Proteomics: Principles and Techniques
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Lecture No. # 18
Applications of two dimensional electrophoresis

Hello. Welcome to the proteomics course. In today's lecture, we will talk about applications of two dimensional electrophoresis.In the last few lectures of this module, we discussed about two dimensional electrophoresis technique. We discussed in much detail the workflow, how various steps are involved in performing two dimensional electrophoresis and difference in gel electrophoresis.

Now today, we will talk about how these techniques can be applied for understanding some of the biological questions. Now this is time for us to assimilate all the knowledge which we have acquired during the last several lectures for example, what are the things one need to take care of during protein extraction then isoelectric focusing, second dimension protein separation followed by gel analysis.So, all of this knowledge actually integrates when you want to apply these techniques in any biological applications.

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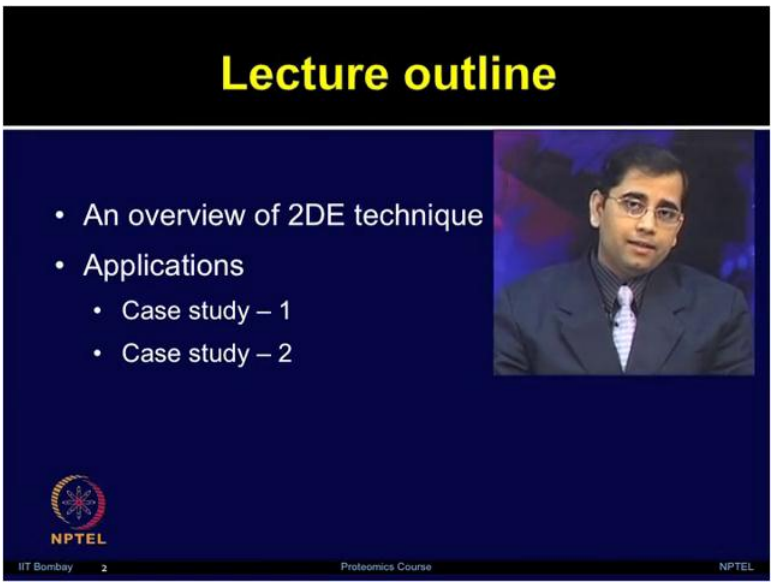

So today, I will just first give you an overview of two DE technique just to refresh you about various things which we discussed in this module and then, I will give you few example applications; we will take two case studies. Now, this two dimensional

electrophoresis technique is now widely applicable in almost all the areas of biological sciences. You name any application and you will see, there are some published report on using two dimensional electrophoresis or difference in gel electrophoresis to apply for such applications, whether it is looking at the human different diseases, looking at drug responses or investigating about plant stresses or any drug treatment in bacterial species or any other microbes.

Sothat is like you name any application and you will see that, several published report where people have used these techniques and try to look for different type of proteome changes. Now, one can look at the differential protein expression which is most commonly used if you want to compare a control with the treatment. You have certain level of exposure to disease or a chemical or a drug, and you want to know, what are all the proteins have been modulated due to that particular stimulus or treatment.

Now, this is known as differential expression. Now, if you are interested to investigate all the proteins of a given sample that will be considered in the global proteome profiling. So many times, if a proteome of a given species is not known then, proteomic techniques have been used for global proteome profile. But, most commonly they have been used for differential proteomics. So today, we will discuss two papers where authors have applied differential proteomic analysis by using two dimensional electrophoresis.

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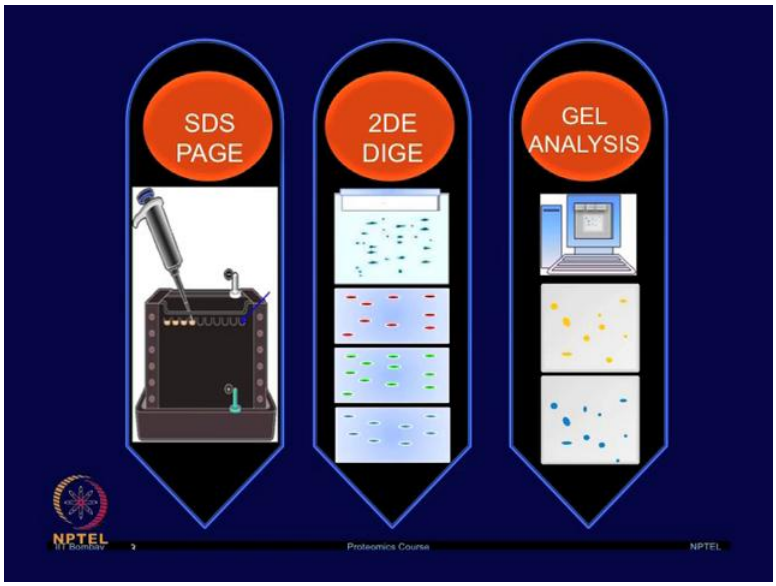

So, before Idiscuss the application, let us just refresh the whatever things we have discussed in the previous lectures on various gel based proteomic techniques. So, this slide remains you that we have talked about different approaches used in the gel based proteomics including SDS page analysis, including two dimensional electrophoresis and various advance level of protein electrophoresis including difference in electrophoresis. Then, data analysis that becomes common for all of these gel based approaches and that becomes actually very challenging aspect of it.

So performing of proteome experiment is not so tedious as compared to analyzing a large amount of data which is generated from these techniques. And most importantly, how to get some meaningful information from theseexperiments;therefore, data analysis and statistical considerations are very important parameters for obtaining any meaningful information.

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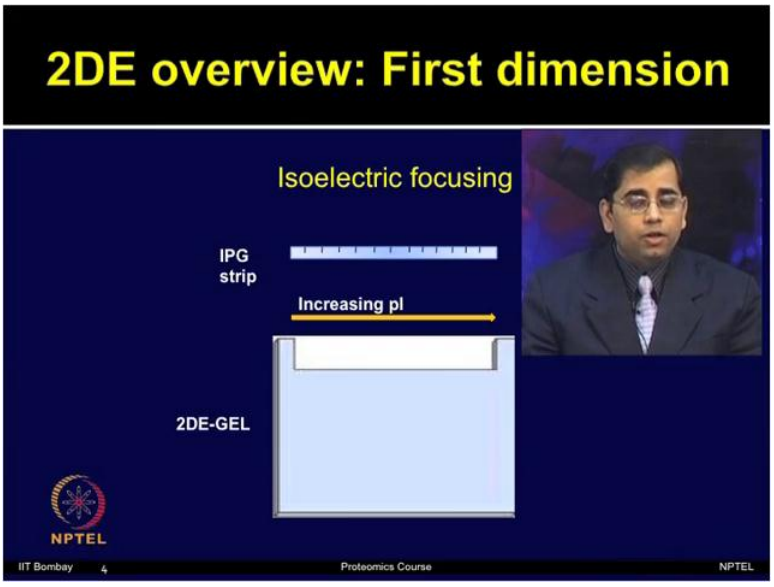

So first of all,you need to extract the protein from a given sample and that actually becomes very determining factor for various applications regardless of whatever questions you have in your mind which you want to apply by using two dimensional electrophoresis. First of all, you need to make a very good protein extract and when I said very good extract it means, it should have minimal interference with any interfering compounds. Ideally, you want to separate as many proteins as possible it means, you want to get the very high coverage of whole proteome.

So, considering the diversity of sample which one can apply on these techniques, it becomes very important that people first (( )) very good protein extraction. Once good protein extract has been obtained, and you have determined aquantity of protein then, you can apply that on the first dimension, which is iso electric focusing or i e f. So in this, you can separate the proteins based on their pI values as shown in this slide here, you can use different length of IPG strip or immobilized pH gradient strip at separate the proteins in the first dimension based on their isoelectric point.

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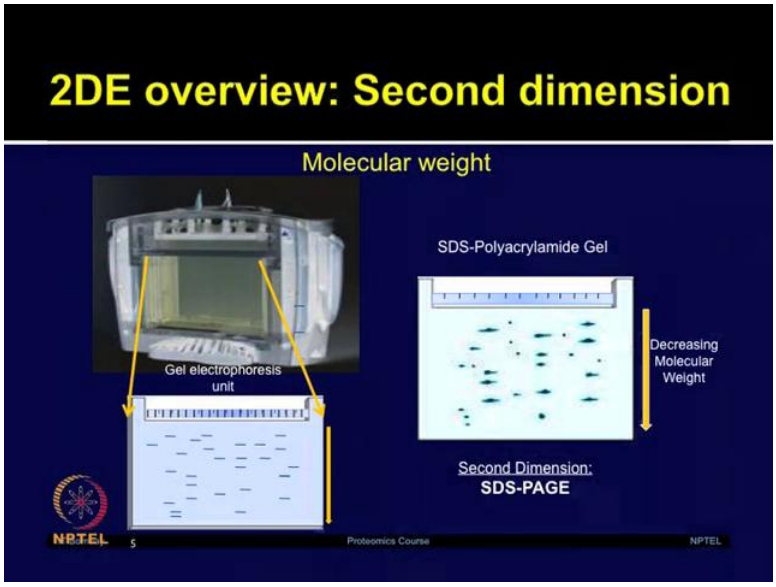

After first dimension separation, if you remember then you need to do some treatment which is equilibration, you want to prepare your strips for the second dimension separation. So in equilibration, there were two components which were very essential and if you recall, we talked about DTT and iodoacetamide. So after doing the equilibration treatment then, you can separate the protein in the second dimension based on molecular weight of the proteins.

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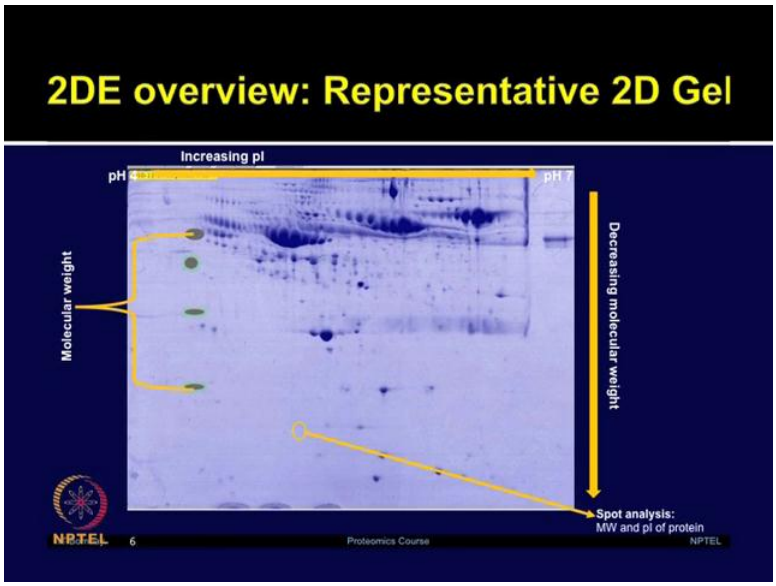

Now,as you can see in the slide, you can separate the proteins in two dimension in two different properties based on their isoelectric point and molecular weight, which is a representative 2D gel image which showsthat large number of protein spots can be separated on these gels, and immediately by looking at a given protein spot, one can obtain two information; what is there molecular weight and what is there isoelectric point. So this information one can obtain immediately and then, after comparison of the different gels one can know, what are the proteins which are differentially expressed. Now as we discussed in much detail infact, in almost complete lecture earlier that, data analysis is very crucial aspect of two dimensional analysis experiment.

