate on these 2-D gels. But serum contains a lot of salt so Isoelectric focusing itself becomes challenging if you have crude serum. So, we also tried whether we remove the salt component of it and after desalting if we do the IEF.

Since serum contains highly abundant proteins and this commercial column which can deplete these abundant proteins selectively through the quite costly and considering that large number of patient's sample you have to process. So, we thought can we disturb 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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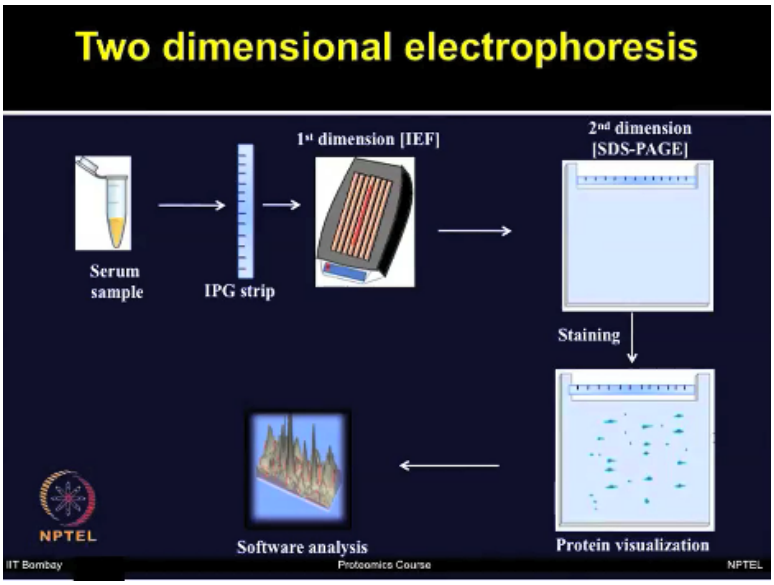

Now sample was collected and processed with all these four variables which I talked to you and after that the standard procedure of two-dimensional electrophoresis was followed.

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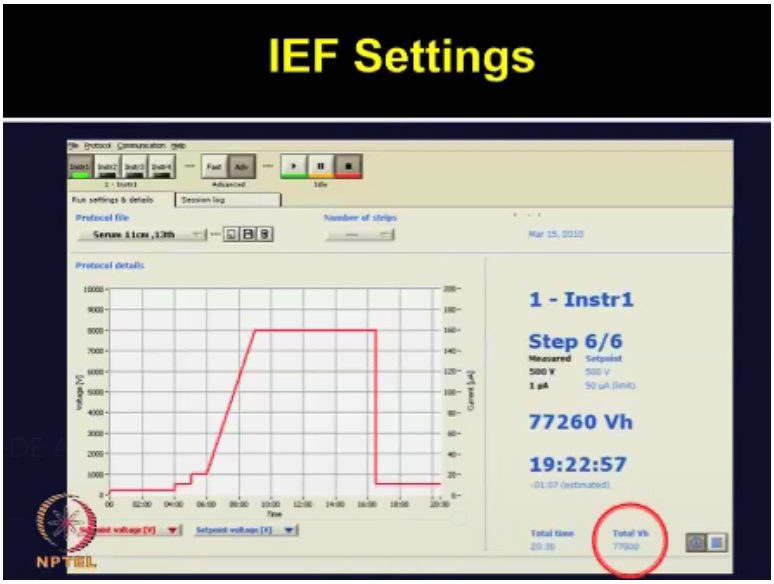

Now we are doing this serum protein separation the IEF becomes very critical because you have a lot of salt component in it and if you salt removal is not effective your Isoelectric focusing run may not be very smooth. So, for the software you can monitor how smoothly your Isoelectric focusing is progressing.

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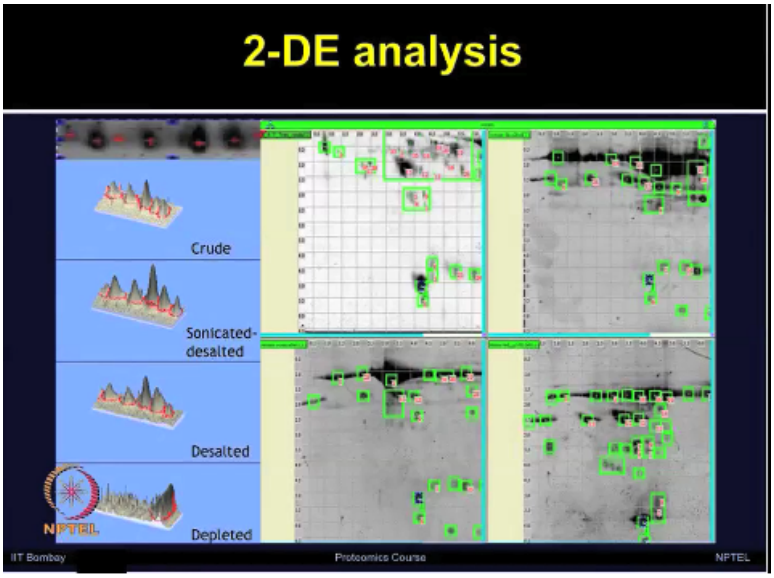

So, we tried various type of effects crude serum alone, sonicated and desalted, desalted alone and depleted and Desalted and then tried to compare the effect of these type of processing on overall proteome coverage. We also tried to see that how many proteins we can separate on the small gels.

