Proteomics: Principles and Techniques
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Lecture No. #17
Difference Gel Electrophoresis (continued)
Discussion and Data Analysis

Welcome to the proteomics course in the last 2 lectures, we have discussed about gel based proteomic techniques. You have seen how 2 dimensional electrophoresis can be performed; there are certain limitations of this technology. So, then there are new creative ways more advanced methods have we merged to overcome those challenges. In the last class we talked about difference gel electrophoresis or DIGE and you have seen how to perform the DIGE experiment in detail.

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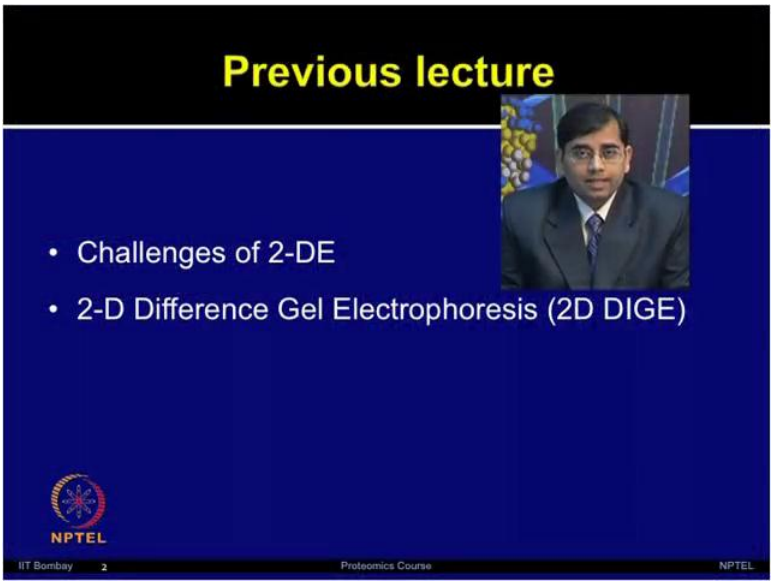

So, the previous lecture we discussed about different challenges which are associated with 2 dimensional electrophoresis. I gave you few examples, how sample preparation different type of reagents or even isoelectric focusing settings all of those factors can contribute towards a very bad gel. So, if you have perform everything properly and all your regents and protocols are good at the end you can expect a good gel, but out of 10 there are various gel to gel artifacts and variations in traditional 2 dimensional electrophoresis method. So, we discussed about how to overcome those method, those problems new method such as a DIGE have we merged

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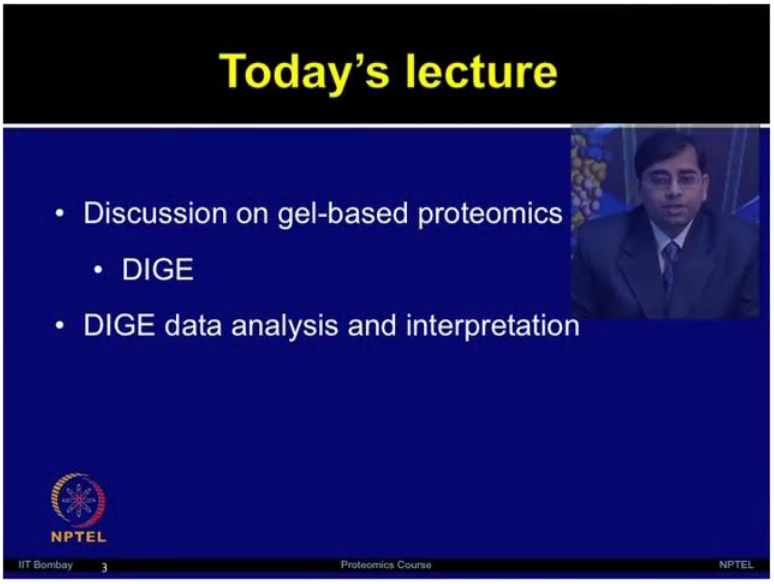

So, in today's lecture we will continue our discussion on gel-based proteomics certain advantages and challenges of performing DIGE experiments, how to analyze DIGE data by using few very specialized software. And how to interrupt that data how to obtain some meaningful biological information from those analysis. So, today I have invited a guest Dr srinivas from g e healthcare who is going to talk us about DIGE technology. And give us a demonstration on software to perform DIGE gel analysis. This is my great pleasure to introduce Dr Srinivas Rao from g e healthcare; his is an application specialist in the research product division of g e healthcare. Dr Srinivas thank you for coming for discussion about 2 d and DIGE technologies. So, what are the major advantages and disadvantages you see by using 2 d approach?

It is very user friendly as well as very low instrumentation cost, there the complete proteomic the differentially expressed proteins we can see exactly during this complete 2 d electrophoresis technique as well as in the normal proteins we can able to isolate fromonly from this technique. This we can identify very easily the softwares as well as very user friendly this is what we can do.There are some disadvantages the reproducible will be not there in most of the times. This is a major disadvantages of this 2 d electrophoresis and the again softwares they are very user friendly still there is lots to be need to be developed. So, that we can use very effectively. So, as you rightly mentioned so 2 dimension electrophoresis still is a very powerful tool to resolve a 1000s of proteins on the gel. One can also analyze isoforms and post-translational modifications including

phosphorylation relation and overall the processes very user friendly. So, still it is 1 of the very powerful technologies currently available for proteome analysis Now, I also agree with your comment thatthere is some reproducibility issues.

Because, users have to run the gelsfrom the control and treatment separately and then there will be lot of manual artifacts, regardless of how meticulous they are doing the experiment. So, in that regard what is your suggestion like how user can overcome the limitations of traditional 2 dimensional electrophoresis technology.

One should have prepare very good sample preparation. So, that the reproducibility thing will overcome, the second point is these days the new generation 2 d electrophoresis systems. That is the DIGE technology is available where you can use a 3 samples in a single strip, and we can co-migrate them in a single strip and we finally, there will be an internal control as well as control and treated these three you can run in a single strip.So, that these kinds of reproducibility issues we can overcome as well as there is scanner very powerful scanner is available in this technology that is the typhoon trio. Where you can have this is the based on the laser based technology is completely.So, you will have very efficient gels you will have in your hand.

So, as you mentioned that DIGE technology is 1 of the very powerful solution, to overcome several challenges, which people face in running traditional 2 d gels. And so in that regard I think if you elaborate little bit about DIGE technology. So, that would be useful to the students. Yes I have a smallvideo regarding the DIGE technology. I will show you that video so that everyone can able to easily understand what exactly DIGE technology.

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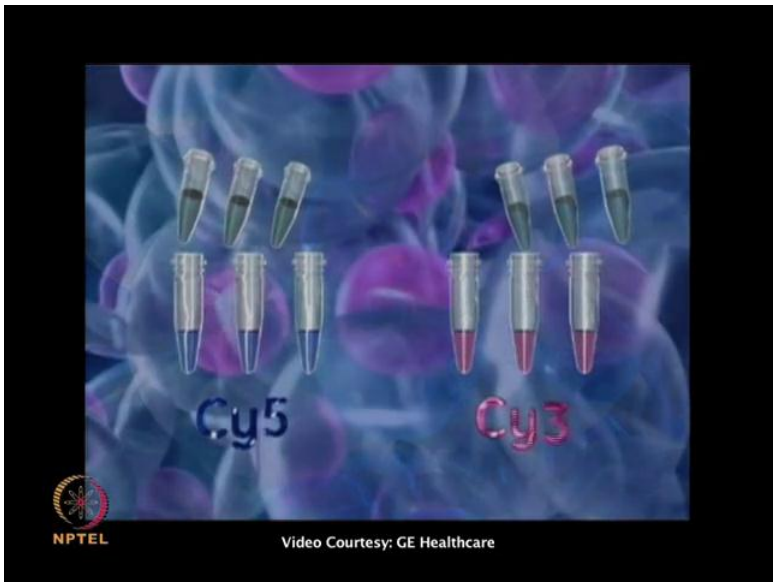

Yes as we were talking about the 3 samples we can loadlabel with cy2, cy 3, cy 5. Now we have 2 different samples from different source. Now you are taking these 3 samples into 3 different eppendorf tubes, and you are labeling with cy3 DIGE as well as again another with cy5. Then you are taking those two samples cooling them and labeling cy2.

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Then you are mixing those 3into a single tube,then you are running in a single strip.

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There you will be analyzed in first dimension the i e f where as in first dimension after analyzing I e f.

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You will be placing into second dimension, where you are completely analyzed to the second dimension.

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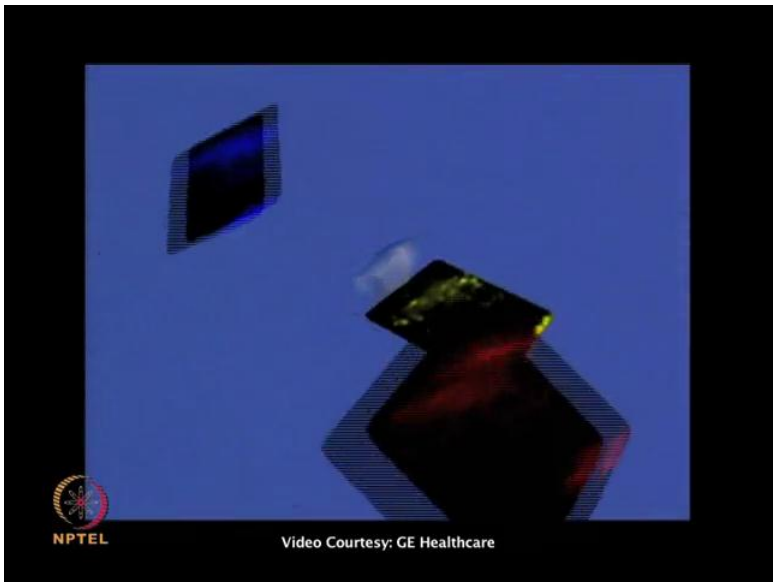

Now this gel can be scanned in the typhoontrio plus, where you will have 3 images from a single gel.

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Then this can be analyzed that to with the help of decyder software, then where the complete analysis can be done with the help of this decyder.