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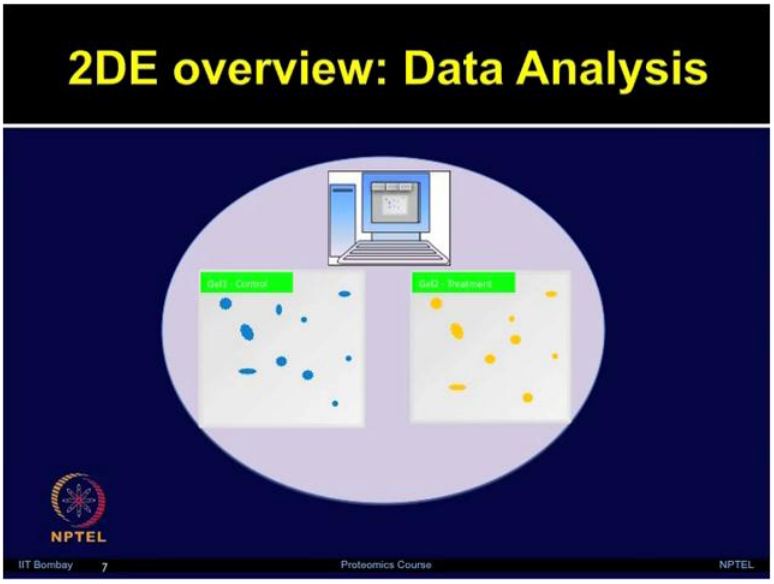

You can learn large number of gels, butwhen it comes to compare these gels, it takes lot of time; you have to very careful to obtain some statistically significant spots which could be meaningful for that biological application.So, having discussedthe broad overview of the two dimensional electrophoresis,

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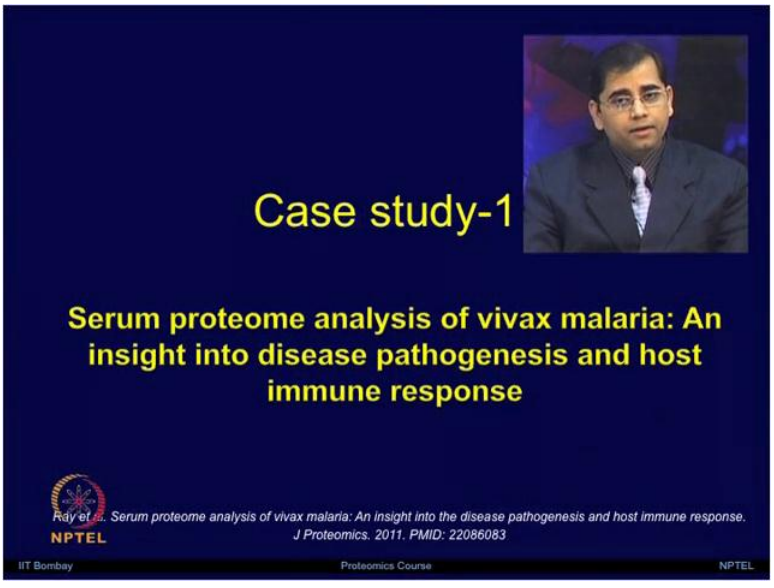

nowlet us move on to the case study.In first study, we will talk about serum proteome analysis of vivax malaria, an insight into disease pathogenesis and host response study

by ray yet all. So, this study was performed by microbe and we have tried to investigate the host response against malarial pathogen vivax.

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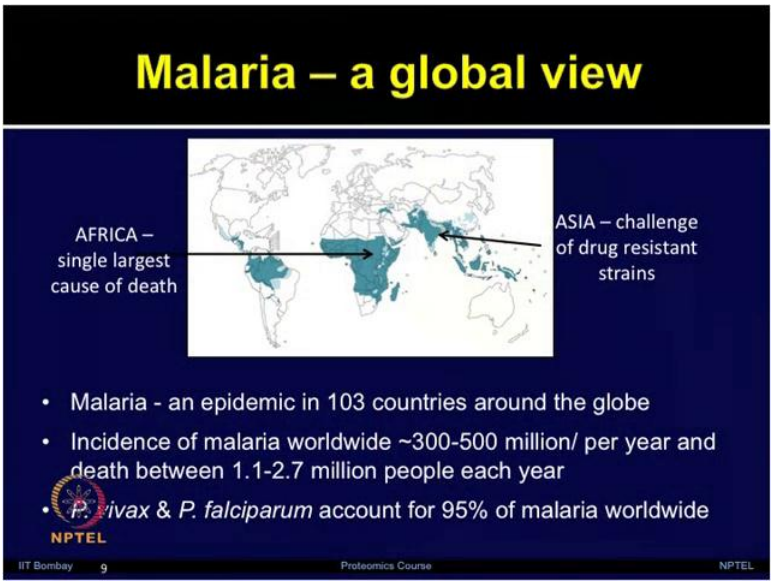

So, if you look at this map of world population overall Asia and Africa are mostly affected due to malaria. Asia is suffering from challenges of drug resisted strains of malaria, and Africa is still the single largest cause of death which is due to malaria, but not only in Asia and Africa, butmalaria is an epidemic in 103 countries around the globe. An incidence of these disease causes more than 300 million per year and death almost between 1 to 2.7 million.So, this stats gives us an indication that, how severe this problem is, and among various species of malaria, plasmodium vivax and plasmodium falciparum these are the accountable for over 90 percent of malarial cases worldwide.

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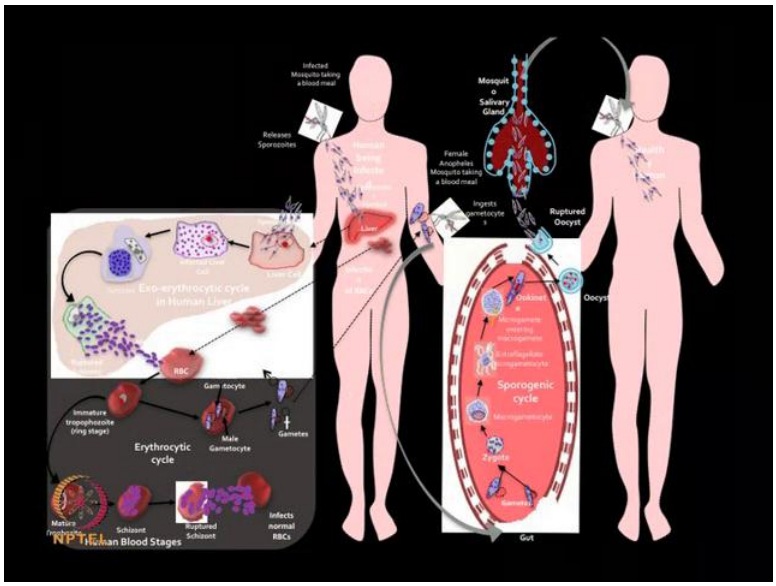

So the estimated annual number for the clinical cases of vivax malaria ranges from 70 to 390 million and that is actually outside of the African contents. Now, the Pvivax accounts for more than 50 percent of all the malarial cases worldwide, but the morbidity associated with this infection andits spectrum of disease still remains neglected. Nearly 2.6 billion people around the globe are at the risk of plasmodium vivax infection, even slightly greater than people who are addressed with the plasmodium falciparum infection. Although, the plasmodium vivax infection is historically regarded as benign, but recently the severe and fatal incidents of vivax malaria has been reported from different regions of the world which rendered this clinical paradigm quite deceptive.

So, in the complex lifecycle of malaria now investigating, how plasmodium falciparum and plasmodium vivax behave differently in human, and what are different type of proteins which are elicited due to the response against these pathogens. These are some of the very challenging questions in the malaria field. This slide gives you various species of malaria parasites which causes different type of malaria; plasmodium vivax causes benign tertian malaria, plasmodium falciparum, malignant tertian malaria, plasmodium ovale, ovale tertian malaria and plasmodium malaria equartan malaria.

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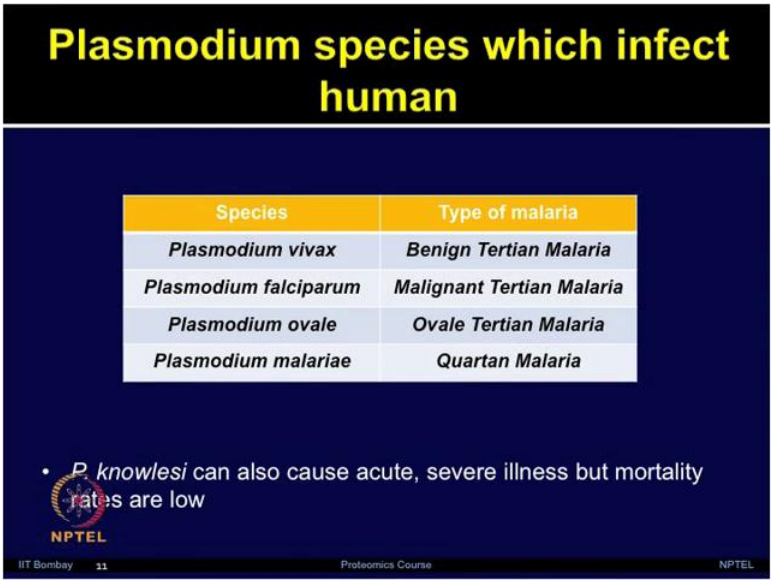

In addition to these, plasmodium know lesi can also cause acute and severe illness, but mortality rates are quite low for this species. So, there are different pathogens of malaria, but plasmodium falciparum and vivax are the prime targets for investigation because as we discussed earlier, more than 95 percent of malarial cases worldwide are due to these two species.

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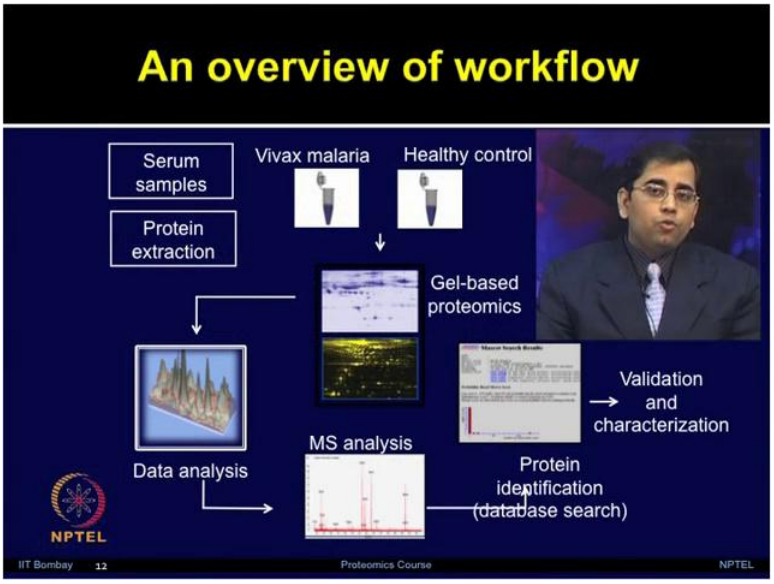

So, this slide gives an overview of the workflow which we followed to investigate the host response against plasmodium vivax in human serum. So, serum samples were

obtained, proteins were extractedand 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. Compare 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 analyze the data for various type of pathways which could be involved in such disease. So before we move to a details of various experiment performed in the study, let me show you this animation to give you an overview of the workflow followed in this experiment.

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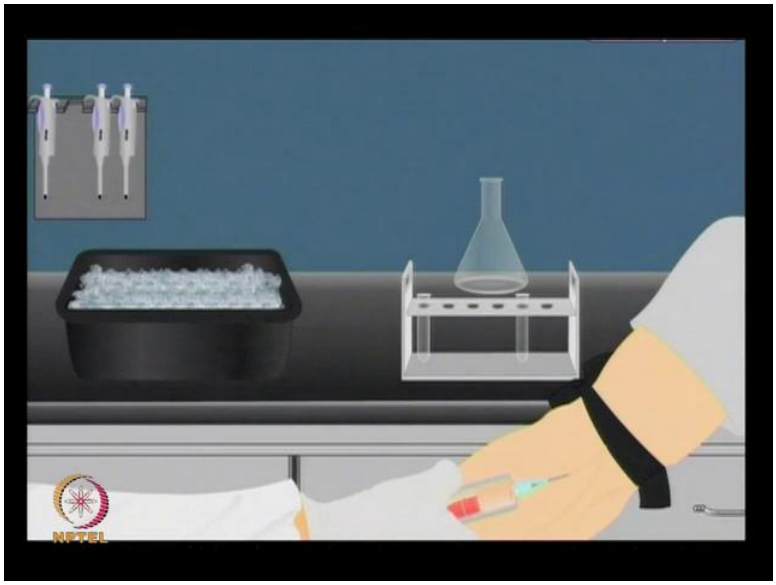

So let me give an overview of all the steps involved in performing such an experiment.