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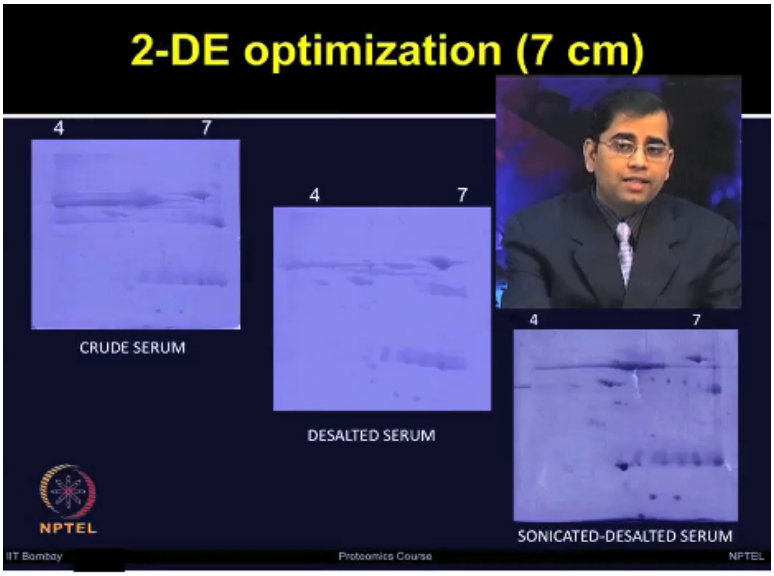

And then as expected the small gel of 7 centimeter cannot provide us very large number of proteins but even these gels can be used for extended digestion process and as you can see it is starting from the crude serum to the Desalted and then finally sonicated and Desalted gives us better coverage of the proteome.

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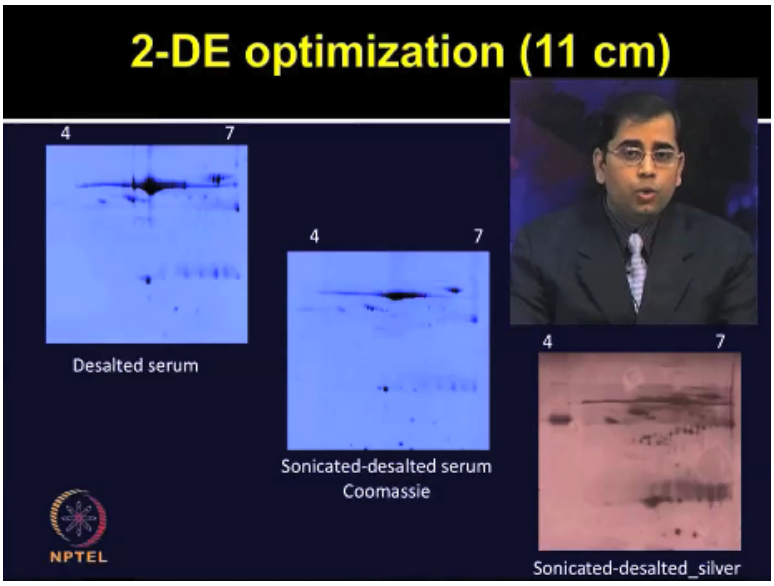

We tried the same with the even largest strip 11 centimeter and it was again quite convincing then we move on to the very largest group 24 centimeter for the clinical studies. So, in general we took 40 micro liter of the serum which was precipitated with the 4 volumes of ice-cold Acetone.

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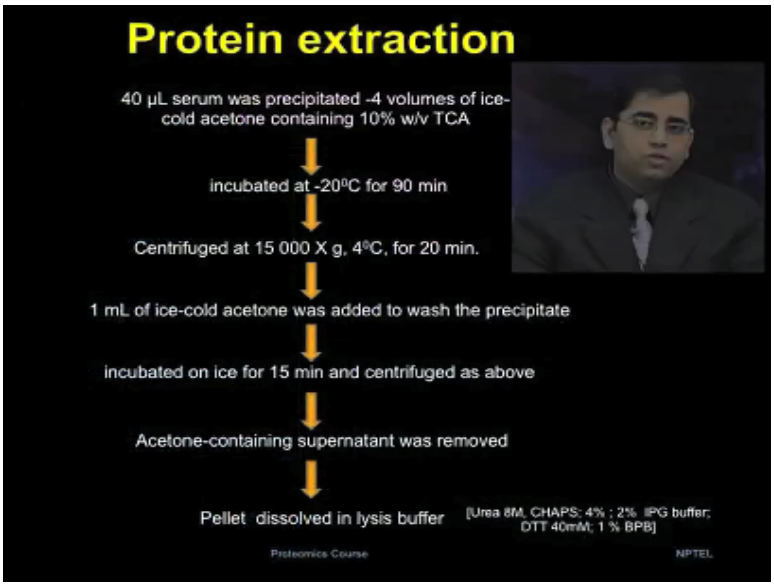

Containing 10% of TCA intubated at -20 degrees centrifuged followed by added 1ml of ice- cold acetone to wash the precipitate then incubated it on the ice for 15 minutes and again centrifugation was performed. Acetone containing supernatant was removed and then pellet was dried in the lysis buffer. The recipe for lysis buffer we have talked earlier when we discussed about sample preparation.

Which is also mentioned in this slide here you can use urea, chaps, IPG buffer, DTT and BPV. So, from the previous study we looked at replicate gel and it is three here and we found that these desalted, sonicated and depleted sample give us largest number of a spot on the gel as compared to crude alone or desalted alone or desalted and sonicated.