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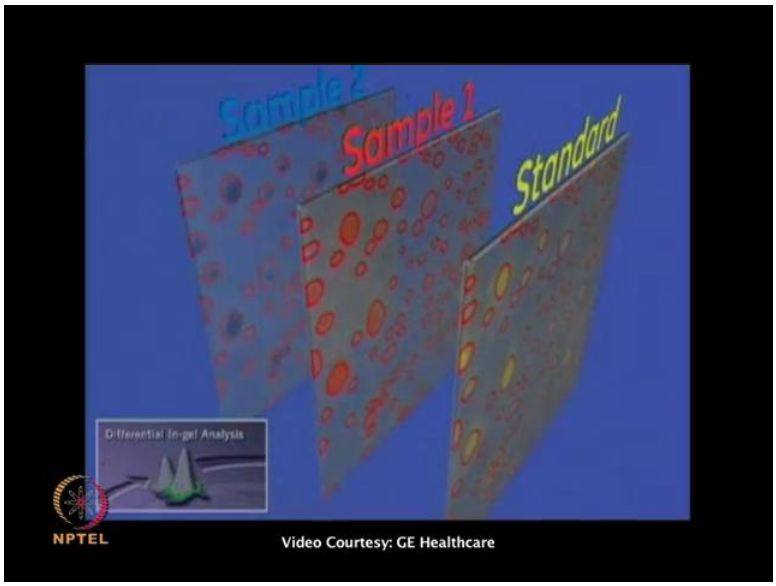

Then in d i a there will be aco-detection the co-detection nothing, but there is a standard gel which is labeled with Cy 2. From the same area we are expanding remain 2 image also.

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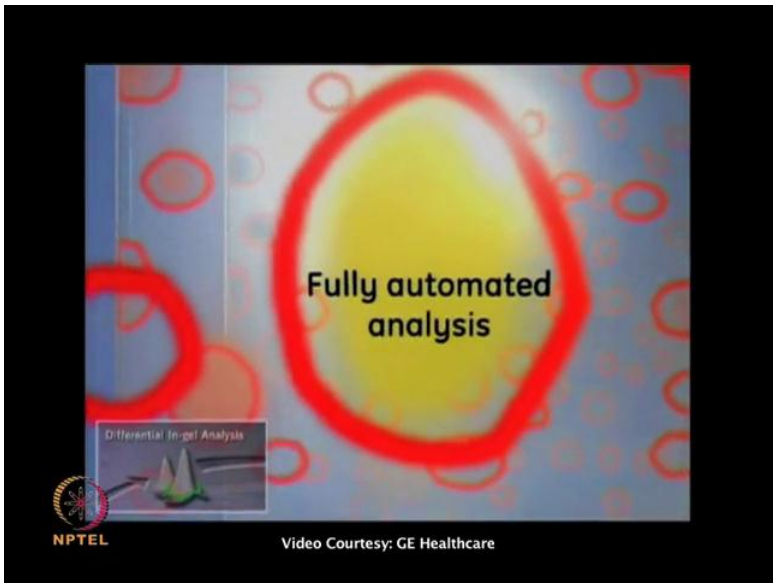

So, that the artifact should can be minimized, and this is fully automated analysis.There will be very less manual interference would be there in this analysis.

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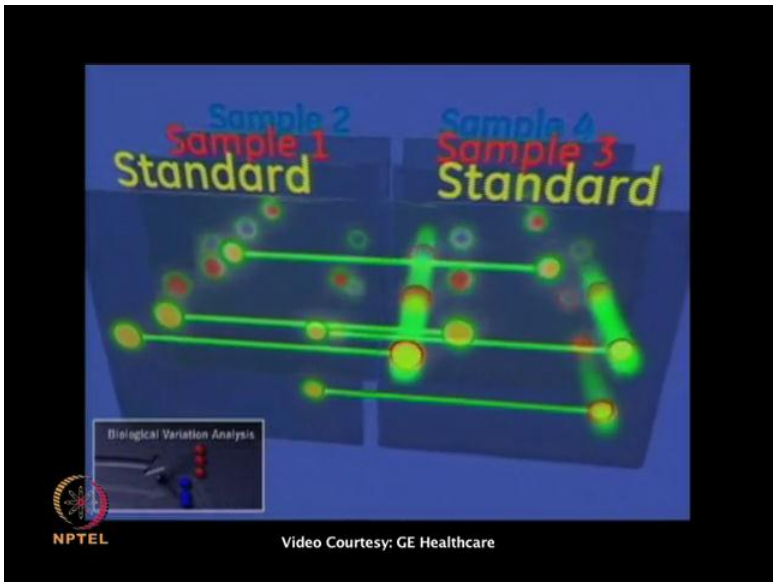

Andafter finishing of d i a we can go to b v ain b v a we can compare all d a s together. Like as you can see in this video this complete first of all it matches between the standard gel and from standard gel to again the corresponding d i a, it matches each and individuals spot and it will give you the final data.

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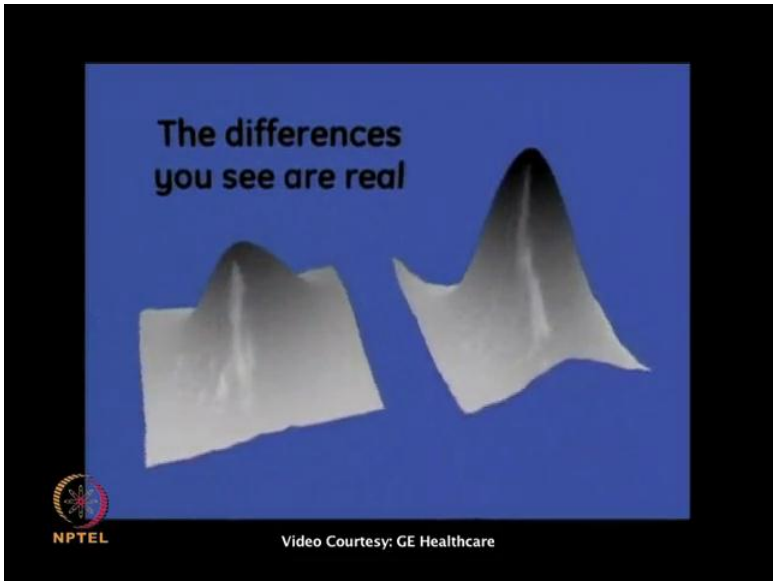

Then these d v s can be analyzed in the further in e d s that is extended data analysis.

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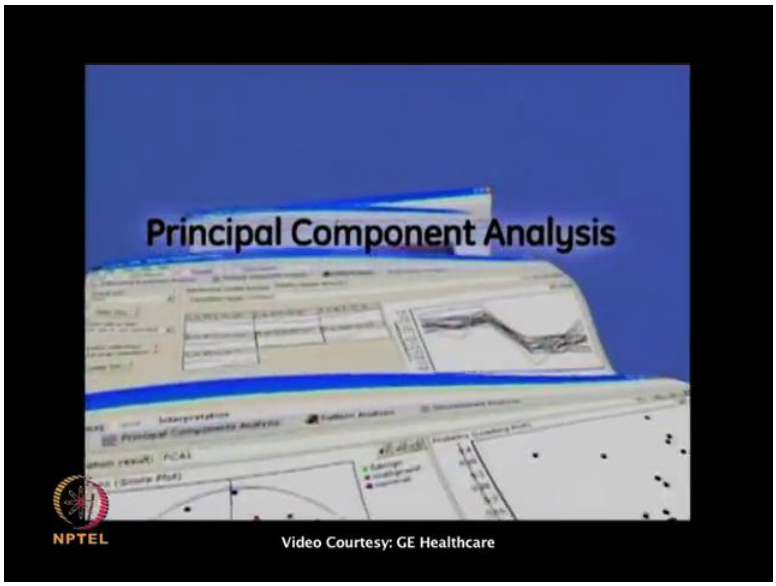

Here you will have lots of stringent this was stringent statistical data, that is a differential expression analysis, pattern analysis, discriminant analysis, and p c a analysis. So, Dr Srinivas it was very useful to get the glimpse of the overall process in DIGE technology. Would you like to elaborate or demonstrate some more detail aboutsteps involvedand doing the labeling of this technology?

Yes there is another video where you can see now, this is the second video which you can see more elaboratively, it is a particularly a protocol which is developed for membrane protein analysis now we can see this video.

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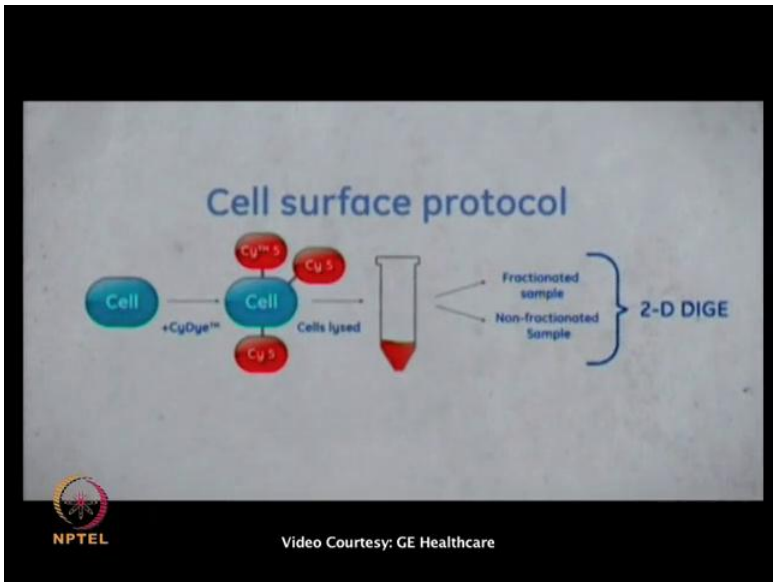

In the cells of is labeling protocol seen on top you label cells while they are still intact. During the labeling process the dye will only have access to the cells surface proteins. After the labeling step the cells are lysed, to verify cells surface specific labeling the label sample was fractionated into membrane and cytosolic proteins. And non-fractionated sample was prepared in parallel for comparison. This fraction analysis it is not necessary, butwas done here just to show that the cells surface protocol is specific forcell surface proteins.