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So first of allcarefully, we draw the intravenous blood into a vacutainer tube and store the tube on ice to allow the blood to coagulate; centrifuge the contents to separate coagulated blood cells, and clotting factors from the serum which forms the clear supernatant. After centrifugation is done then, collect the obtained serum in a fresh tube. Sonicate the serum to breakdown any large protein complexes.

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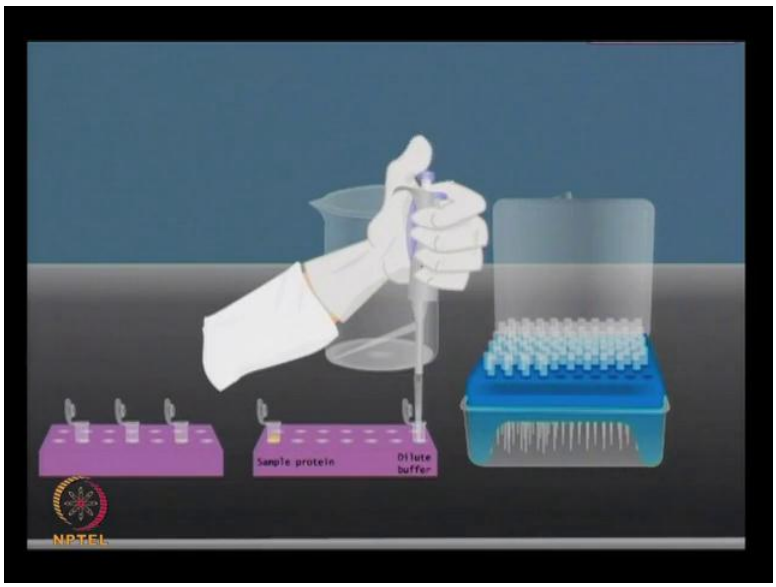

Serum contains several proteins in wide range of concentrations of which albumin amino globulin g are the most abundant ones.

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So presence of such high abundance proteins can interfere with the experiment. Therefore, we remove to we need to remove this high abundant protein. So, this experiment we tried both sonication and depletion of the high abundant proteins. After sonication is done then, we can add this serum sample on the columns usually these commercial available which can deplete various high abundant proteins.

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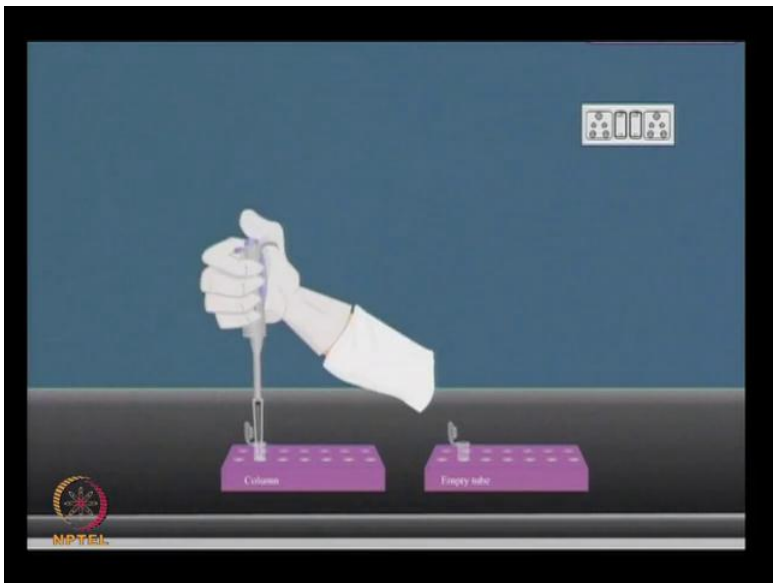

Now, these depletion column bind only the high abundance proteins on to their matrix through the affinity interactions; once the serum has been processed using a depression column, precipitate out the remaining proteins by using trichloroacetic acid and acetone.

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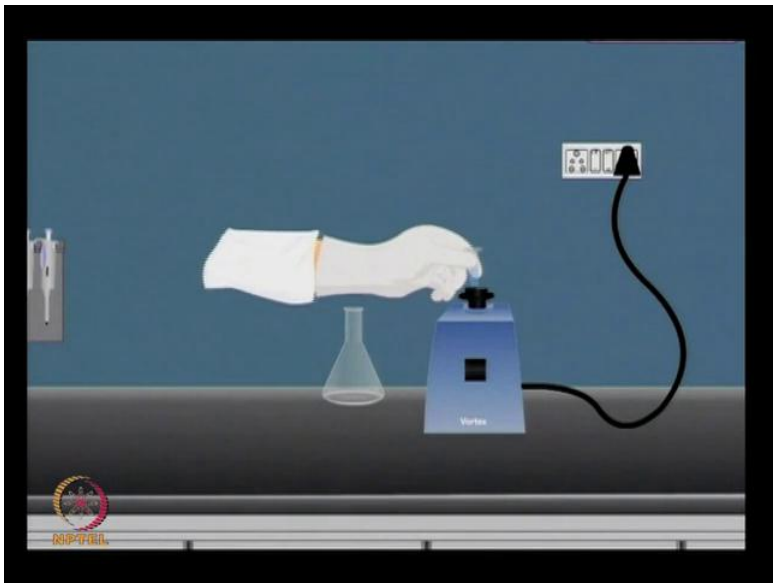

Once the protein extraction is done, then we need to add the rehydration buffer which contains urea, thiourea chaps, DTT and BPB. After overnight incubation then, we can centrifuge and collect the supernatant. Now, we watch the quantification of the proteins.

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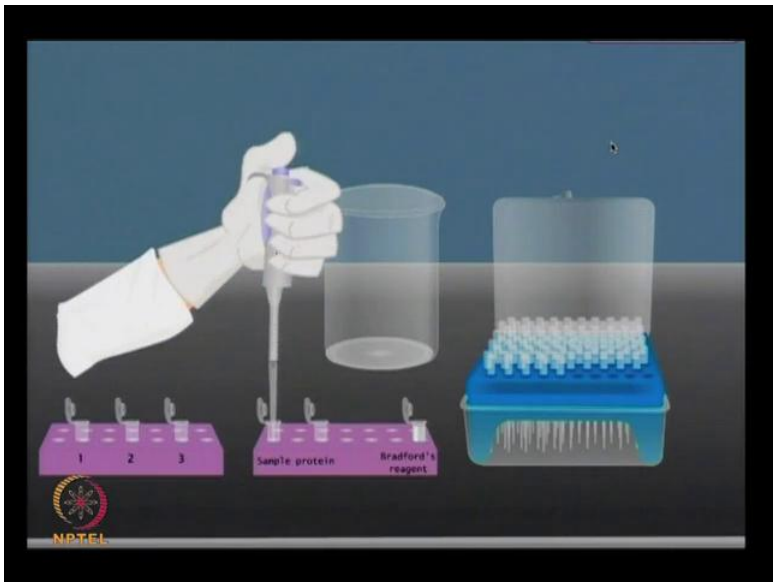

So, prepare the brat for dye region and label the tubes suitably for the standard and test samples; at these standard and sample solutions to their respective tubes. Now add the Bradford color reagent to the tubes and mix them thoroughly.

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Once the color reaction has occurred then, perform the absorbance measurement at 595 nanometer. Once you have obtained good amount of proteins which was determined by the protein quantification.

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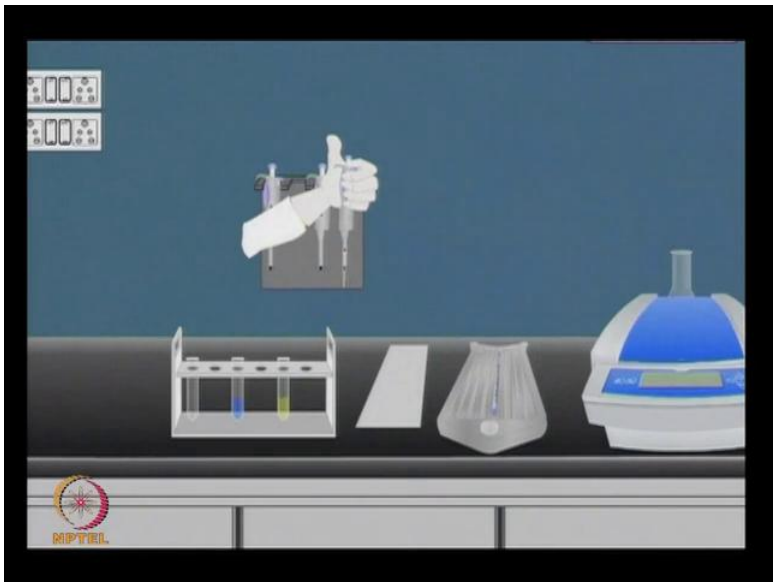

Now proceeds for the rehydration ofimmobilized pH gradient strips and focusing of these rehydrated strips. So remove the IPG strip form the cover, place it in the rehydration tray at the reconsidered protein sample on the strip, pour mineral oil to prevent it from the dry.

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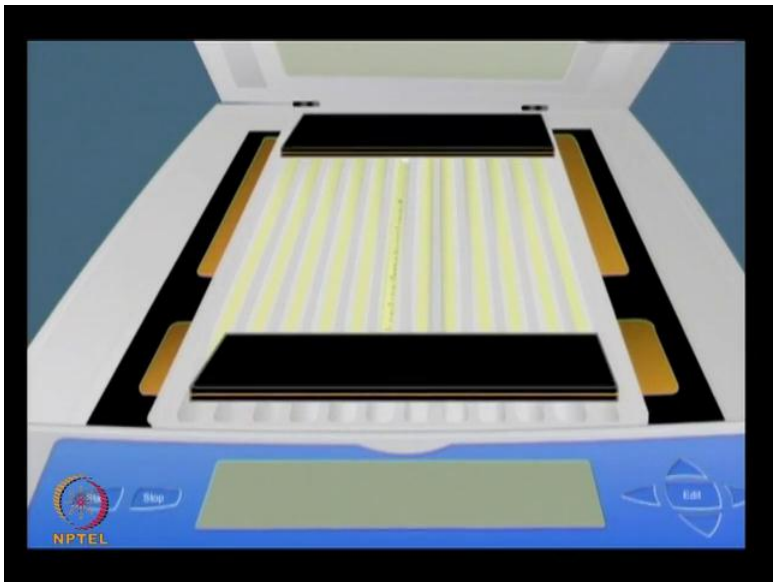

And then, move forward for the isoelectric focusing. So place the width at the either end of the IPG strip followed by an electrode at each end; fill all the adjacent wells with the mineral oil to ensure the uniform current flow. You can input the desired protocol on the instrument software along with the details of strip length, pH range, number of strips etcetera and start the focusing process.

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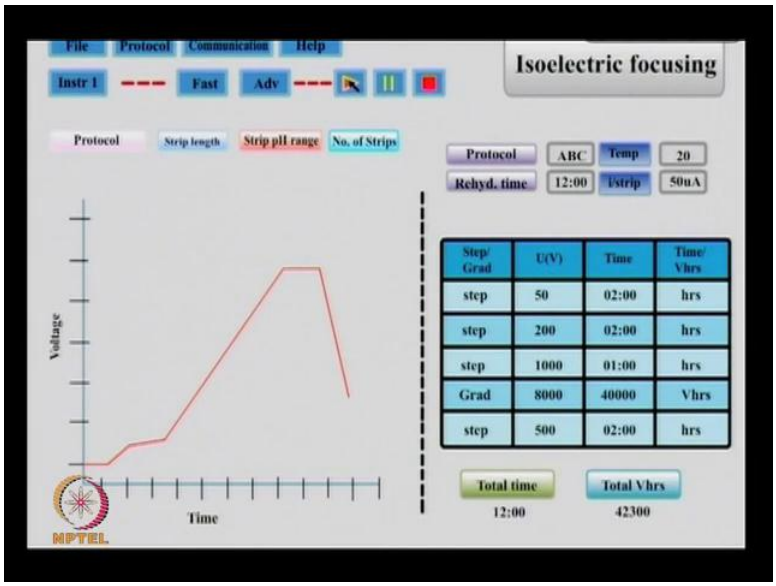

You can monitor the focusing progress on the software, and you need to ensure that focusing is proper. During the i e f proteins will migrate along its strip and come to rest at a point when there net charge becomes zero which is known as their iso electric point. After the i e f is done then, we need to separate the proteins in the second dimension on SDS page.