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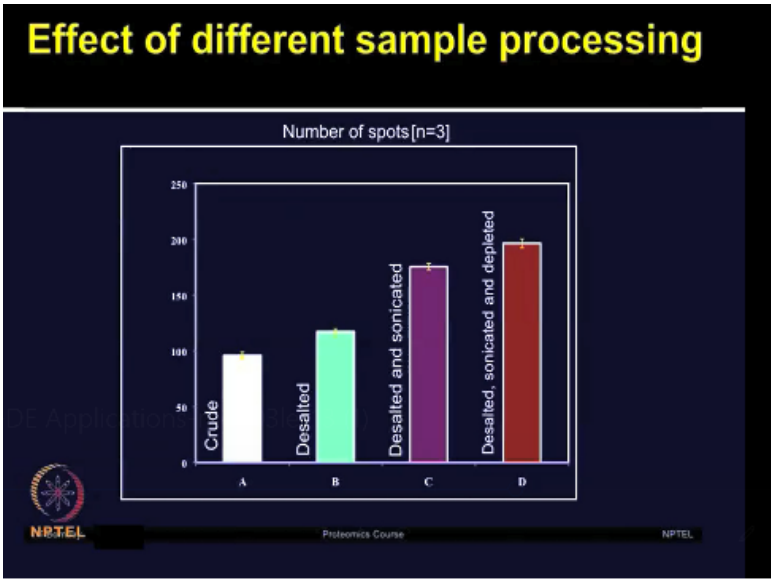

So, these studies were performed on the small strip designed the procedure but were able to draw the conclusion based on the reported bill pattern obtained in these gels and then we applied the desalted and sonicated and depleted. This conditions for the processing all the samples.

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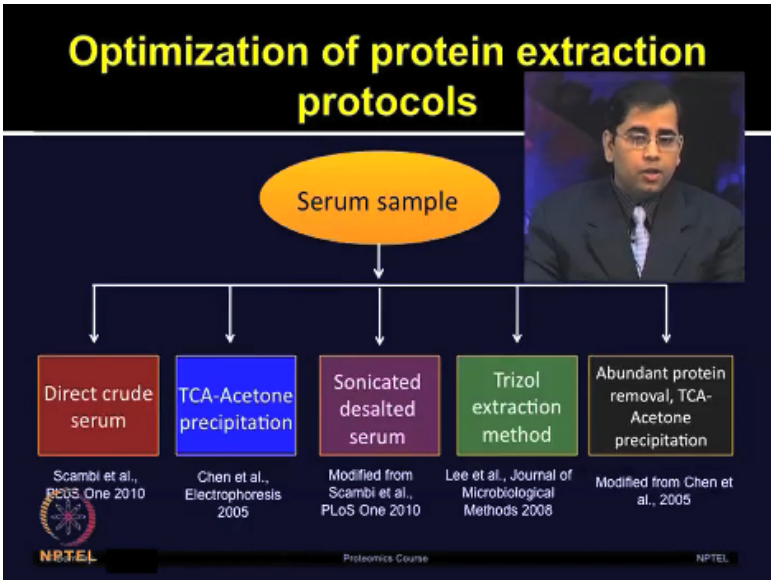

Now after processing off all these samples now how to extract the proteins. So, protein extraction protocol for those optimized and to optimize these protocols first of all we looked at what are different methods available in the literature. So, people have applied right crude serum, TCA Acetone precipitation, sonicated desalted serum, Trizol extraction method as the less abundant protein removal and TCA Acetone precipitation.

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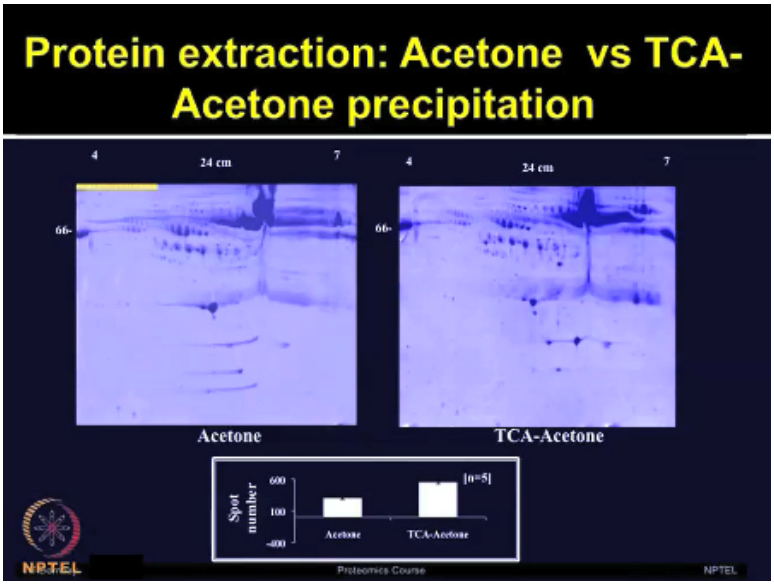

So, we used this modified procedure of TCA Acetone precipitation where we used depleted and desalted and sonicated serum. We also compared the effect of Acetone alone or TCA Acetone precipitation. As you can see in the slide the left one is showing the over all proteome spot present in the Acetone and right-side slide showing the TCA Acetone precipitation. Overall more number of spots were resolved on the TCA Acetone precipitated gel.