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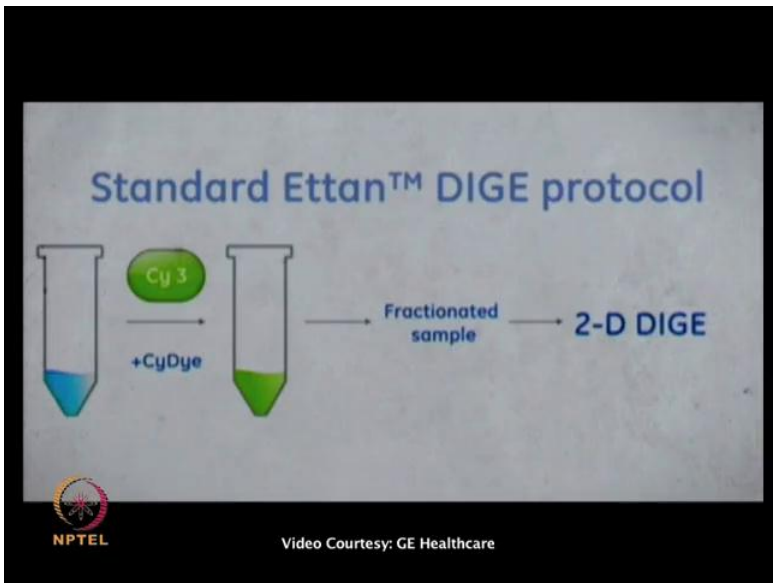

We also preformed the standard ettan DIGE labeling protocol same below. The cells are lysed before labeling and then these way whole cellular proteins are for accessible for labeling. After the labeling step the samples are subjected to 2 D electrophoresis.

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Adherent cells are detached using a non-enzymatic procedure to avoid the distance of the cells surface proteins targeted in this protocol. We used to rubusing enzyme-free cell dissociation media is also an option, come to divide the cell suspensions into aliquots of 5 to 10 million cells per tube. The cells are then pelleted and washed in h b s s p h7.4 to remove traces of cell culture media. Contamination from serum proteins and fresh and components can interfere with labeling and detection, cells growing in suspension or directly pelleted and washed before the labeling step.

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After the wash the cell pellet is resuspended in 200 micro liters ice cold labeling buffer containing h b s s p h 8.5 and 1 molar urea for optimal labeling conditions of cells surface proteins, always check p h before labeling. We used 600 pick mole cydye for 10 million c h o cells, the optimal ratio of cydye to cell number will vary depending on the cell type since, we do not know the exact protein concentrations on the cells surface. How to determine the optimal conditions for cydye labeling of proteins is described in the 2 D electrophoresis principles and methods handbook.

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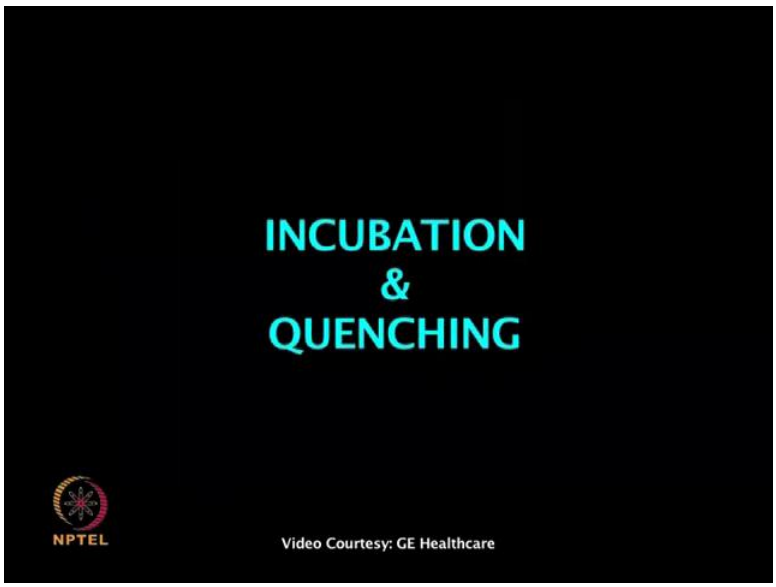

The cells are incubated with cydye DIGEFluor minimal dyes for 20 minutes on ice in the dark.

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After the labeling reaction the unreacted dye is quenched by adding 20 micro liters of 10 millimolar lysine. The label cells are now washed twice in cold h b s s p h 7.4 buffer to remove the access cydye. There will therefore, be no free dye left for unwanted intracellular labeling of proteins

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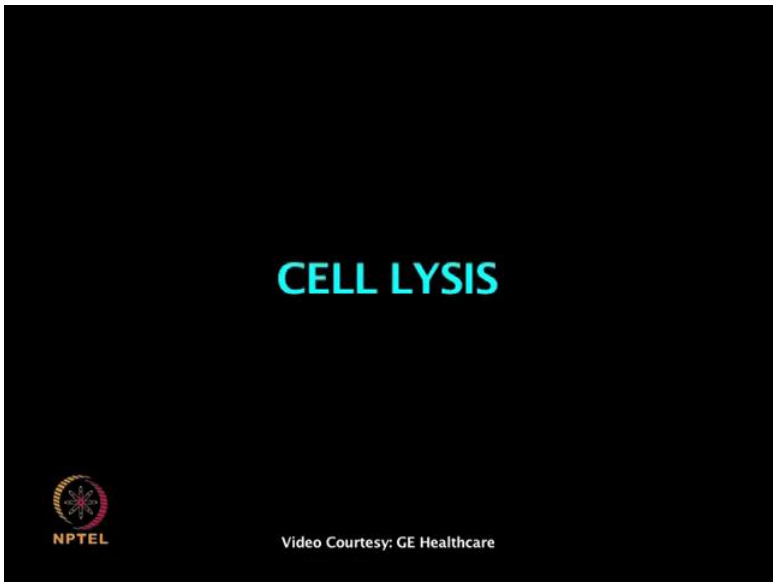

In the next step which is cell lysis. The proteins on thecells surface are now labeled and the cells are washed and ready to be lysed. The pellet from the last washing step is resuspended in 150 micro litter cold lyses buffer containing 7 molar urea, 2 molar 5 urea,4 percent chaps, 30 millimolar trace, 5 milli molar magnesium acetate p h 8.5 and left on ice for at least 1 hour with occasional vortexing.

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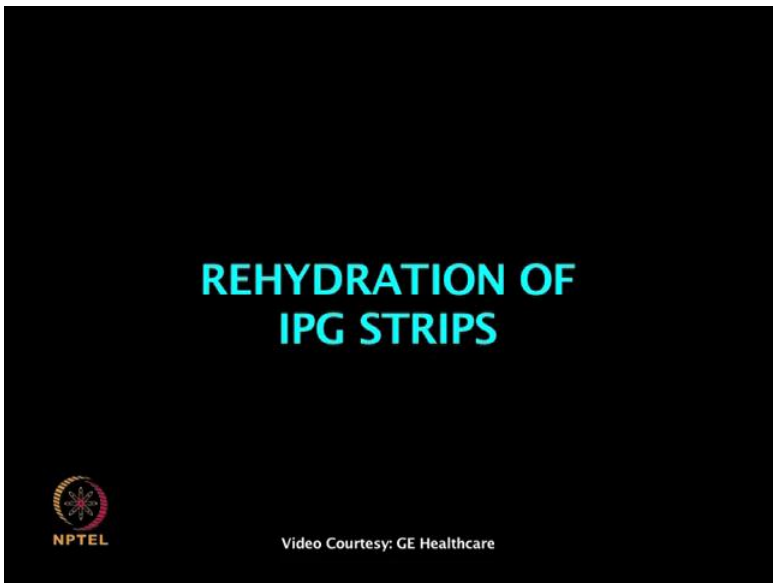

The samples are now ready for 2 DIGE gel separation. The first step in 2 D electrophoresis is to prepare i p g strips for rehydration.

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Prepare this rehydration solution by adding i p g buffer corresponding to the p h interval of the strips used, and add the solution in the lens of the rehydration tray, remove the protective film of the I p g strip and place the strips carefully with a dry gel facing down in the rehydration tray containing the rehydration solution. Close the lid of the I p g box and rehydrate the strips overnight.

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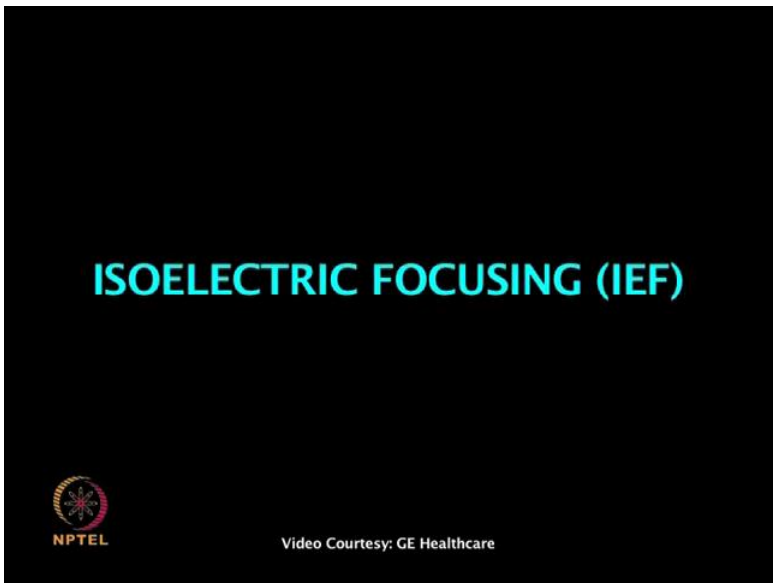

In the first dimension isolectric focusing the proteins are separated according to their p i. This is performed using the I P G for strip, the rehydrated strips are placed in the manifold and the electrode is mounted on top 50 micrograms from h sample where applied using sample application caps.

We have either directly applied non-fractionated samples without prior fractionation old fractionated a sample into membrane and cytosolic fractions before they were applied. The lid is closed to protect the fluorescent samples from light; the instrument was programmed according to recommendation and run overnight.