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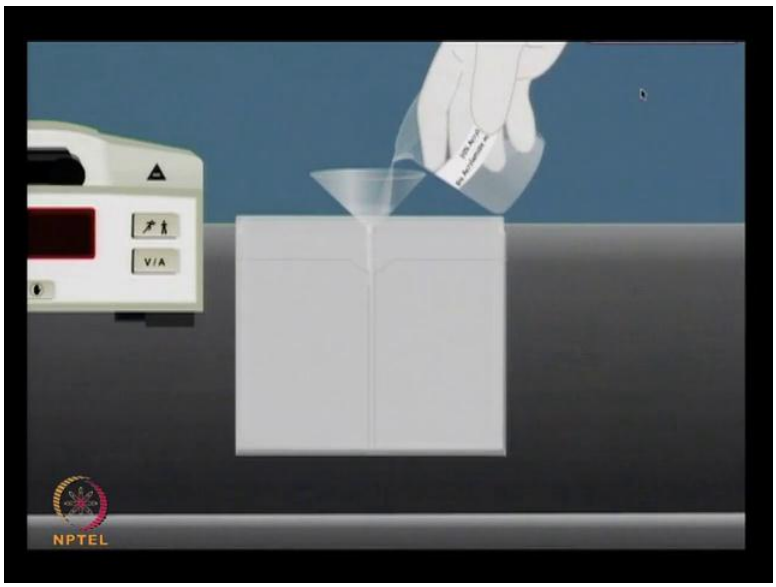

Remember, in between you have to do the equilibration strip as well. The SDS page which constitute the second dimension of two dimensional gel electrophoresis involves

assembly of the gel apparatus, gel casting, equilibration of IPG strip followed by placement of the IPG strip on the gel and protein separation.

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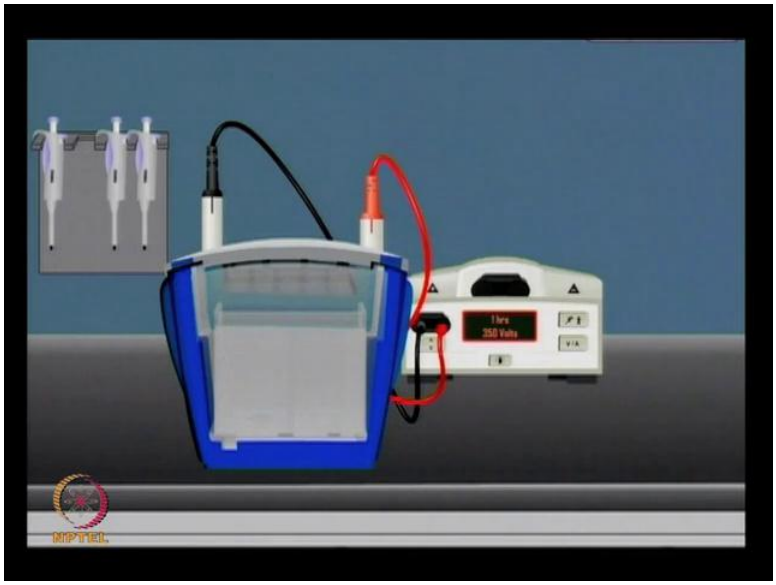

So prepare the gel casting solution consisting of acrylamide, bisacrylamide, tris chloride, SDS, APS and timid. Once the gel is polymerized then, you can add the IPG strip and now, ensure that assembly is properly sealed and buffers are properly placed so that proteins can be separated based on their molecular weight. Depending on the gel length, you can perform the second dimensional separation for few hour.

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After second dimensional separation is done then, we can perform the staining and distaining.

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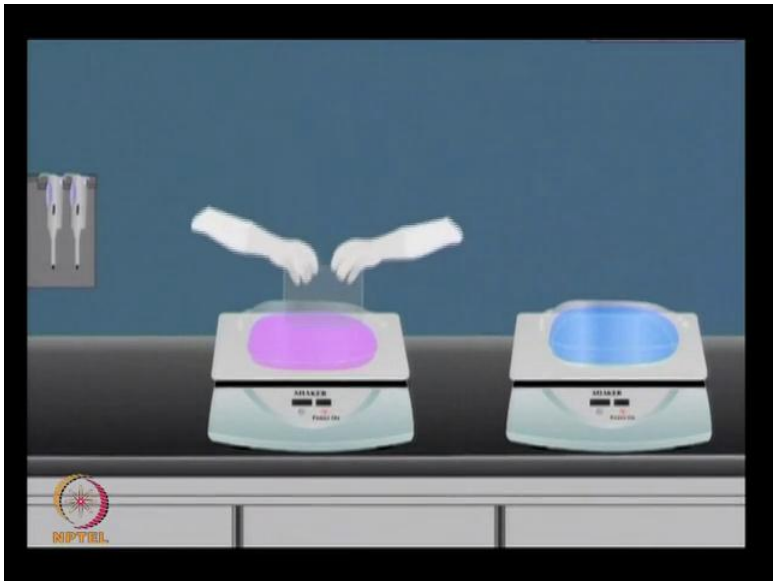

So this process involves removal of the gel from the electrophoretic assembly followed by treatment with a fixing solution, staining solution and finallythe distaining solution. You need to ensure that proper shaking conditions are maintained, and you need to allow 10 to 14 hours usually overnight strep for the staining as well as, distaining steps.

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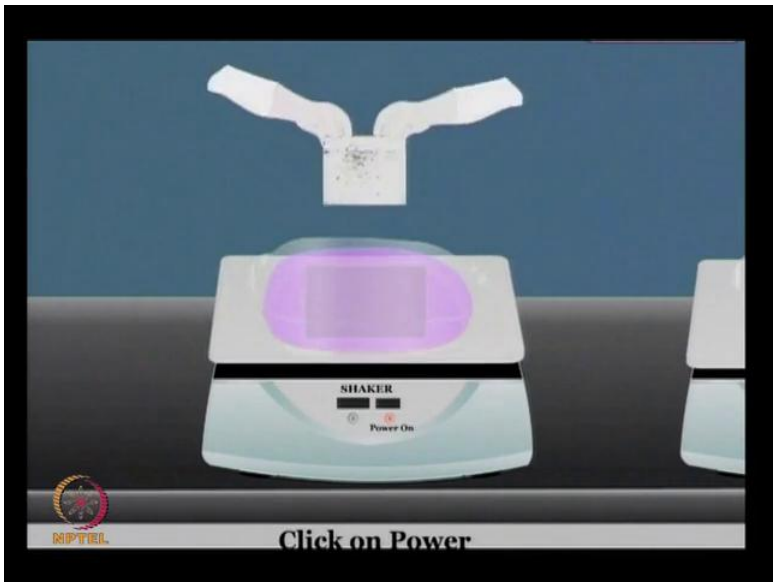

After distaining is done, then you can see a protein spots on the gel with the very clear background. After watching this animation now you must be clear of various steps which are required to perform this experiment. In clinical studies first of all, obtaining the clinical samples and getting the right type of clinical sample that is very challenging question.

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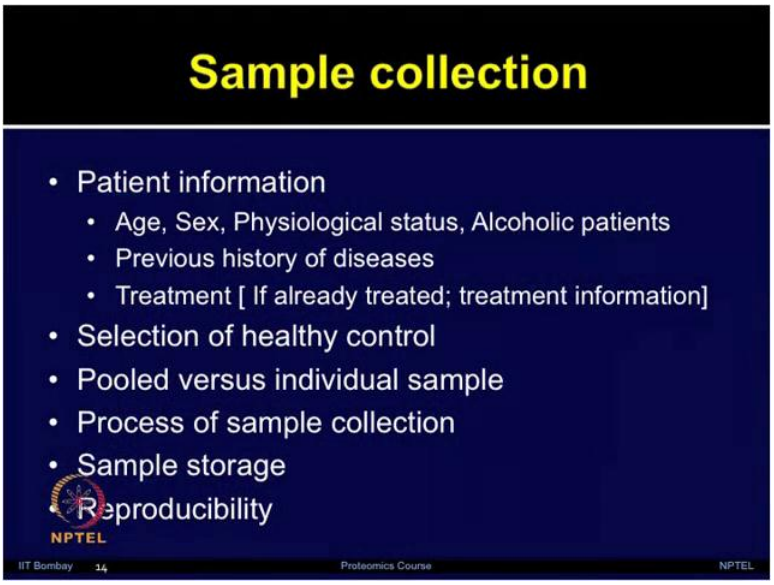

So to obtain the samples first of all, one need to do the ethics committee approval; scientific committee has to look at the proposal and review and approve it and then, patients which fall into those categories, which you want to investigate have to be enrolled after taking their consent and ethically, it should be approved. So various type of information for such patient are tabulated and then, these patient sample can be compared for the proteomic analysis.

So in addition to the age, their gender, physiological status, whether patients have anyalcoholic background, any previous history of diseases or they have taken any treatment earlier. In addition to all this, various type of clinical test which are performed, they are tabulated which eventually helps in analyzing the data when you want to compare the proteomic response for various patients. Selection of healthy controls become critical because, you want to compare healthy with the disease sample. Now, if you are healthy suffering from disease then, that may give you some artifact information. So that finds the clinical studies you need large number of samples then, there are issue

whether somebody wants to analyze only individual sample of this clinical patients or they want to pull it and then analyze. Consider you have 200 cases and 200 controls.

Now running individual gels for all of this number of patients and the healthy control can be very challenging and then finally, running the gels in triplicate and analyzing the data it becomes very tedious. So, there are other discussions going on whether, for such large number clinical cohorts with their group of patients and controls can be pooled and then analyzed for example, rather analyzing 200, if you make pool of 10 patients then, you have 20 samples, but that all depends on the investigators and kind of experimental design which people have in their mind for performing such a studies. Then sample collection process becomes very important. If you recall previous lectures, how different type of a storage conditions can also affect your proteome.So all these parameters one need to take into the consideration.

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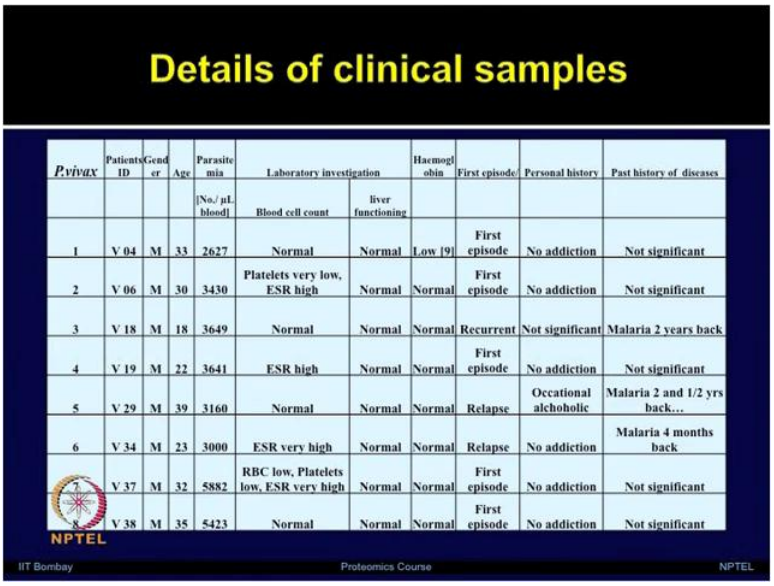

So similarly, we alsotabulated various information for different vivax patients and healthy controls, this slide just gives you an overview that, before when starting your proteome experiment, lot of thought has to go into your experiment, into your experimental design and what type of samples one need to collect and then, you need to obtain various clinical information for those healthy controls and your patients. So as you have seen in the animation first of all, you need to collect the blood sample then serum has to separated from that, and after serum is collected then, you need to store that

in a small aliquots at minus 80 degrees. So, this slide gives you various steps involved in this process.