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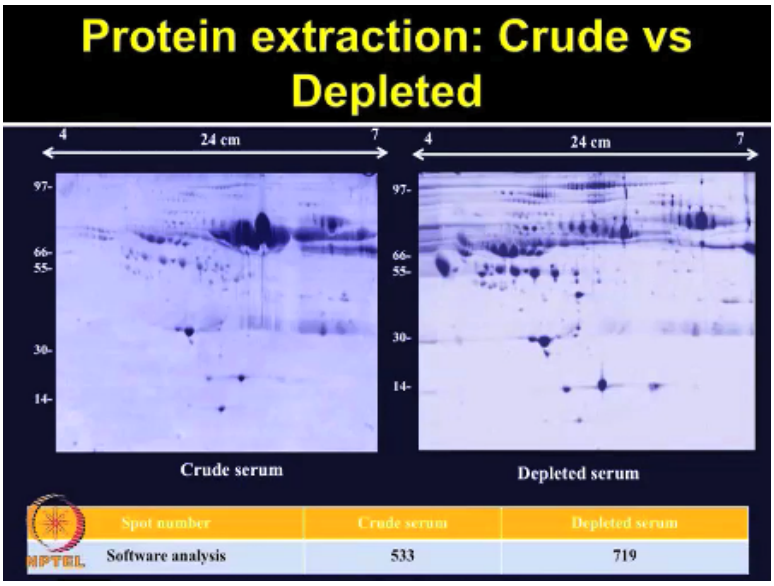

Since there were conditions on the small gels earlier we also tried to apply that on the large gel of 24 centimeters because that is where we have to perform all our analysis. So, we again compared crude versus depleted protein response of the serum samples on the larger and as expected the depleted serum sample showed more number of a spots as compared to the crude serum samples.

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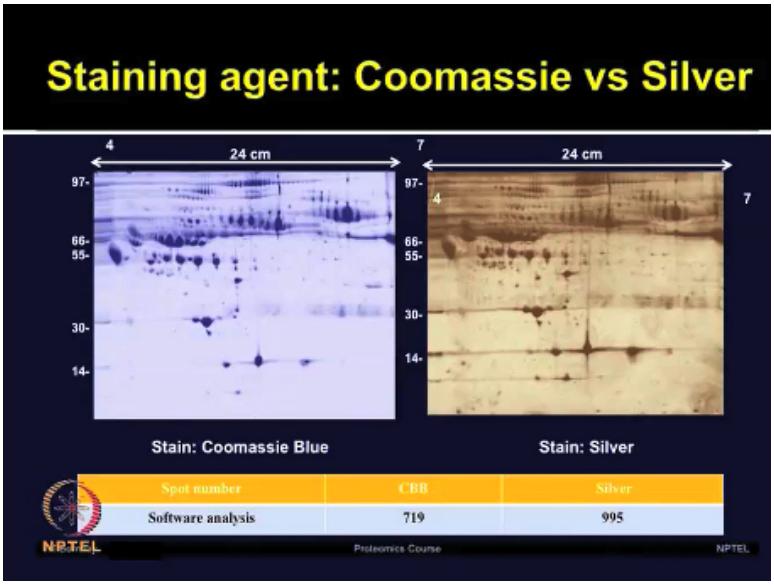

We also try to look at effects of various type of a staining whether it is Coomassie blue staining or silver staining. We definitely identified more number of spots from the silver staining but just because of we want to compare large number of clinical samples with the different healthy controls it was very difficult to standardize the conditions to keep it uniformly staining for the silver.

So, to overcome this limitation we use the Colloidal Coomassie and Bio safe Coomassie staining and we standardize the same condition for all the gels.

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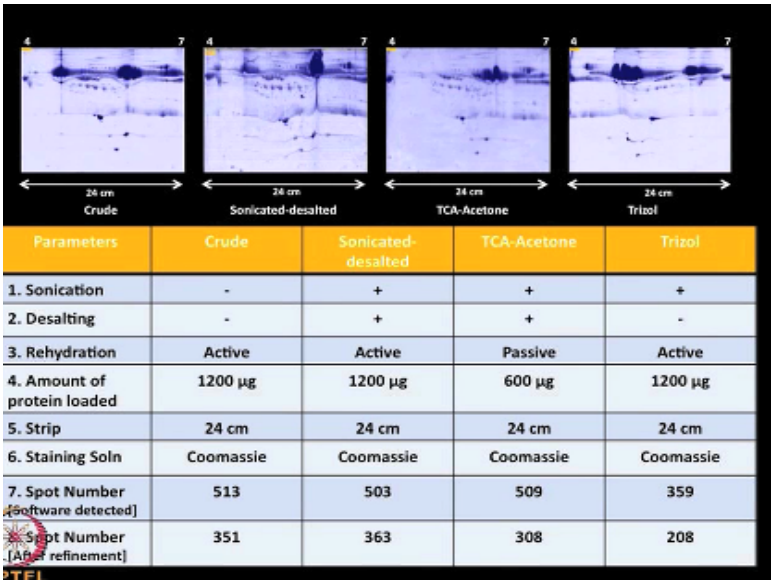

Parameters	Crude	Sonicated-desalted	TCA-Acetone	Trizol
1. Sonication	-	+	+	+
2. Desalting	-	+	+	-
3. Rehydration	Active	Active	Passive	Active
4. Amount of protein loaded	1200 µg	1200 µg	600 µg	1200 µg
5. Strip	24 cm	24 cm	24 cm	24 cm
6. Staining Soln	Coomassie	Coomassie	Coomassie	Coomassie
7. Spot Number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

So, this slide gives you an over view of various types of parameters which we investigated from different type of treatments crude serum, sonicated-desalted, TCA Acetone precipitation and Trizol extraction methods. So, we looked at effect of sonication, desalting, rehydration, amount of protein loading, type of a strip, staining solution. How many number of spots we can resolve in these gel and software analysis automated gives lot of artifacts.