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Large 12 percent lemley gels were caused using a dual 12 gel coaster this plating solution was added to avoid polymerized acryl amide gels in the tubing's, the gels were allowed to polymerize overnight at room temperature prior to use .

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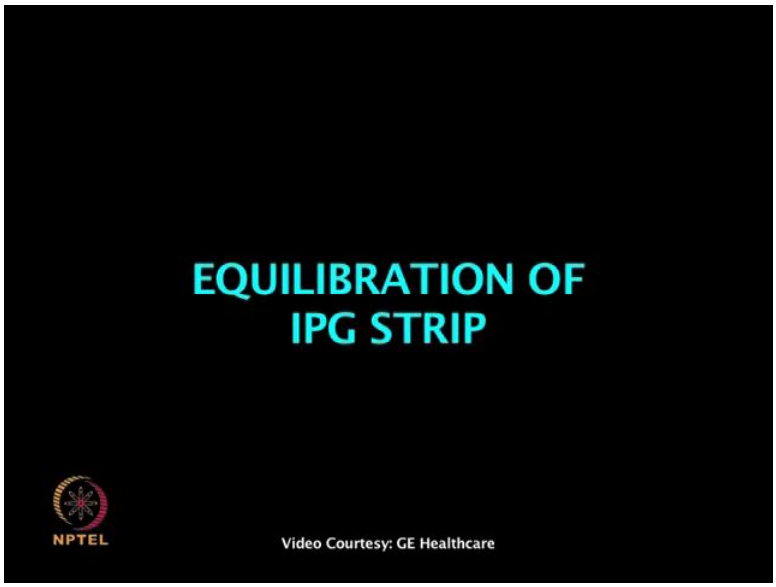

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After the isoelectric focusing the strips are removed and equilibrated in S T S containing buffer in two steps using D T Tto reduce the disulphide bonds of cysteine residues followed by argulation with acetoacetamide to avoid modification by acryl amide.

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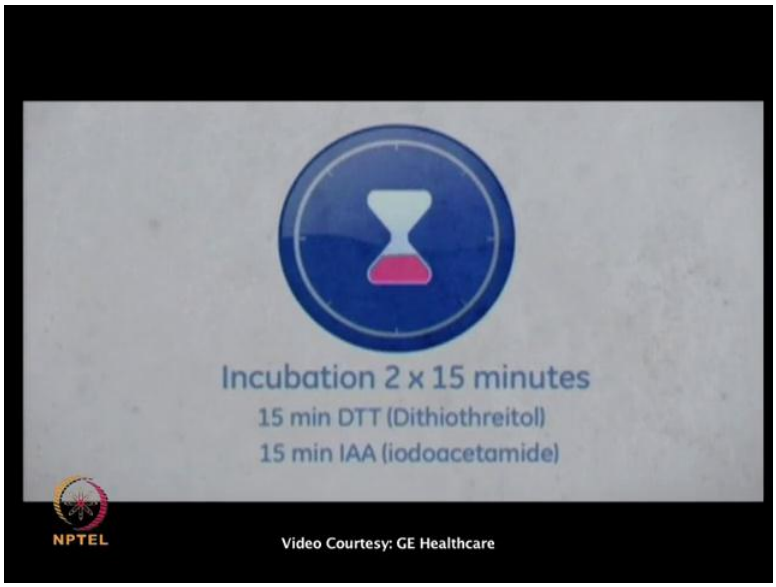

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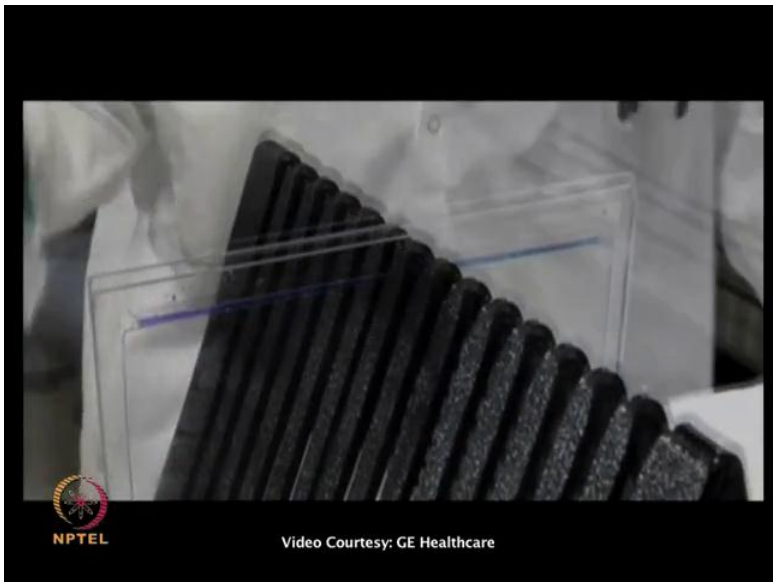

So, I P G strips are dipped in running bufferand carefully placed on top of the large 2 D gels. Avoid trapping air between the strips and the gel.Seal by adding melted 2 percent agarose solution with bromophenol blue on top, the gels are now ready for second dimension s d s page separation.

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In the second dimensionS D S page, the proteins will be separated according to molecular weight.

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And this is preformed with6 systemfill the electrophoresis chamber with a Nordic running buffer, insert the dialysis and fill the top compartment with cathodic running buffer.Program the power supply according to recommendations and run the second dimension protected from light for approximately 4 to5 hours or until the diaphragm reaches the bottom of the gel.

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After the second dimension electrophoresis the gel cassettes are placed by using the grippers in the typhoon fluorescence imager.2 gels and 3 channels can be scanned simultaneously.

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The result from this 2 D gel shows high resolution of membrane proteins in the sample. Even if there are some known restriction for hydrophobic proteins to be detected in a 2 D gel.

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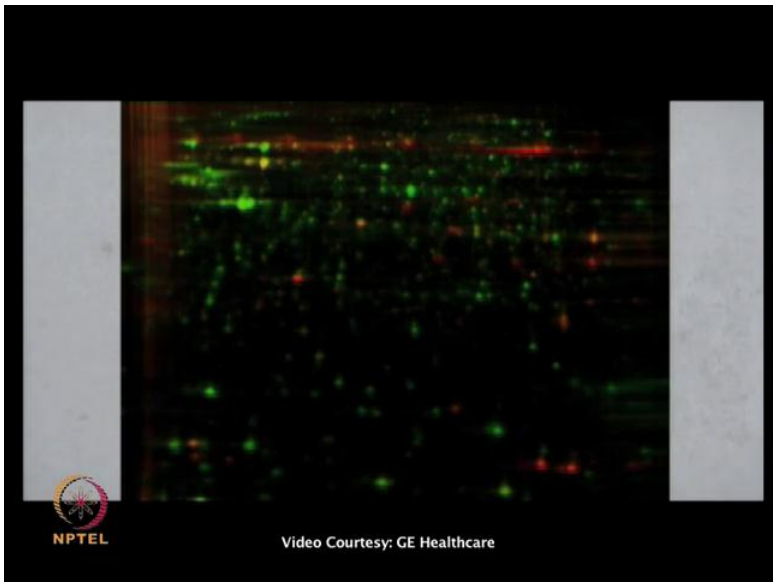

The result show many new cells of its label spot shown here in red.

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That are not detected using this standard labeling protocol show here in green.

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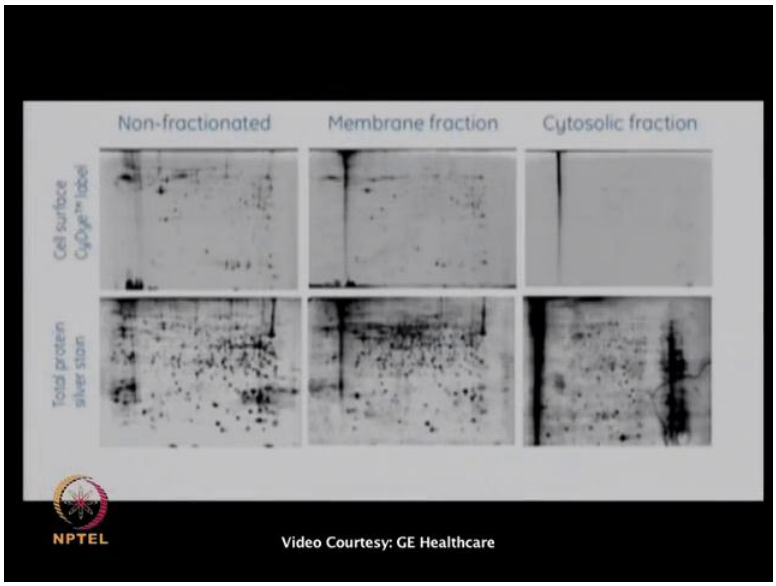

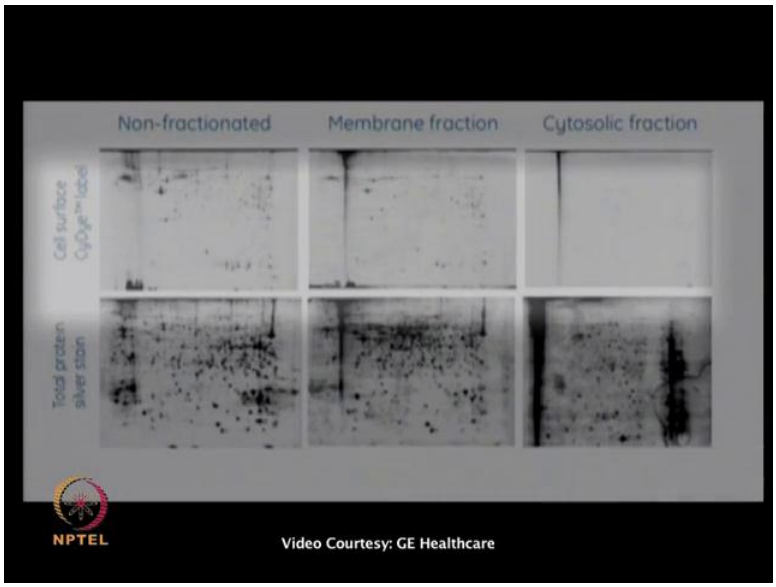

This result show that the cells of its labeling protocol is highly specific for labeling cells of its proteins, since cells of this proteins are exclusively labeled they are more easily visualized and that animation by abundant cytosolic proteins is avoided. The fluorescent image of gels with known fractionated membrane fraction or cytosolic fraction is shown on top. Below is an image of the same gel for stain with silver. The result has shown no fluorescent labeling of cytosolic proteins, but the silver staining shows that there are proteins in the gels. The results also shows similar spot map patterns from known fractionated and membrane fractions demonstrating that there is no need for fractionation prior to 2 Delectrophoresis.Which makes this protocol both simple and convenient Cy 2,

Cy 3 and Cy 5 show similar, labeling patterns and are all compatible with the cells of its protocol.