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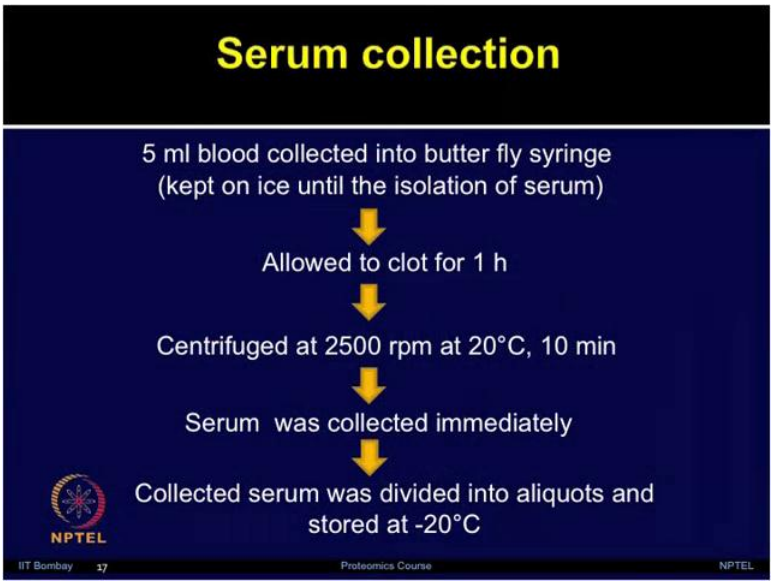

So, ideally a 5 ml of blood is sufficient forgetting enough serum to perform such proteomic investigation. So 5 ml blood if you collect and then, keep it on ice until the serum isolation; allowed to clot for an hour and then centrifuge at 20 degrees for 10 minutes followed by separate the supernatant, the serum layer immediately; collected serum can be stored at minus 20 degrees or minus 80 degrees; minus 20 if you want to use that immediately for your further use or if you want to store for long time then, you can store in minus 80.

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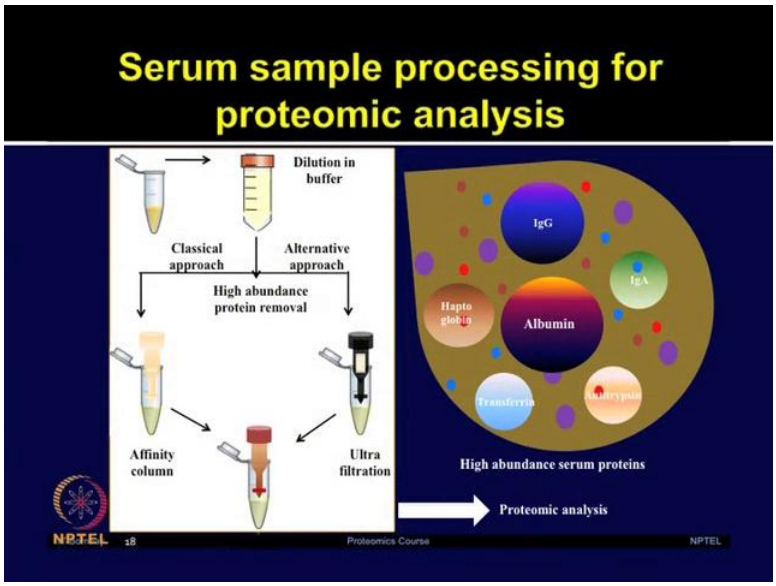

Now, serum poses challenge of abundant proteins. So first of all, we had to remove the abundant proteins from the serum and there are standard columns available from various commercial manufacturing from which, one can remove selectively the very highly abundant proteins such as, serum albumin, IGG and 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 proteomic experiment. So first of all to address this question, we tried separating the proteins from various type of samples.

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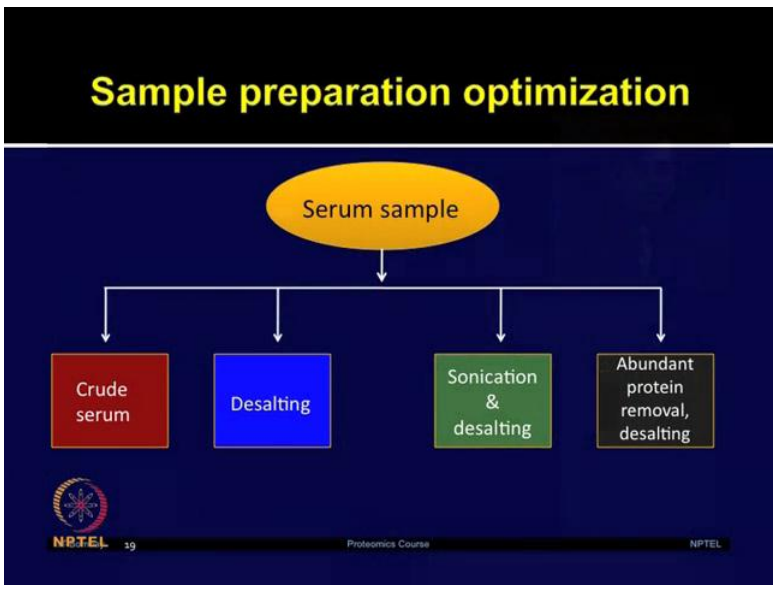

We took crude serum because, serum will have mostly the protein content so we thought, can we directly apply the serum on the IPG strip immobilized pH gradient strip. Separate the protein and see, how many proteins we can separate on these 2-D gels, but serum contains lot of salt so, isoelectric focusing 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 columns which can deplete this abundant protein selectively those are quite costly, and considering that large number of patient sample, 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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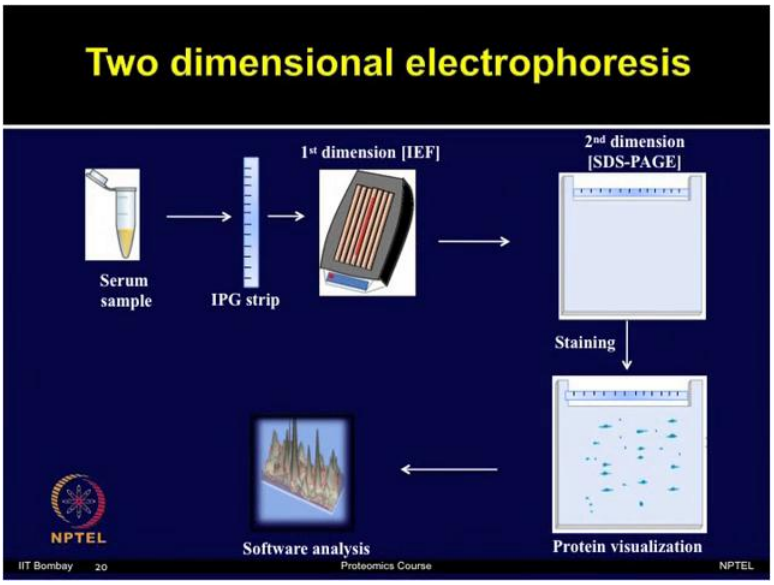

Now sample was corrected and processed with all these four variables which I talked you and after that, the standard procedure of two dimensional electrophoresis was followed.

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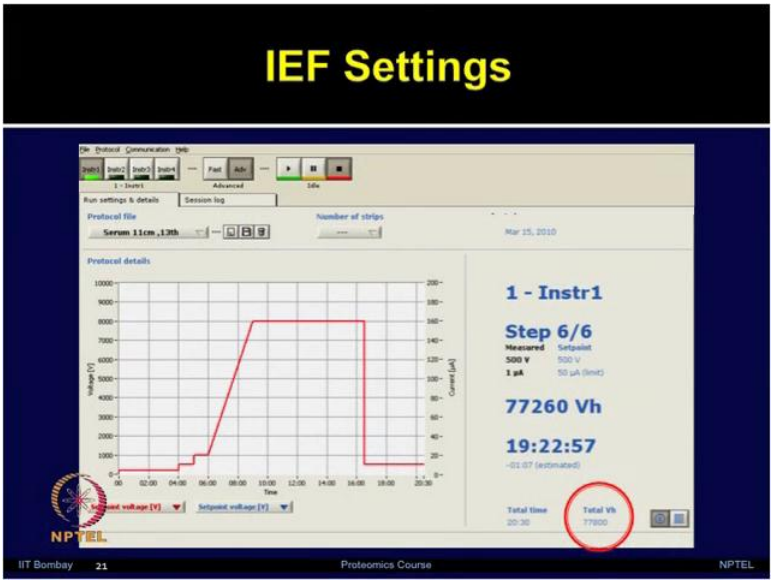

Now, when you are doing this serum protein separation the IEF becomes very critical because, you have lot of salt component in it, and if your salt removal is not effective, your isoelectric focusing run may not be very smooth. Sofor the software's you can monitor, how smoothly your isoelectric focusing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

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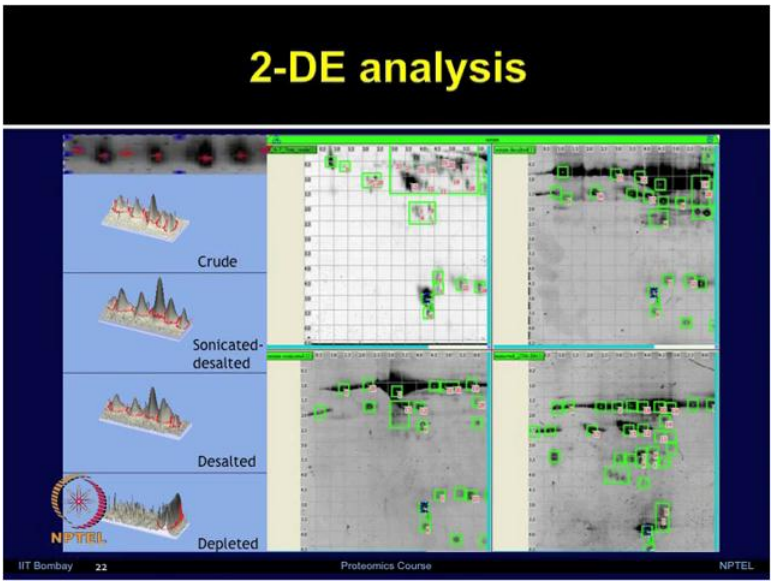

So, we tried various type ofeffectscrude, serum alone or sonicated and desalted, desalted alone and depleted and desalted and then, try to compare the effect of these type of processing on overall proteome coverage.

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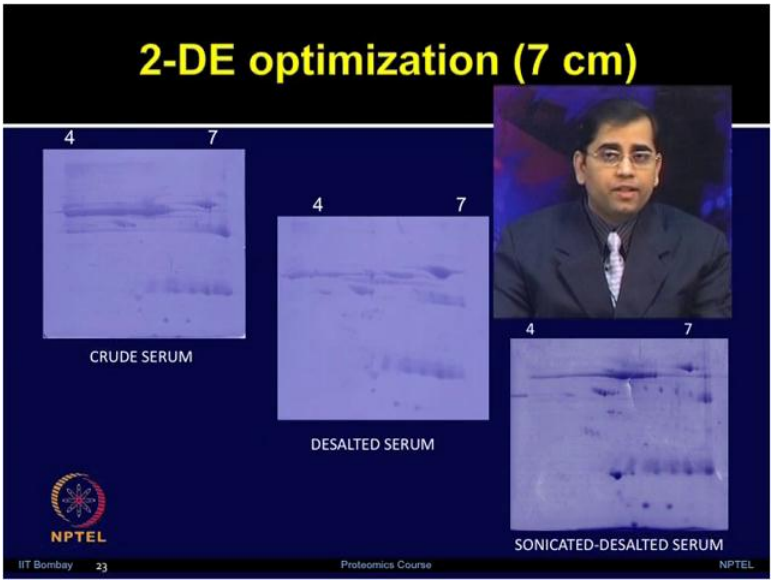

We also tried to see that, how many protein we can separate on the small gels. And then, as expected the small gel of several centimeter cannot provide us very large number of proteins, but even these gels can be use for standardization process. As you can see, starting from the crude serum to the desalted and then finally, sonicated and desalted gives us better coverage of the proteome.