How many real spots we can obtain after the manual refinement? So, I am trying to give you all the details what one need 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 type of concepts which we have talked in the sample processing. And in different lectures of two-dimensional electrophoresis.

So, now after doing a lot of comparative analysis and standardizing the protocols for sample preparation and protein extraction.

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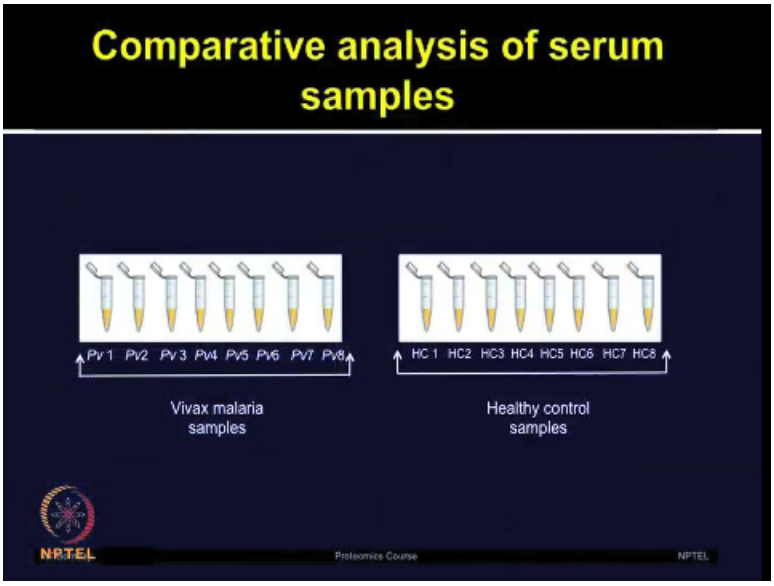

Now we are ready to perform the comparative study on serum samples of vivax patients with healthy controls.

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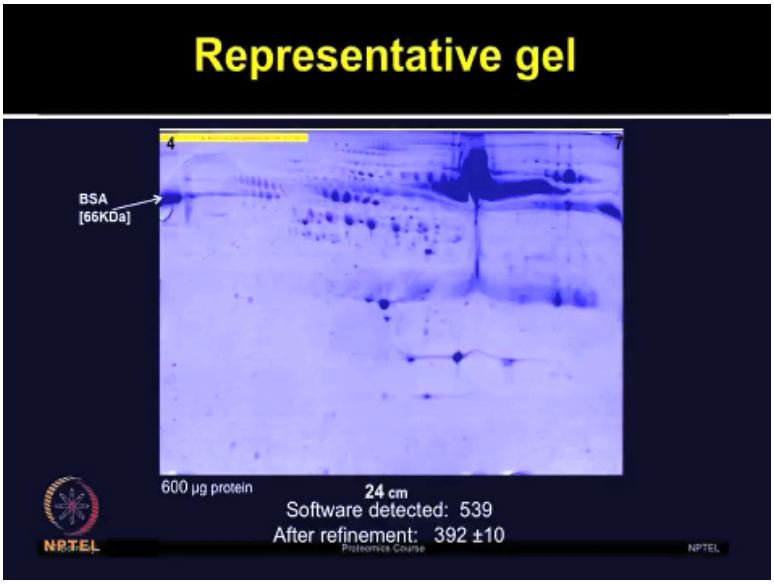

This is one of the gel image showing that that is what is expected on 24 cm large 2-D gel.

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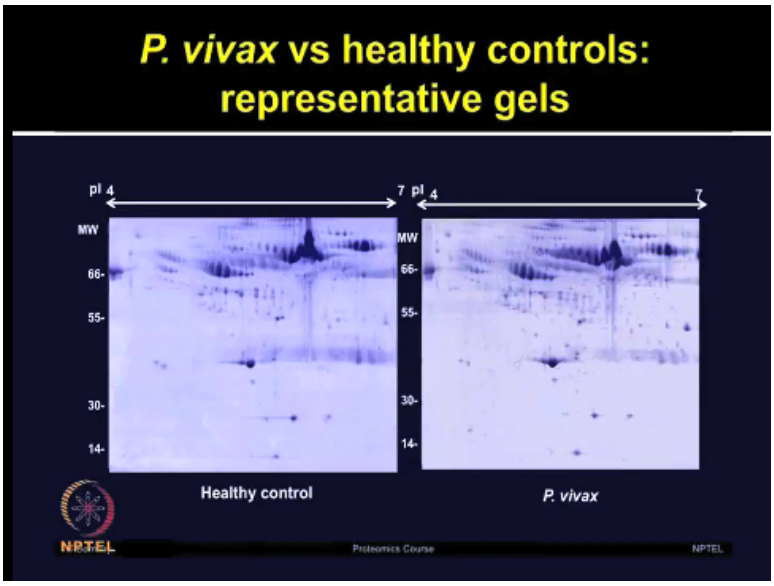

And then the representative gel will show the healthy control and plasmodium vivax treated samples. So, these samples as you can see were showing large number of protein spots resolved on these gels.