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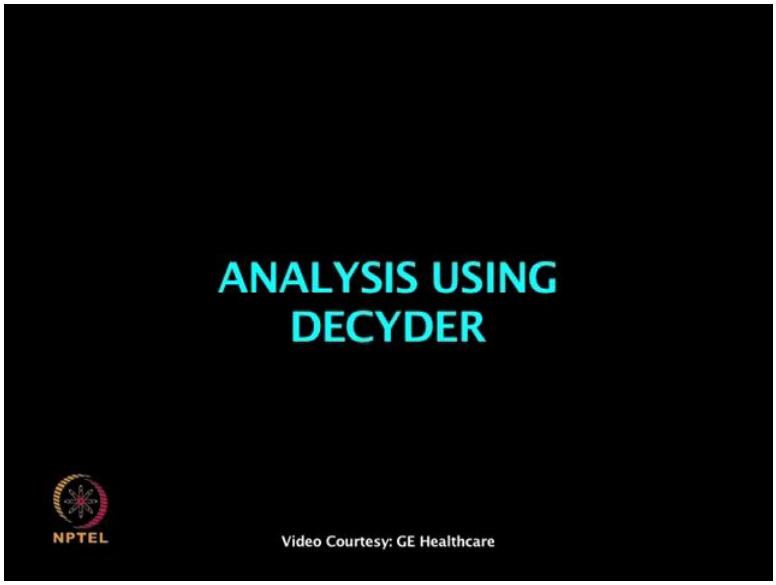

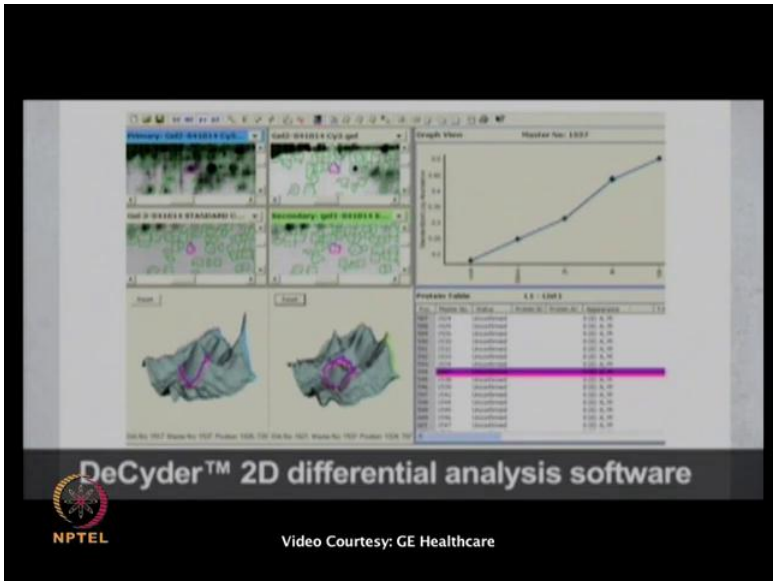

At DIGE 6 be a meant was performed using all 3 decyder DIGE for minimal dyes. For studying differential expression of cells of its proteins in c h o cells of the serum starvation for different lengths of time. Cy 2 cells of a samples from all sample in the experiment performed and usedas an internal standard.

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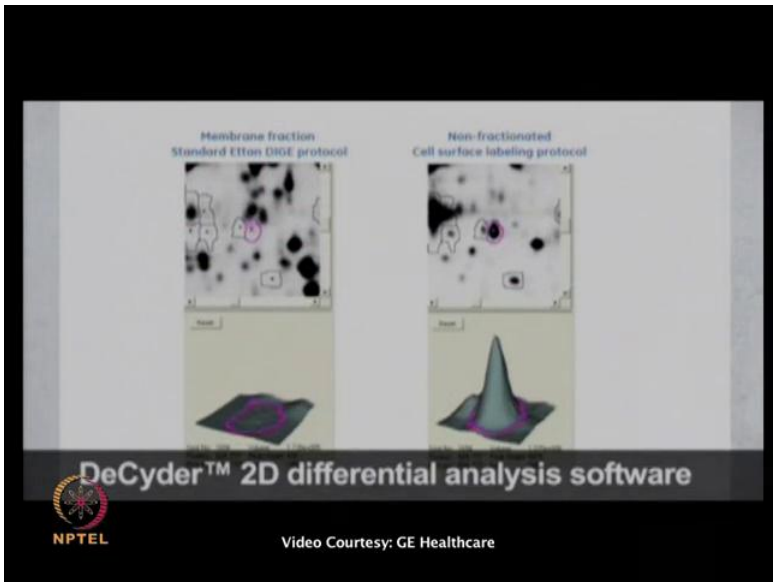

The differential changes of several cells of these proteins could be followed during this starvation period, over eighty novel membrane proteins were detected using the cells of this protocol that were not detected with a standard at an DIGE protocol. For finding their identity of the proteins in the preparative gel, it was necessary to spike with the cells of this sample to facilitate matching back to the spots on the analytical data set.

So, rotation was it was veryuseful to see all the steps which are involved in doing this DIGE technology. There is that discussion going on in the proteomics field, that due to advancement in mass spectrometry and microarrays. How to foresee the feature of 2 dimensional electrophoresis and DIGE technology? Do you think it is going to simultaneously working along with m s pack and microarrays, or do you think it is going to be behind, because now more advanced approaches have immersed? Yes. So, what is your suggestion on that?

Yes are the m s and microarrays develop differently they have their own advantages as well as disadvantages. Like disadvantages like very high instrumentation cost and high protein requirement as well as technical variance would be there, that is why still 2 D DIGE will be the core technology to analyze propteomics one can easily analyze. So, you are still very convene thatby using2 d DIGE approach that will still remain 1 of the core proteomic technology for proteomic analysis. C s dash. And it will be use simultaneously along with mass spectrometry and micro arrays. Yes. So, at the end I

wouldlike to request you togive a brief over review of the decyder software which is being used for analysis of DIGE gel will show decyder is the software to analyze DIGE gels where we can analyze DIGE gels in different stages.

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In first stage we have to upload or gel into or software that this is basically works on the oracle database, where we are incorporating our gels into database, there you can add these gels into software by this way and you can select what are the gels you want to upload. And once we have upload then you can able to addict these gels here itself, like you want to crop (( )) things any sort of things you can do here, it will allow you to edit these images.

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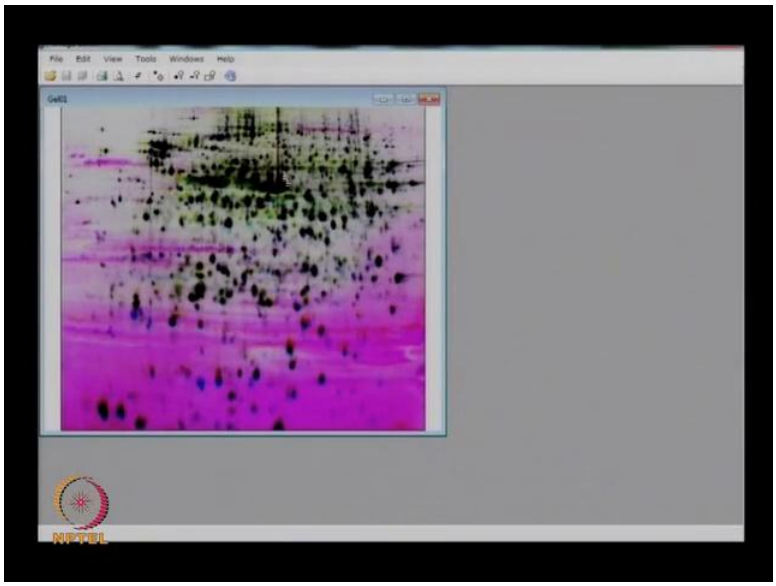

So, now you can see this is the overlay of 3 images. Now you can crop all these images like what are the portion you want to take, if you have to remove any area which is not your interest of then you can directly crop those images, where if once you directly editing you can save it. Yes save it and close it this is what you can edit these images then you have to create a new project. Suppose your project name would be according to your project name this is demo.

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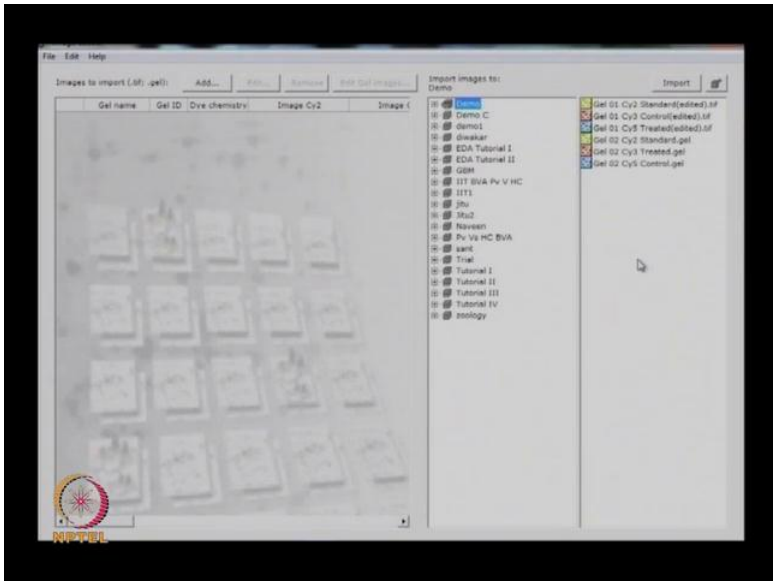

Now we have the project mane of demo. Now, you can able to import these 2 gel image into it is a new project. Where we create a just click on import now, these gels will be imported into this particular project. Now, here you can see all six images through these are the basically 2 gels inside we think these 2 gels we have Cy 2,Cy 3, Cy 5 kinds total 6 gel images. This is the image editor and image loader. The next one is differential in-gel analysis shortly we call as D I A in this you can say we can create a new project new D I A here like here there is an option create work space from here.