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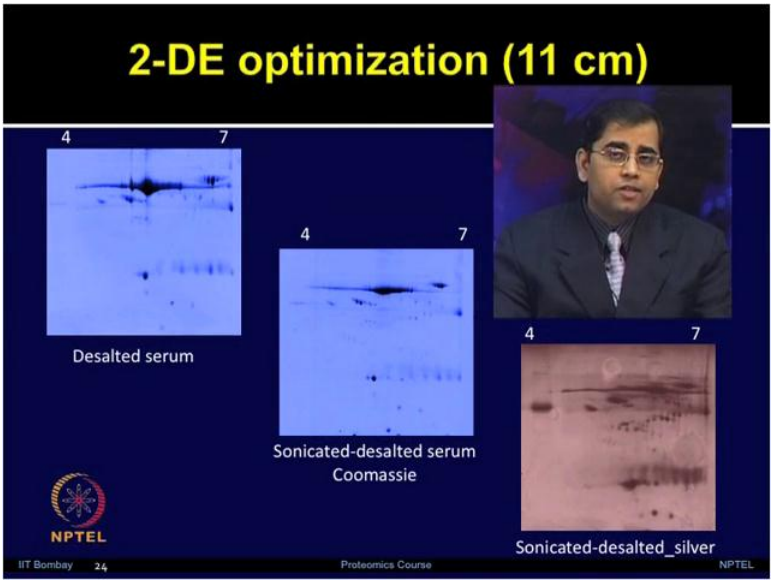

We try the same with the even larger strip 11 centimeter and it was again quiet convincing then, we moved on to the very larger strips 24 centimeter for the clinical studies.

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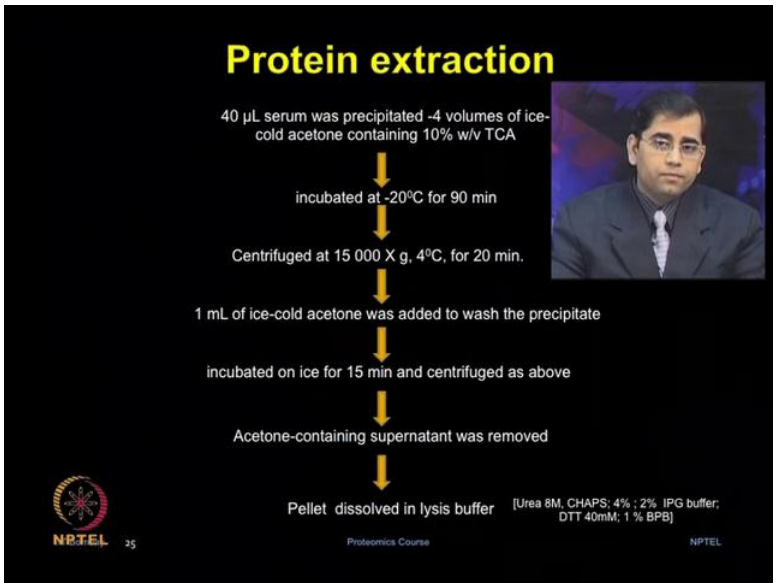

So in general, we took 40 microliter of the serum which was precipitated with the 4 volume of ice cold acetonecontaining 10 percent of TCA incubated at minus 20 degrees, centrifuged followed by added 1 ml of ice cold acetone to the to wash the precipitate; then incubated it on the ice for 15 minutes 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 the slide here; you can use urea, chaps, IPG buffer, DTT and BPB. So from the previous study, we looked atreplicate gels andit is three 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.

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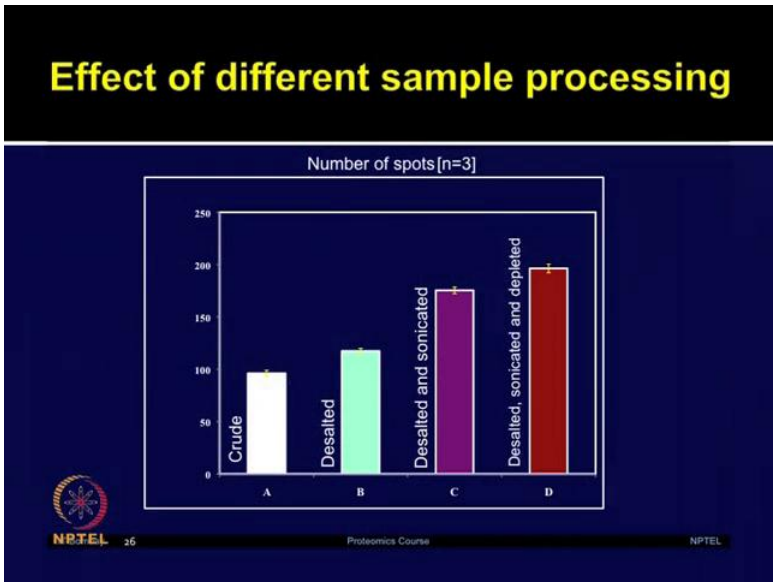

So, these studies we have performed in the small strips to sindrile the procedure, but we are able to draw the conclusion based on the reproducible pattern obtain in these gels and then, we applied the desalted sonicated and depleted. This conditions for the processing all the samples.

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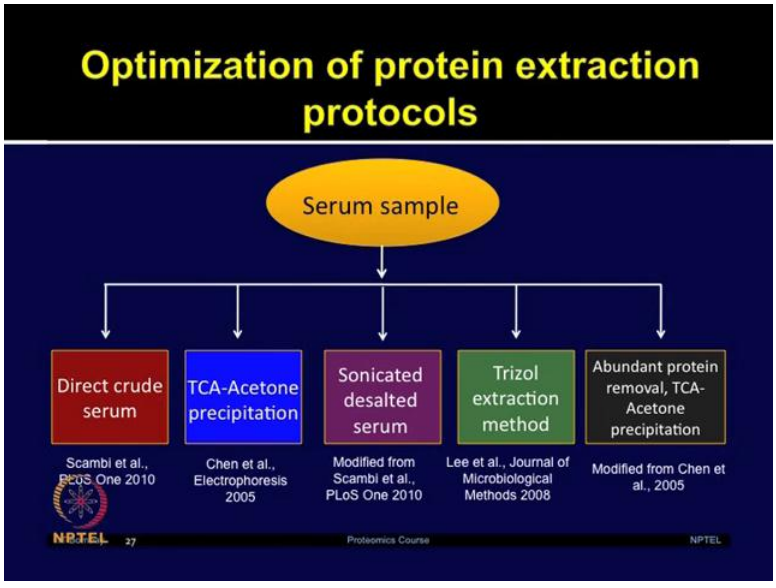

After processing of these samples now, how to extract the proteins? So, protein extraction protocol was also optimized and to optimize these protocols first of all, we looked at what are different methods available in the literature. So, people have applied

direct crude serum, TCA, acetone precipitation, sonicated and desalted serum, trizol extraction method as well as abundant protein removal and TCA acetone precipitation.

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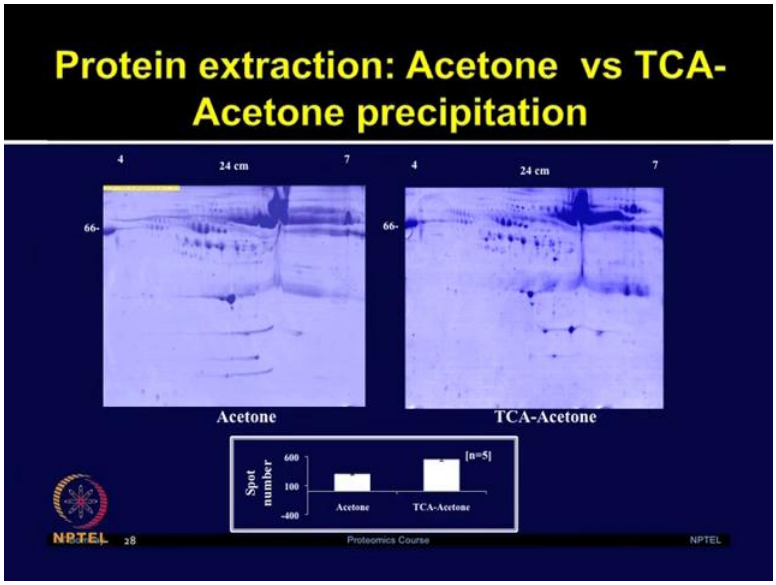

So, we used this modified procedure of TCA acetone precipitation where we used depleted and desalted and sonicated serum. We also compared the effect of acetone alone or TCA acetone precipitation. As you can see in this slide, the left one is showing the overall protein spot are present in the acetone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

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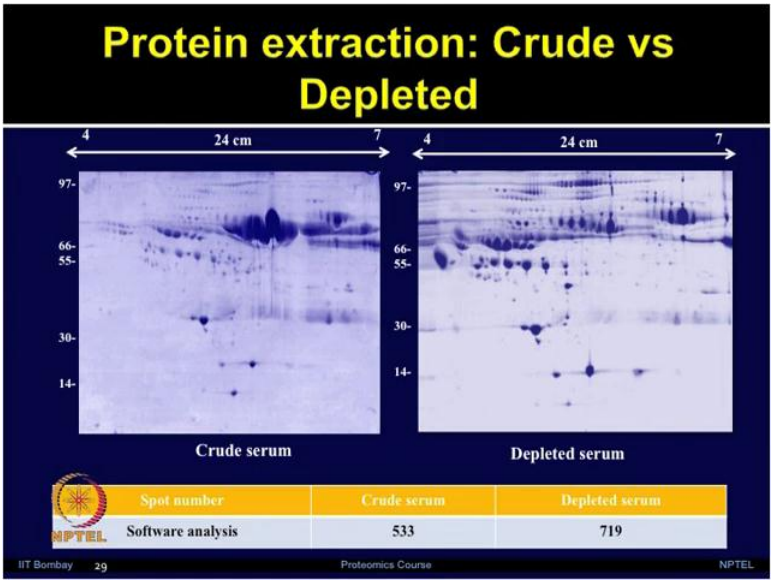

So, since we standardize our condition on the small gels earlier, we also tried to apply that on the large gel of 24 centimeters because,that is where we have to perform all over analysis. So, we again compared crude versus depleted protein response of these serum samples on the larger and as expected,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

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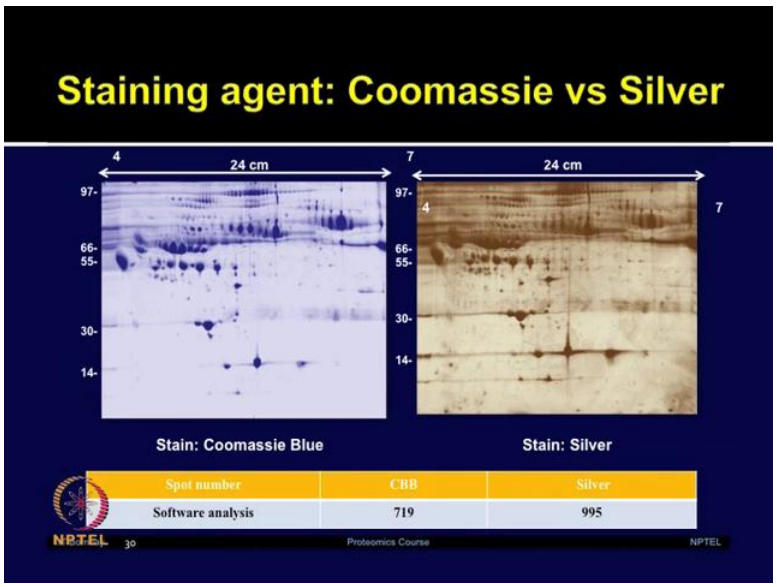

We also tried to look at effects of various type of staining, whether it is coomassie blue staining or silver staining. We definitely identified more number of a spots from the

silver staining, butjust because of we want to compare a 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 use the colloidal coomassie and biosafe coomassie staining, and we standardize the same condition for all the gels.