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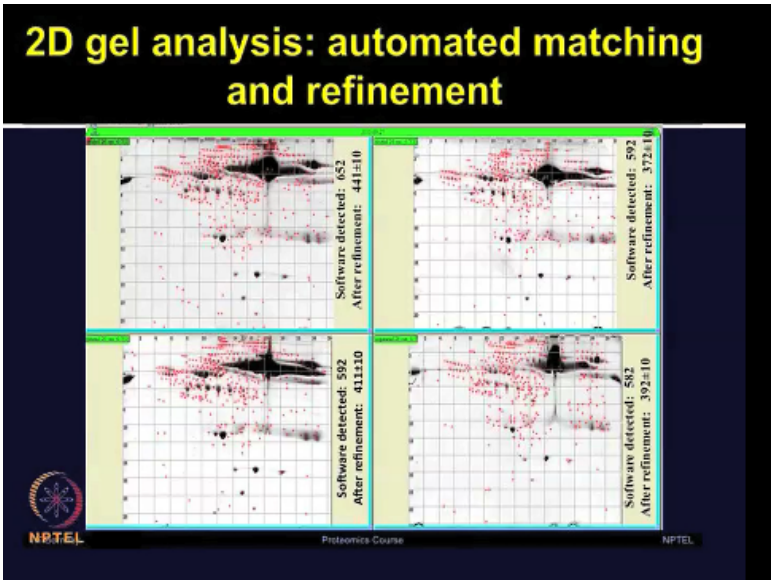

And then after separating these protein samples on these 2D gels from various patients and controls then most challenging task was the data analysis. So, automated matching as well as manual refinements were perform to obtain the how many spots are significantly modulated due to vivax infection. As you can see in the slide we had looked at various controls and various vivax patients.

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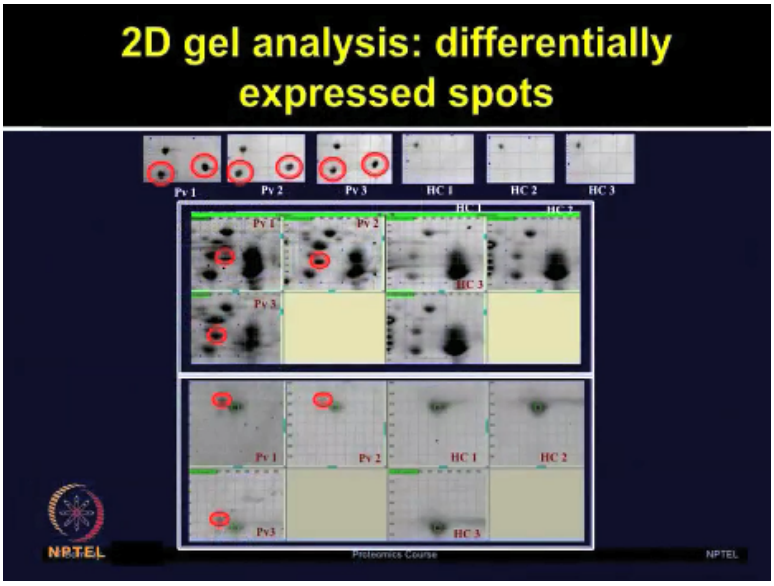

These are only few patient's samples here but we analyzed large number of samples but those spots which were reproducible in all the patients for example one you can see in the top panel 2 spots are showing significant alteration in the vivax as compared to the healthy controls. So, these types of a spots were considered for the mass spectrometry analysis.

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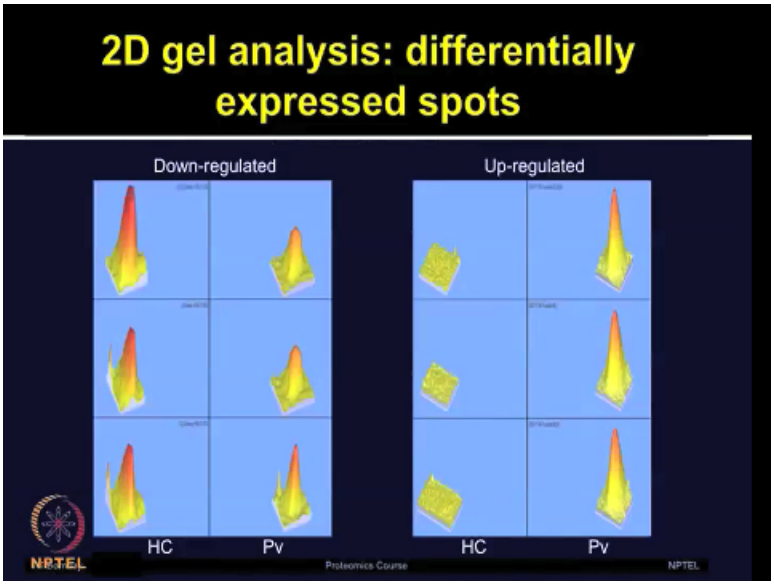

Now these spots which the phone which we found were showing good differential expression. We looked at the three-dimensional views of those spot to ensure that those are not artefacts and those are the real spots showing up or down regulation of these proteins in human's serum. Now after completing analysis we were able to obtain a large number of proteins which were differentially expressed and as you can see in this graph.