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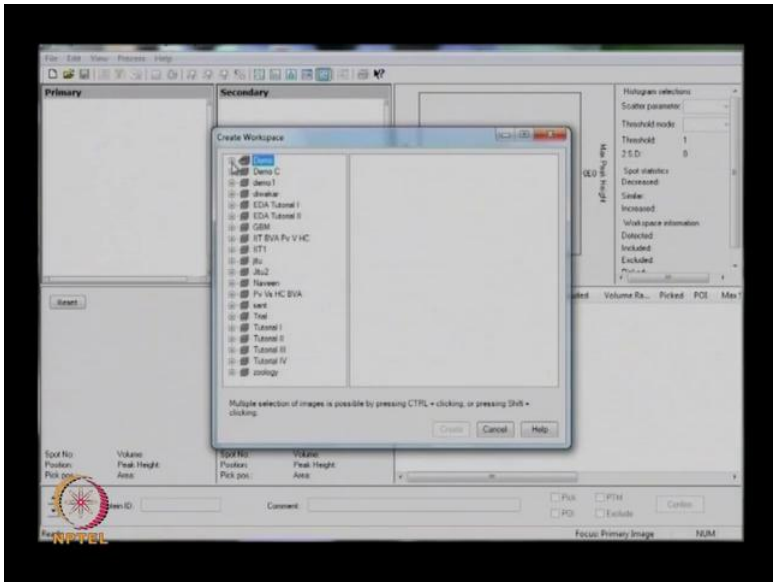

You can now it will take you to where we already saved our gels in our database. Now from the database we can select any particular project, and from there I have I am selecting gel number 1 as we saved this 1.

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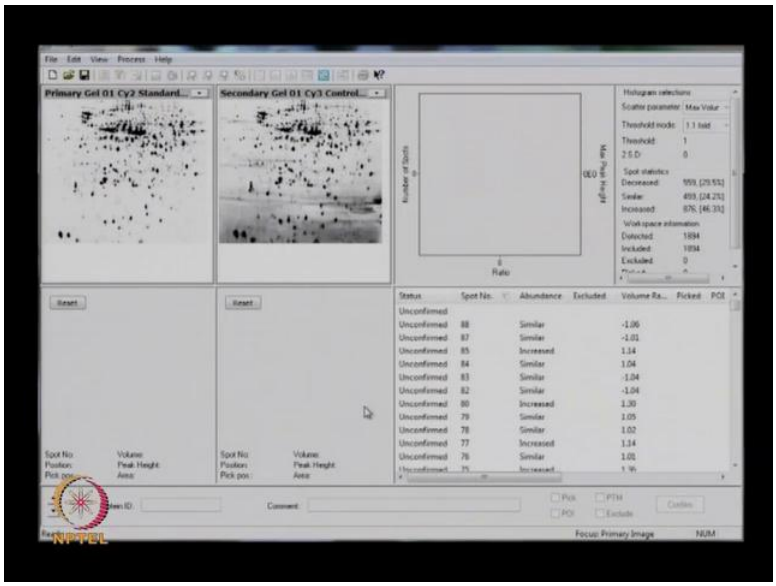

Now, you can see these gels where we have uploaded. Now after uploading here you can process these gels. Then during process you have to give some number, this is some threshold which you are giving here actually this may be 2000. So, that it will takes care of background issues also then basically in decyderitco-detection will be had, but I would like to explain you some more about what is co-detection this co-detection is uses a information of all three channels.

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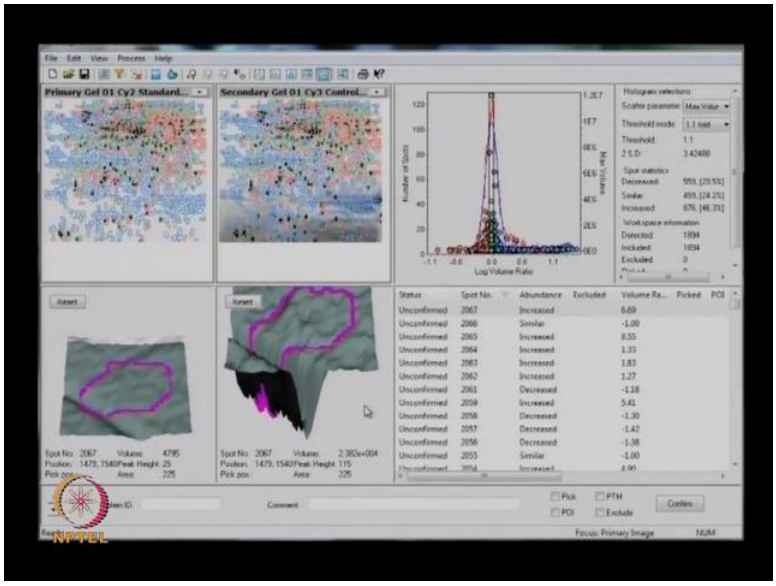

And will create the geometrically identical spot boundary for your spot across all the channels that means, there are 3 channels Cy 2,Cy 3, Cy 5 out of these three channels with inside two image it creates a particular volume and the same area can be applicable for the remaining 2 gels also this is way it works.In this way quantitative and qualitative results are much more accurate than which a single detection. In D I A each image is co-detected with its internal control producing 2 images pairs. The ratio of standard sample is calculated further or the ratio of standard sample is calculated for each protein in each image.So, as we see here these are all the number of spots which isit has been detected in which there are we can see some red color spots here, these are all down regulated and compare with control with treated or and this blue color spots where you can see these are all up regulated spots when comparing with control and treated in between these there is a blue there are some blue color spots these are all similarly, regulated so this is what we can see in D I A.

Now you can go through each and individual spot and you can see the 3 D view of that particular spot. If suppose if we can select any one you can go to 1 by 1 and you can see whether it is exactly spot or some background or if it is a background you have you have to remove that.

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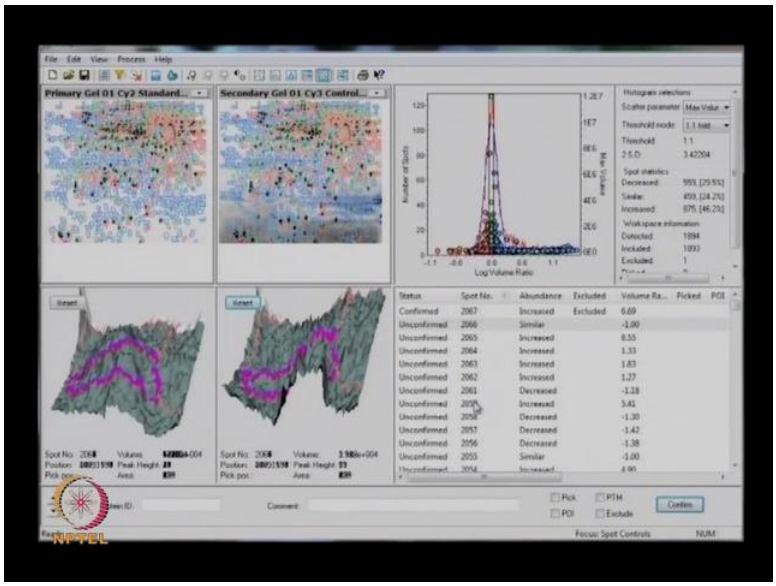

So if suppose this is the background. So, there is no spot at all still it is detected some background. So, you can exclude it from here by clicking, then confirm it. So, this

protein has been removed from the gels. So, the same way you can go each and individual then exclude it confirm it by this way you can check all spots and you can have more accurate data with you. Like you say can see the how accuracy would be there like the spot detection. Now you can see this is what we will get in D I A that is D I A creation we have not finished. This is the b v ab v a is nothing, but biological variation analysis. one of the internal standard image is selected as a master image and all internal standard image matched into this, samples standard spot ratio for each protein each sample then compared giving t test value fault changes anova values for each and individual protein. How to create b v a work space can open the b v a and create a b v a work space and go to or D I A workspace where we have our b D I A s from there you can create actually, and edit 1 D I A I have added and this is another D I A and I am adding here minimum 2 D a s we require for the b v a. So, we have 2 D as here and click on create so it creates a new b v a for you.

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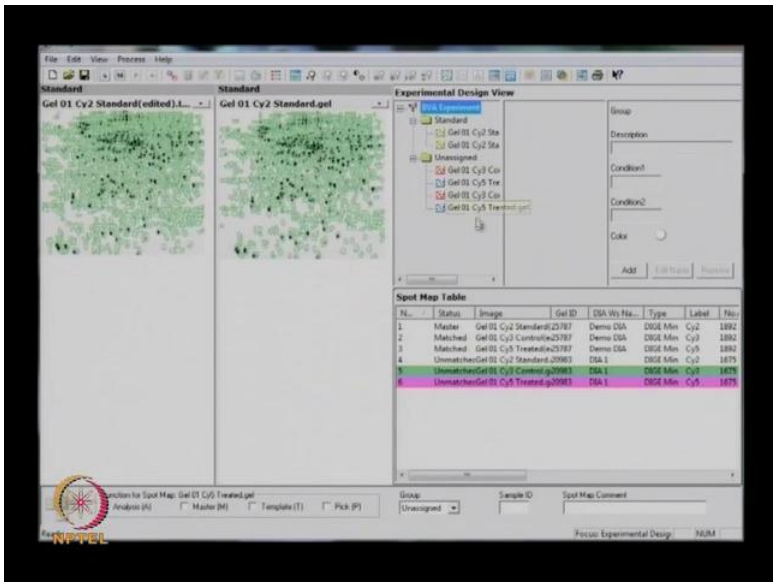

This is the newb v a from here the first all Cy 2 gels are automatically goes to standard folder, there is f standard folder you can see. And remaining all gels remains in the unassigned folder where we need to assign these gels as it as according to the gel type or sample type. Then you just have to click on add option, now create a group it may be control or treated this first 1 is the control and apply some color draft, confirm it then another you can create like treated then give some color draft confirm it.