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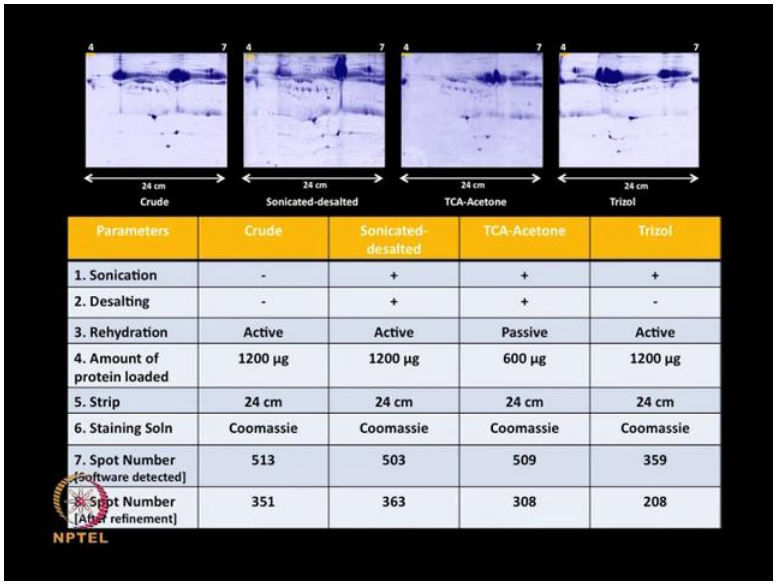

Parameters	Crude	Sonicated-desalted	TCA-Acetone	Trizol
1. Sonication	-	+	+	+
2. Desalting	-	+	+	-
3. Rehydration	Active	Active	Passive	Active
4. Amount of protein loaded	1200 µg	1200 µg	600 µg	1200 µg
5. Strip	24 cm	24 cm	24 cm	24 cm
6. Staining Soln	Coomassie	Coomassie	Coomassie	Coomassie
7. Spot Number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

So, this slide gives you an overview of various type ofparameters which we investigated from different type of treatments crude serum, sonicated and desalted, TCA 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 a spot we can resolve on these gels, and thensince software analysis automated gives lot of artifacts, how many real spot we can obtain after the manual refinement. So, I am trying to give you all the details what one need to actuallyperform 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 lot of comparative analysis and standardizing the protocols for sample preparation and protein extraction.

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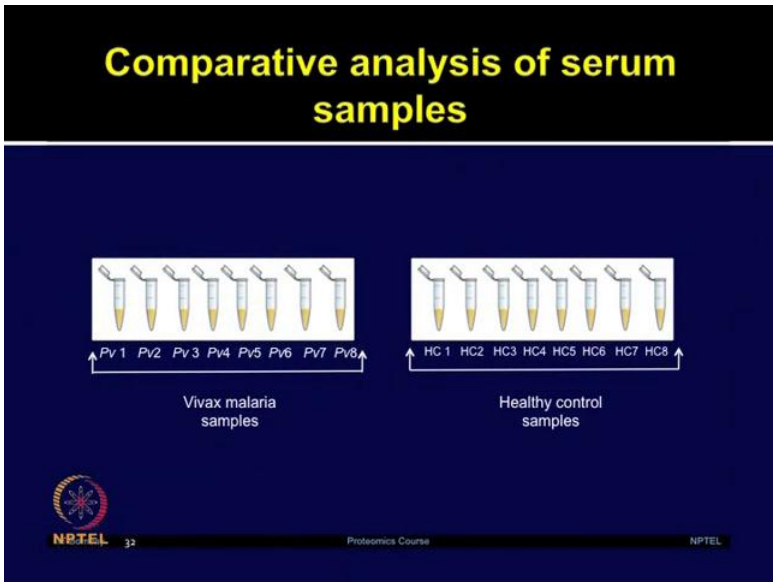

Now, we were ready to perform the comparative study onserum samples of vivax patients with the healthy control.

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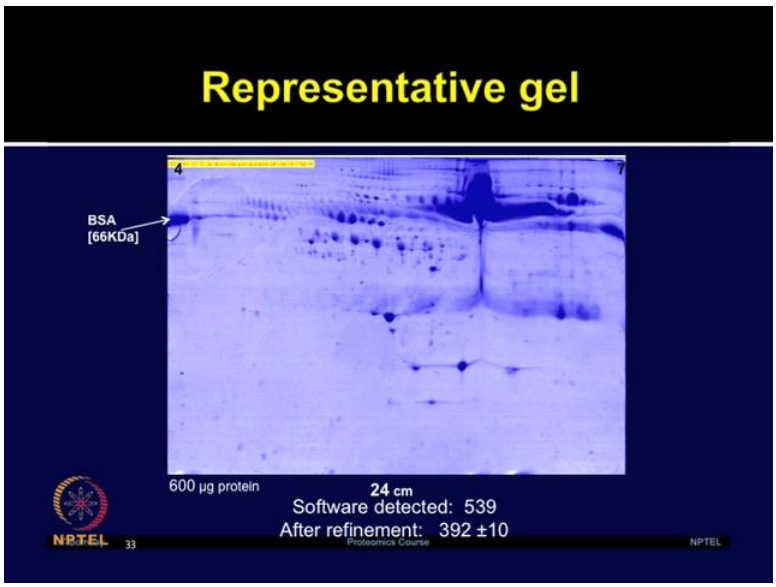

This is one of the gel imageshowing that is what is expected on 24 centimeter large 2D gel.

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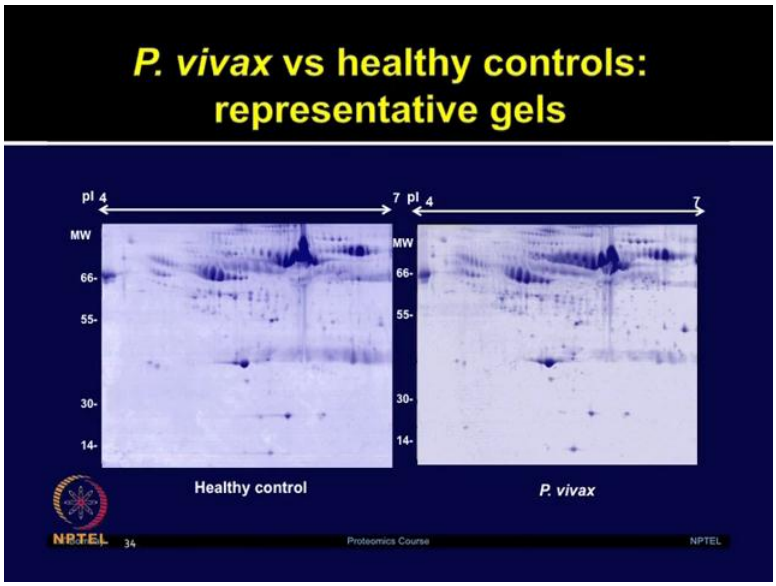

And then, a representative gel showingthe healthy control and the plasmodium vivax treated samples. So these samples as you can see, whereshowing large number of protein spots resolved on these gels.

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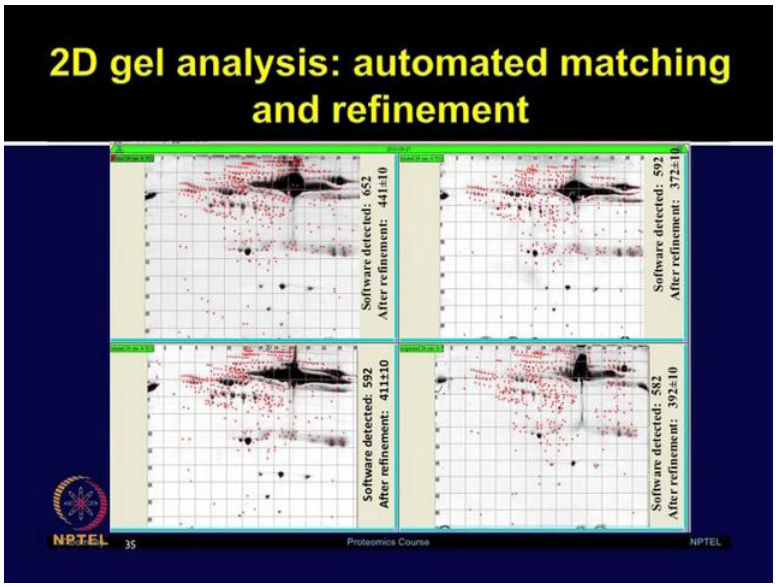

And then after separating these protein samples on these 2D gels from various patients and controls then most challenging task was the data analysis.So automated matching as well as manual refinements are performed to obtain the, how many spots are significantly modulated due to vivax infection. As you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

looked at various controls and various vivax patients; these are only few patients' samples here, butwe analyzed a large number of samples.

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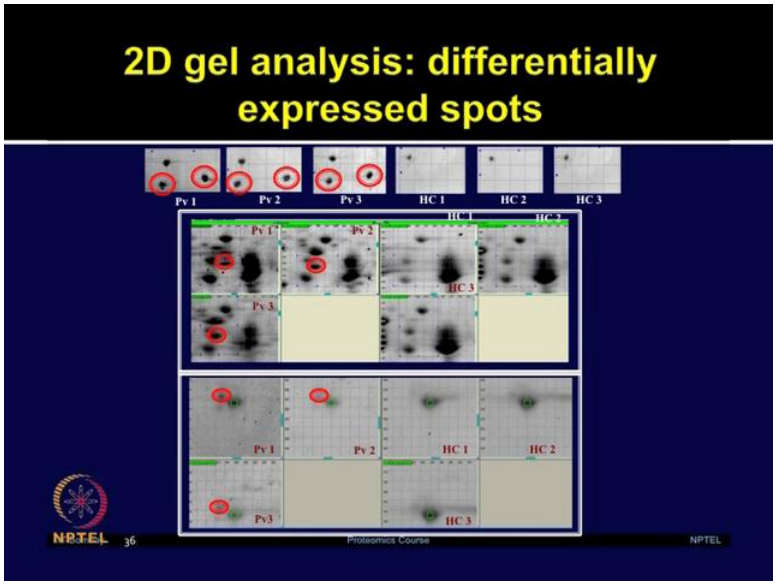

But, those are spots which were reproducible in all the patients for example, one you can see in the top panel;two spots are showing significant alteration in the vivax as compared to the healthy control. So these type of spots were considered further for themass spectrometry analysis.

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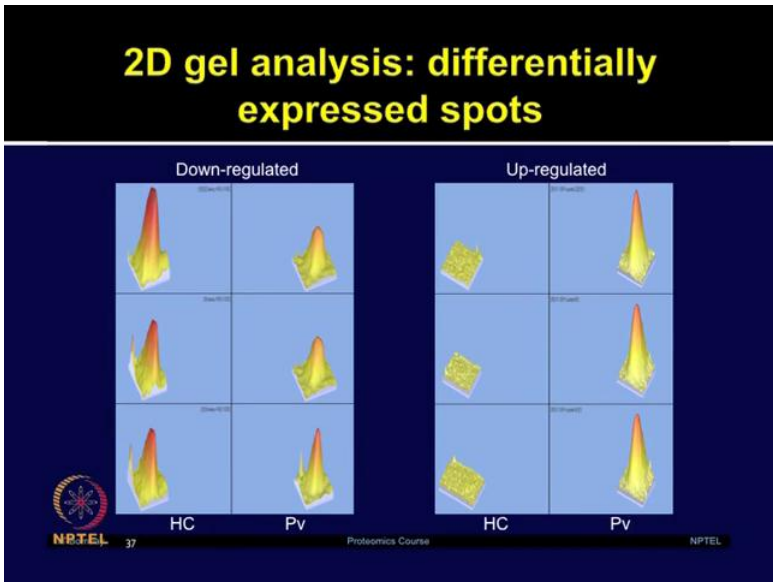

Now, those spots which we found were showing good differential expression, we looked at the three dimensional views of those spot to ensure that, those are not artifacts and those are the real spot showing up or down regulation of these proteins in humans cell.