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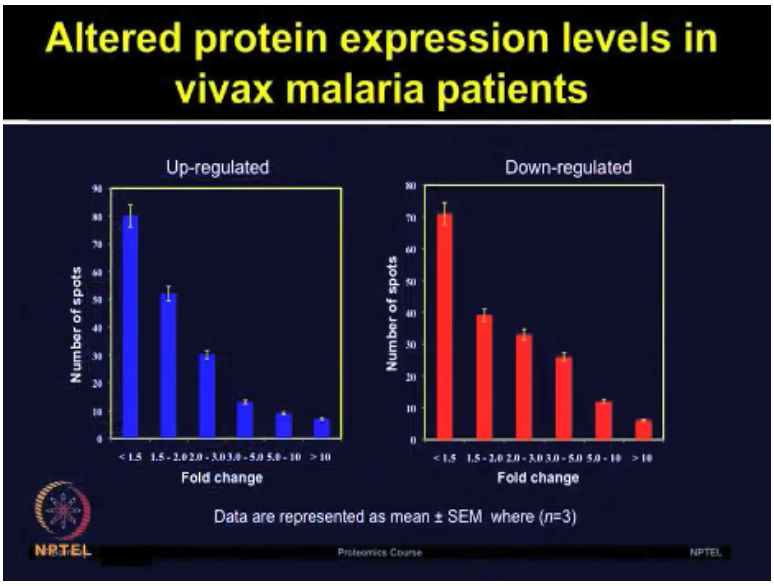

There are proteins which vary in different range of the fold change and many proteins show very less fold change between 1 to 1.54 and there are few proteins which also show more than 10-fold up or down regulation. So, these proteins spots were considered in testing because those were statistically significant and then this spot were further analyzed using mass spectrometry techniques.

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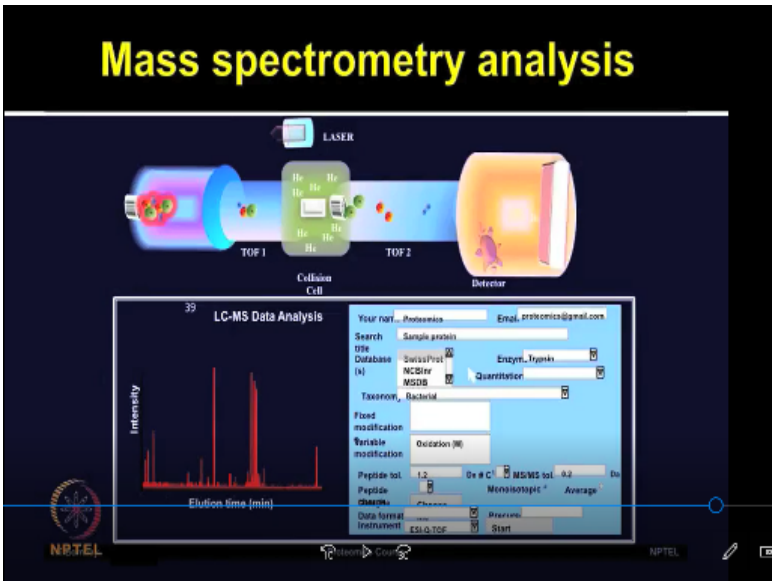

So, this slide gives you an overview of tandem mass spectrometry technique and followed by how LC MS MS data can be analyzed by using mascot search engine. Different type of positivity techniques is available including multi topped off and LC MS MS based approaches and we will

talk about these techniques in much more detail in the next model we will talk about mass spectrometry.

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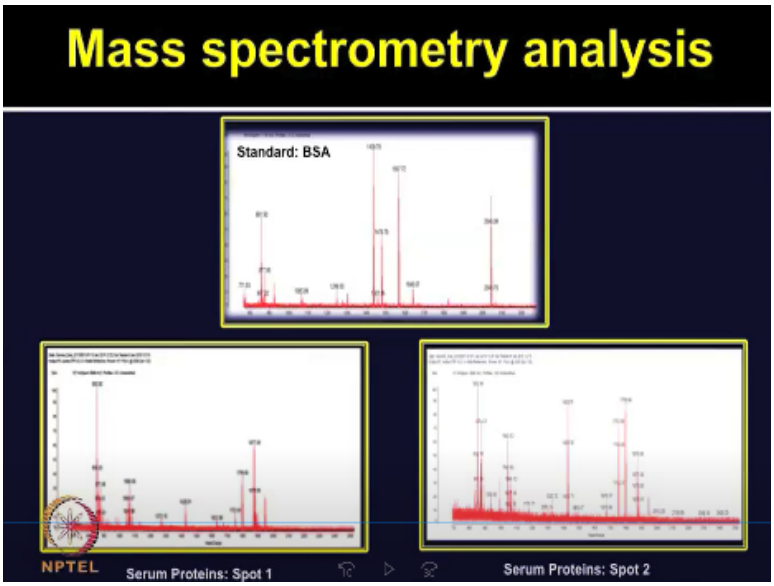

But just to give an overview here that one need to analyze a spectra and also one need to have some standard proteins to ensure that corrected spectra is obtained. So, you have to rule out of quality control checks in mass spectrometry to ensure that your data is of good quality. I will cover the details of mass spectrometry experiment when we talk about mass spectrometry in more detail in the next module

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And again, maybe I will take the study at that time that what are the different detailed procedures

required to perform such analysis. But at the end from these experiment of mass spectrometry one can obtain the identity of these proteins.