Now we have two folders control and treated.So, as we have in assign folder both control and treated. This control gels we can transfer into control folder by dragging those images and treated gels we can transfer to treated folder by dragging them.

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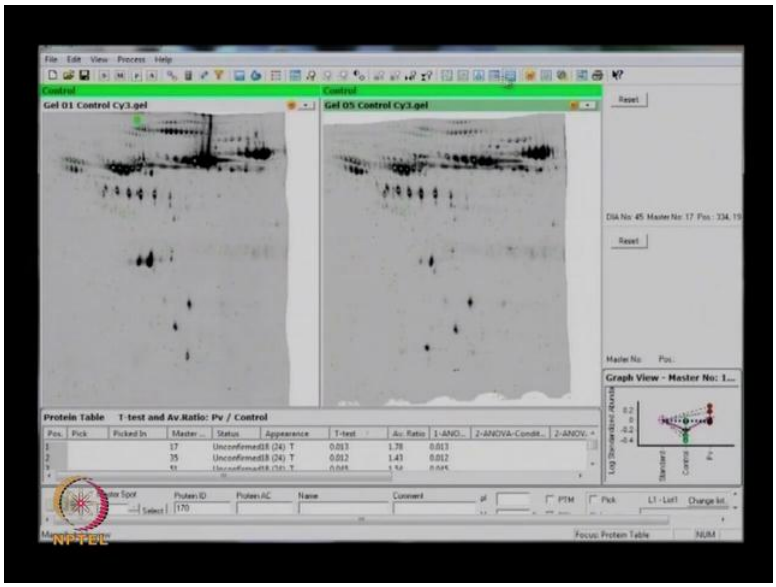

Now, we have images here, after shifting it control to control and treated to treated we have to match all gels. Just click on match and match all let us match the matching process has been finished. Now as we discussed in out of all standard gels 1 gel selected as a master image, as we can see the number 24 gel has selected has been selected as a master and it will compares remaining other is else with this master gel. Now we have this comparison data after that we need to calculate statistical parameters. So, click on statistical parameter button. See now we have some pair statistical parameters like independent t testaverage ratio student t test 1 way anova in between different groups we are doing in between control and treated.

So, calculate them so the statistical parameter has been calculated. Now we can see exact results of statistical parameters say if I can go to that table view here, you can see t test 1 way anova this you can see. So, we can select from here which are all the statistical significant which are all not significant.

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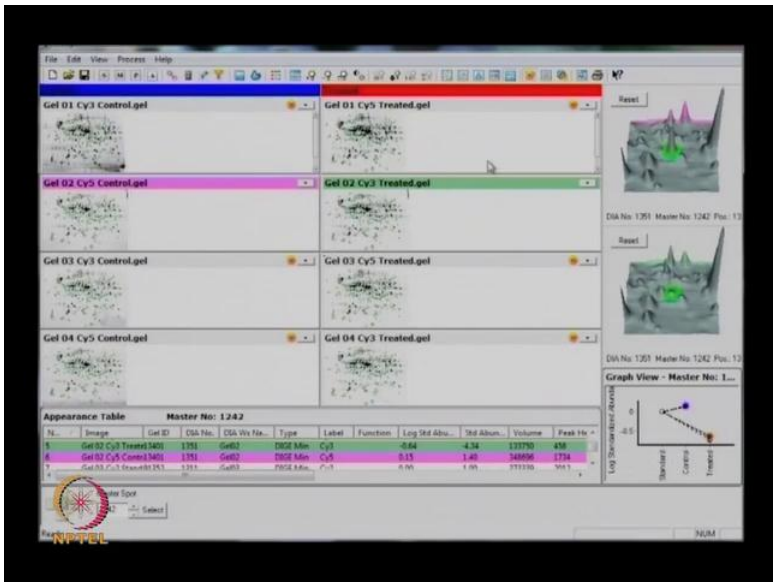

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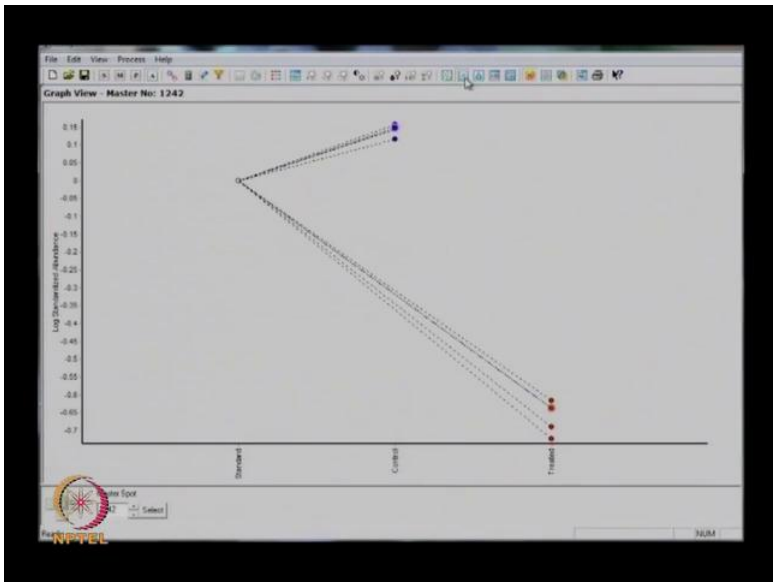

After analyzing the statistical data now we can see the complete results here we can see the 4 views like this is the image view and this is the histogram view in histogram view we can see clearly particular protein how it is behaving throughout control and treated if you can see this is the standard gel that means, this is the mixture of these control and treated.

So, it is somewhere 0 we can consider this 1,then the control is completely it is showing up regulation and after giving the three particular treatments it is showing the down

regulation.So, this kind of data we can see here, then in the table viewas you can see the complete protein data where the t test value average ratio value 1 D anova value these all we can see the we can see here in the table view.

So, the four views at a timeto see this is the four views you can see here. So, after this we can filter then according to our interest. So, select other few parameters which are available this treat to 10 gels which are spot maps, which are present in the particular protein should be there, and student t test as well as average ratio, than one way anova value then filter it.

So, there are 2299 spots are available in this all gels, but 107 proteins only passed all these parameters. So, these parameters we can select as a protein of interest and assign pick list. So, that this proteins can be saved in a file this file can be given further to or spot picker these are all the things we can able to identify very easily in b v a. This is very user friendly there is no much more manual interference, this is what this helps you to analyze your DIGE gels.

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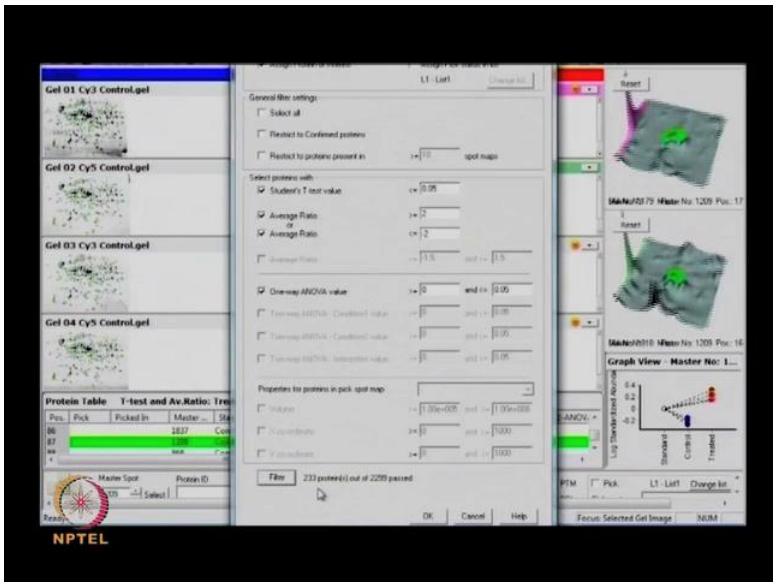

So, can you elaborate onwhat E D A and extended data analysis what it can dowhich we are unable to do it in b v a.So, there is a layers here right 1 is the D I A followed by.

Yeah.

Student: B v a

Yeah

Student: And then ultimately E D A analysis.

Exactly.

Basically, what we can do here is we can compare 2 b v a's together there exactly here we are talking about a particular disease or particular particular set of an data only there we can analyze different b v a's together in d d a. There you will get a majorly differential expression again you will get as well as p c a and discriminate analyze so see you will these kind of statistical data you will get d d a, very shortly as i just show youbriefly regarding the… If I understood correctly probably the statistical parameter more stringent over therein the E D A. Anyway we can have some better biological significance and formation formula. Exactly. Data set. Exactly.

Because, lot of clinical data or different type of treatments people like to do several gels and lot of treatment. Yeah. So, your number of sample to be analyzed. Yes. It is very large. Exactly and really obtaining the meaningful and formation. Is 1 of the major challenge? Exactly. In all the proteomics. Yeah. So, I would like to see in E D A. Just the thing which we can see here is differential expression analysis, in which you can see differences in between control as well as two experimental data.