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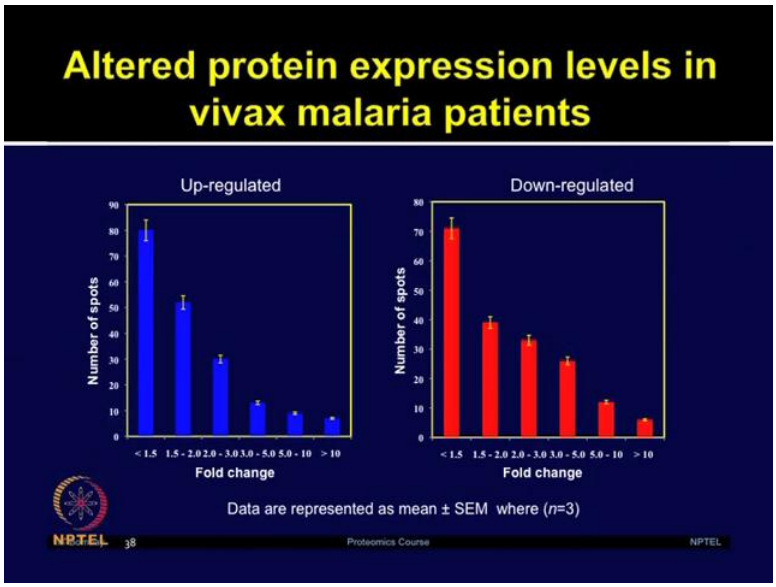

Now after completing analysis, we were able to obtain large number of proteins which were differentially expressed and as you can see in this graph, there are proteins which vary in different range of the fold change; many proteins show very less fold change between 1 to 1.54; another few proteins which also show more than 10 fold up or down regulation.So these protein spots were considered interesting because, those were statistically significant and then, these are spots were further analyzed using mass spectrometry techniques.

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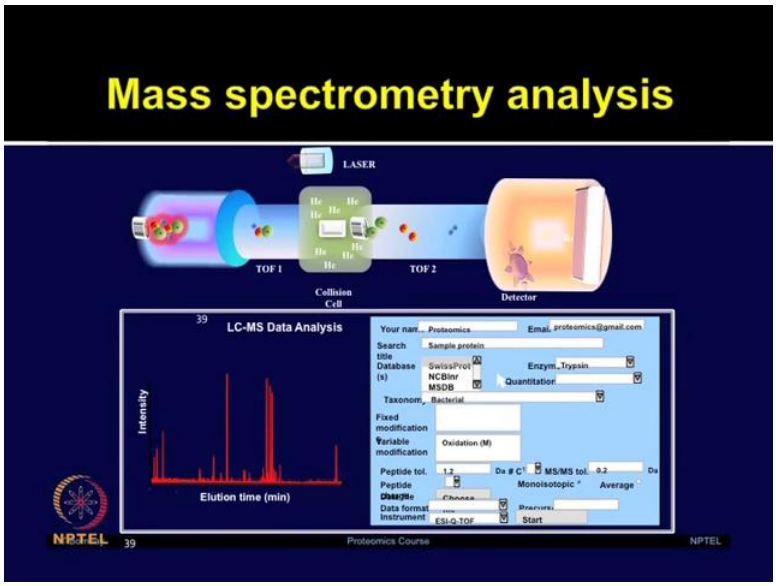

So, this slide gives you an overview of tandem mass spectrometry technique, and followed by how LC-MSdata can be analyzed by usingMascot search engine. So, different type of mass spectrometry techniques are available including Maldi-Tof and LC-MS based approaches and we will talk about these techniques in much more detail in the next module; we may talk about mass spectrometry.

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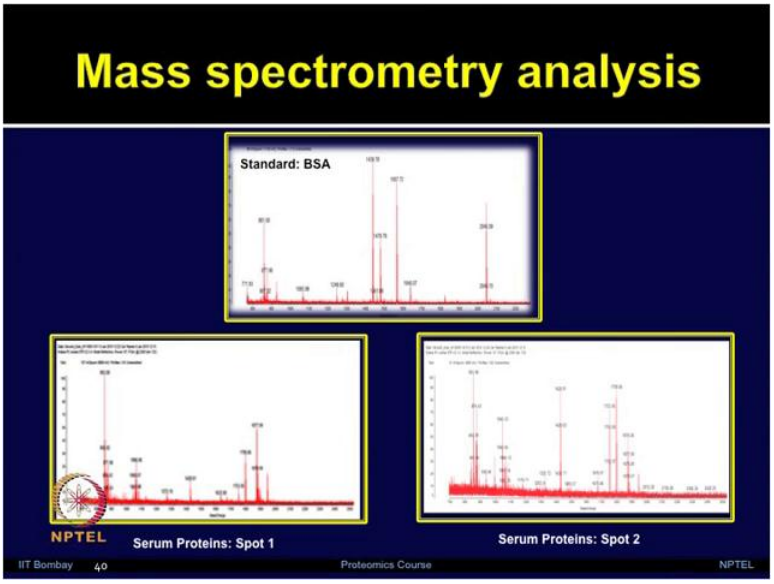

But, just to give you an overview here that, one need to analyze a spectra, and also one need to have some standard proteins to ensure that correct spectra is obtained. So, we

have to do lot of quality controls 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, and again maybe I will take this study at that time that, what are the different detailed procedure required to perform such analysis.

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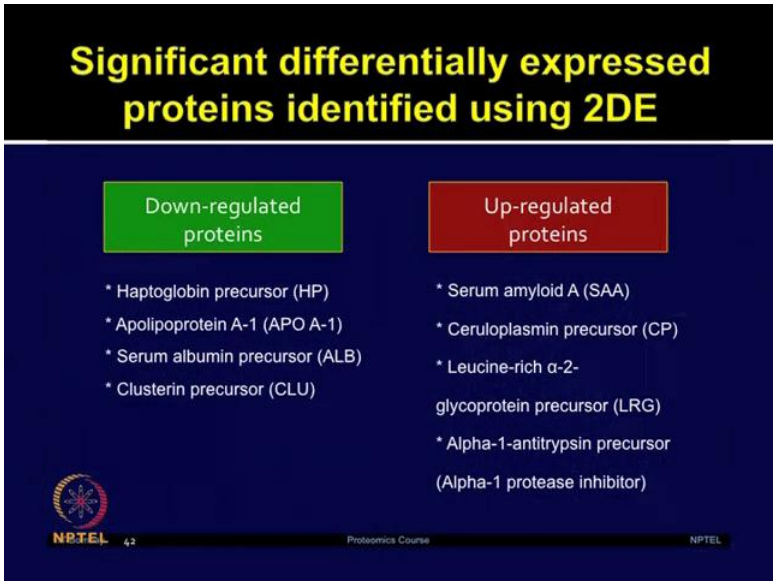

But, at the end from these experiments of mass spectrometry, one can obtain the identity of these proteins and as you can see in the slide, there are several proteins in the host human where differentially expressed due to the plasmodium vivax infection, and these are some of the proteins listed here such as haptoglobin, apolipoprotein A-1, serum albumin precursor, and cluster in these were down regulated and serum amyloid A, ceruloplasmin precursor, leucine-rich alpha 2 glycoprotein precursor and alpha antitrypsin precursor, these proteins were up regulated among many other proteins.

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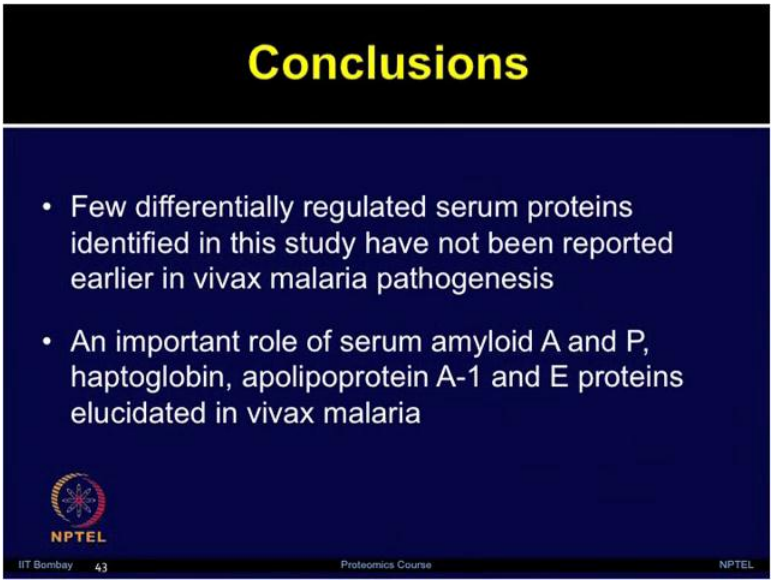

So conclusions fromthis case study that, few differentially regulatedserum proteins were identified in the study including ceruloplasmin, hemopexin, alpha antichymotrypsin, alpha 1anti trypsin which have not been previously reported in the vivax malaria pathogenesis. A very important role of few proteins such as serum amyloid A and haptoglobin, apolipoprotein A-1 and E was established in the vivax malaria and this study finally, enhanced our understanding for the bases of disease pathogenesis and provided few potential targets which could be used for further investigation.

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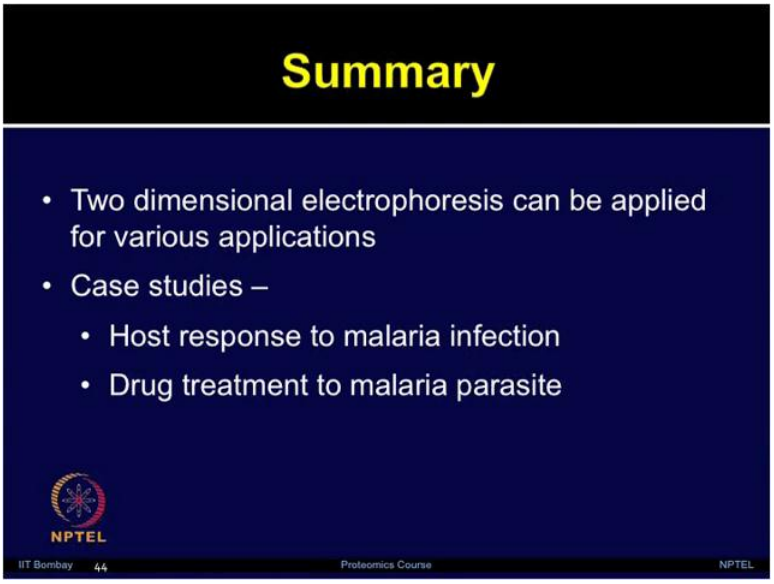

Today, we talked about how two dimensional electrophoresis techniques can be applied for various biological applications.All I thought, I will be able to cover two case studies on malaria; one on the host side like how host response can be monitored indue to the infection of plasmodium vivax and then second, study which we are going to take was based on the effect of a drug on another plasmodium species plasmodium falciparum. So, we could not discuss the other study on the drug treatment of malaria parasite which we will discuss in the next class, but by giving you a case study and details of various type of experiments one need perform, probably you got an idea that before performing that final experiment on you case and the controls, we can lot of optimization has to performed.

And starting from you sample processing, and how you can expand the coverage of the proteome, these type of quality controls 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, we have good chance of identifying various potential targets which could be novel drug target or potential biomarkers. But, if we have not done the proper quality experiment, we have not optimized the protocols properly and your protein 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 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 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. So, we will continue our discussion in the next class thank you.

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