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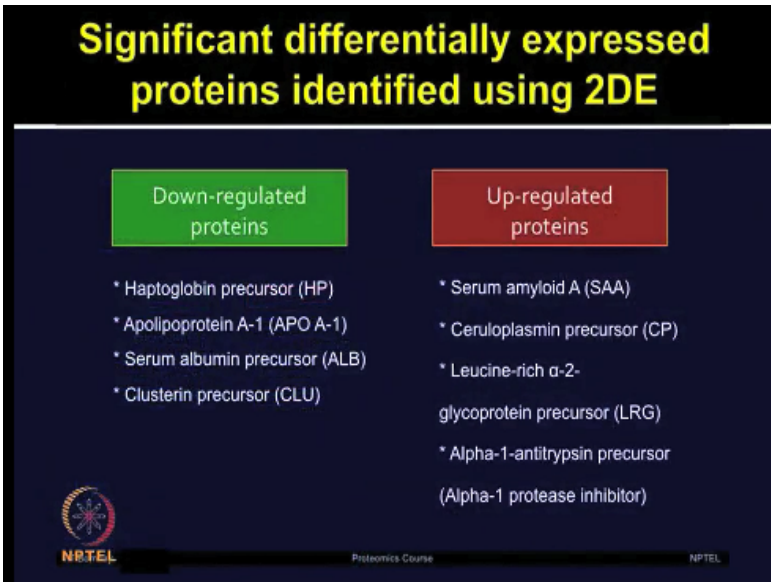

And as you can see in the slide there are several proteins in the host human where differentially expressed due to the plasmodium vivax infection and these are some of the proteins listed here such as haptoglobin apolipoprotein A1, serum albumin precursor and clustering these were down regulated and serum amyloid A, ceruloplasmin precursor, Leucine rich alpha 2 glycoprotein precursor and alpha antitrypsin precursor these proteins were upregulated among many other proteins.

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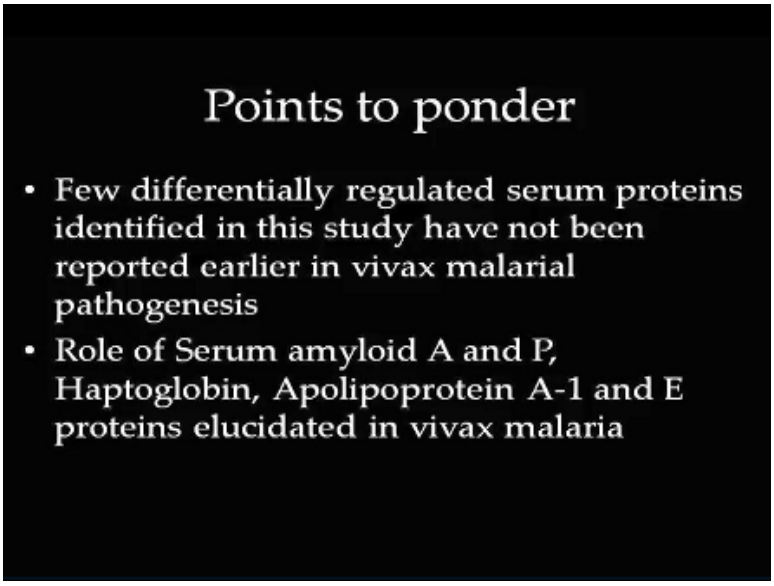

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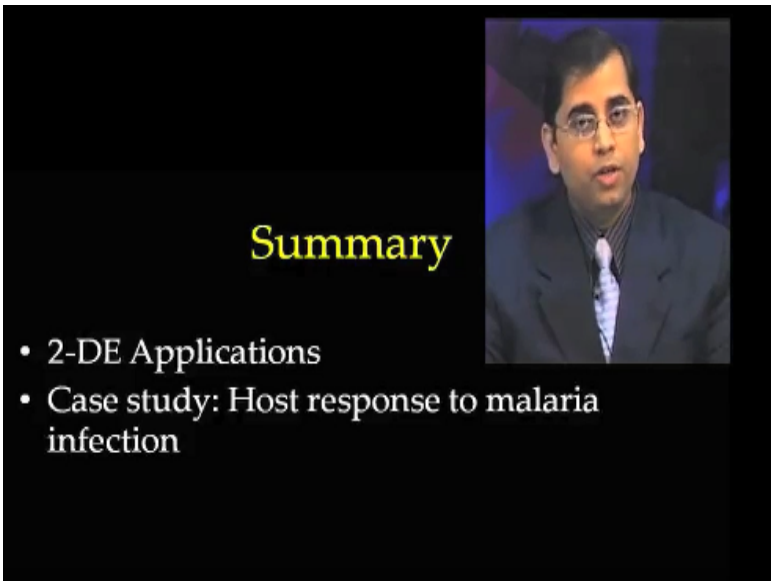

How could a two-dimensional electrophoresis technique can be applied for various biological applications by giving you a case study and details of various type of experiments one need to perform probably you got an idea that before are performing that final experiment on your case and the controls it can lot of optimization has to be performed and if starting from your sample processing.

How you can expand the coverage of the proteome these type of quality control checks are very essential. If you can increase the overall proteome coverage you can separate or 1500 or 2000 proteins on the gel reproducibly then you have a good chance of identifying various potential targets which could be normal drug target or potential biomarkers. 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 do we discuss 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 need to perform to achieve such type of comparison.

We will continue our discussion on some more applications of two-dimensional electrophoresis

and also two-dimensional difference in the electrophoresis. Because due to the variations in the 2-DE people also try two dimensional DIGE experiments and also that is very sensitive. So that also expands the overall proteome coverage. Let us continue our discussion in the next class. Thank you.

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