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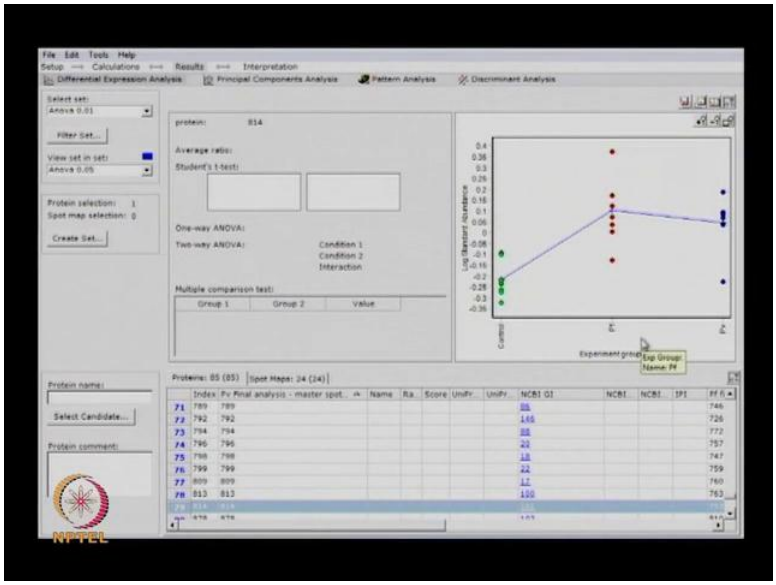

This controls different treatments which given here you can see how the particular protein is we have expressing throughout these control as well as this p f and p b this kind of things. And you can see this kind of data for each and individual protein here. So, that from here you can see which one is your interest and which is not.

So, you are actually… You are actually analyzing the data spot wise now the spot by spot you are looking at that. Exactly here the spot by spot which we are seeing, the number of the even the index number which shows there is a master cellfrom that master gel you can see the exactly this number. This is what which we are seeing here for each and individual spot here we can see the results then as well as if you can seeprinciple component analysis for this data.

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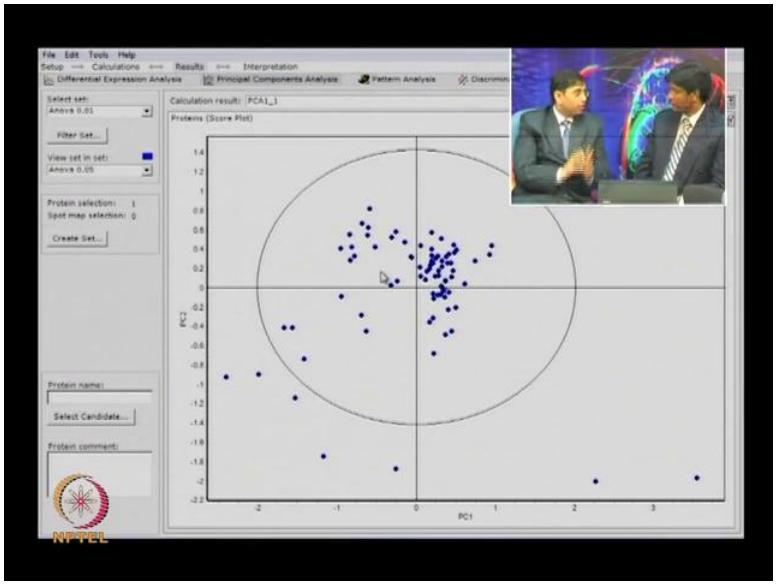

Here there are almost 89 proteins. So, out of these eighty nine proteinsyou can see there are inner the circle there are protein some proteins are present and out of the other out layers are there. The inner circle they are similarly, especially if I can say a 95 percent statistically significant there are. And out layers which you can see are exactly this can be a some non reproducible spots or else what the major thing is this are all very highly up regulated or highly down regulated. So, this can be worked as the marker also then we have to go back to our b v a data, and we check the protein how exactly it is regulating then we can identify that protein and we can use and for further analysis. So this is the powerful statistical parameter. By using which you can identify some outlines. Which

could be the potentialdiscriminate or between the control and treatment. Control and treatment… And once you identify those proteins then actually you can go back to your original data from the b v a.

Student: B v a and get all the analysis done.

Exactly This is very interesting.

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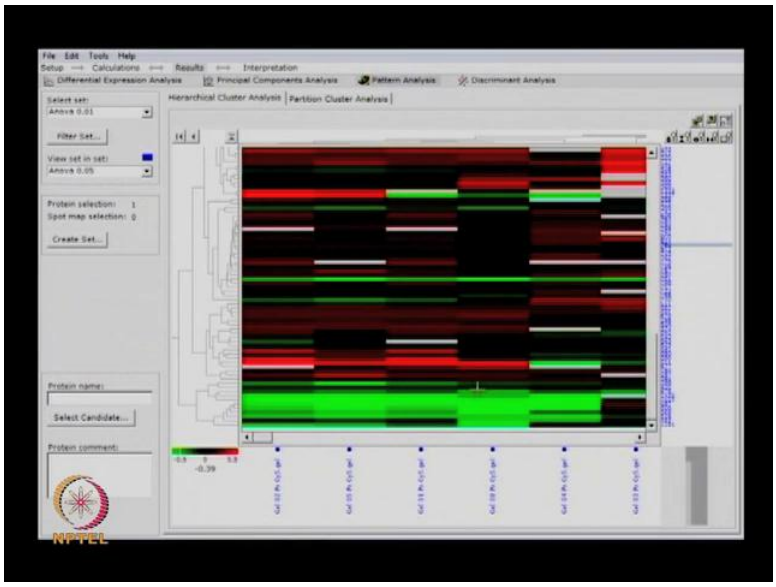

And next pattern analyze is you can see the whole proteome, then how these all are different from each other. So this is the heat map of this is the heat map of the count total 82 proteins are which we are taking into consideration then how they are up regulated. If you can see the blue area sorry the green area exactly we can see is completely down regulated area, and the red color portion where as you can see that is the up regulated portion, and the remaining black color which we can those proteins are similarly, regulated this is what which you can hereyou can see here, this kind of data will help you to represent your complete the whole analysis.

So, rotation wasit wasvery useful to have you here and to get an over view of DIGE technology. How people can use this decyder software, and analyze the data by usingd i a b v a and E D A.And all though there was not enough time, but you gave an very good demonstration in a very short time, to give a glimpse of the processes involved for doing

this analysis. As well as how different type of a statistical parameters can be applied to get some very powerful statistical information from our biological data.

So, thank you very much for coming here and giving this very good interaction about DIGE technology. So, I hope our discussion with Dr Shrinivas was useful. And now you can perform this analysis, by using specialized software and obtain some very useful biological information from your data set. Probably you must appreciate there are lot of meticulous steps, are involved then performing these experiments, but at the end this provides a very useful quantitative multiplexing approach to separate proteins and to analyze different type variations.

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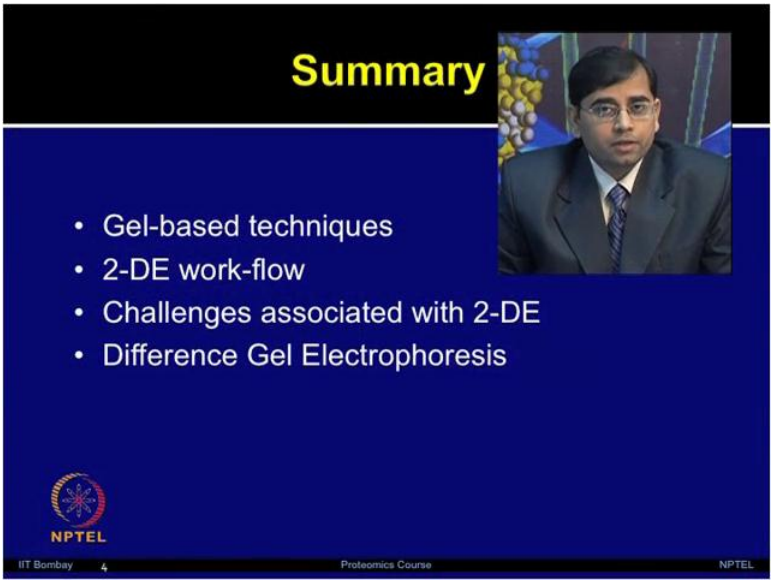

So, we will summary in this modulewe have discussed about gel base techniques. We talked about basic separation by using SDS page blue native page. Then how it would perform isoelectric focusing and by combining those two methods. How to perform 2 dimensional electrophoresis. I give you a work flow for performing 2 dimensional electrophoresis experiments, then we have discussed about different type of challenges for these methods. And I briefly introduce few creative ways of performing gel based proteomics method, including of gel fractionation methods. And then we discussed in much more detail about difference gel electrophoresis.I hope at the end of this module and lecture, you will be able to perform the gel based proteomics experiment, but please keep in mind these protocols and methods are only giving you a feel for performing these

experiments. Each experiment each sample type each biological question brings its own unique challenges, and depending upon those conditions andyour sample type you need to optimize these methods. There is number one technology which can answer all of your question, but it is good idea for you to know that what are different methods which are available for you to use.

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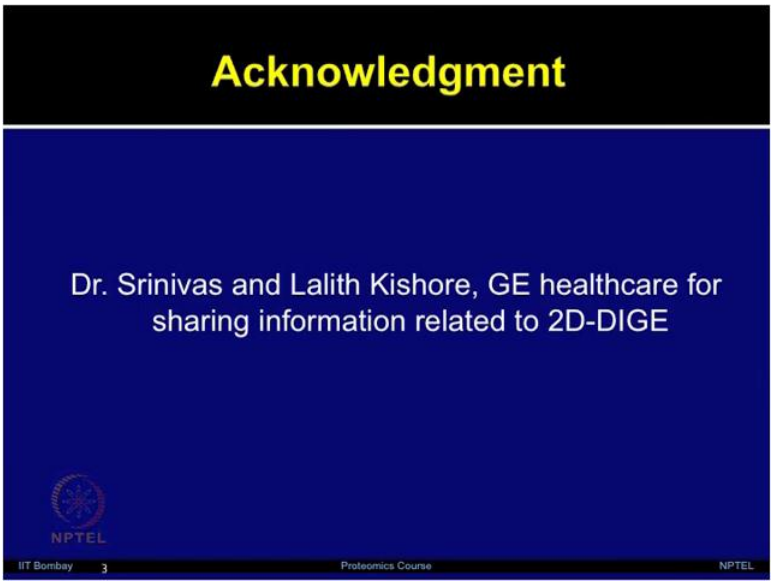

So, I hope by taking this module on gel based proteomics. Now you are familiar with different type of gel based technique, these are only a few there are many other methods as well available, but these are the most commonly used methods which people are applying in the fields of proteomics.Thank you.