Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
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Indian Institute of Technology, Bombay
Mod 05 Lecture Number 18

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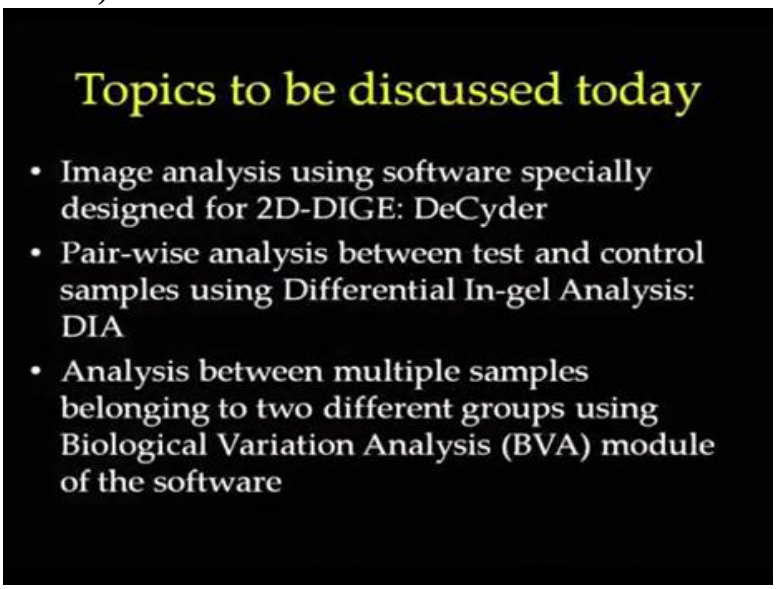

So in today's lecture, we will continue our discussion on gel-based proteomics, certain advantages and challenges of performing DIGE experiments, how to analyze DIGE data by using few very specialized software and how to interpret that data, how to obtain some meaningful biological information from those analysis.

So today I have invited a guest Doctor Srinivas from GE Healthcare who is going to talk to us about DIGE technology and give us a demonstration on software to perform DIGE gel analysis.

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Professor - expert conversation starts

Professor: This is my great pleasure to introduce Doctor Srinivasrao from GE Healthcare.

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Professor: He is an Application Specialist in the Research Product Division of GE Healthcare. Doctor Srinivas, thank you for coming for discussion about 2D and DIGE technologies. So what are the major advantages and disadvantages you see by using 2D approach?

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Expert: It is very user-friendly as well as very low instrumentation cost. There, the completely proteomic, the differentially expressed proteins we can say exactly…

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…during this complete 2D electrophoresis technique as well as the novel proteins we can able to isolate only from this technique. This we can identify very easily the software as well as very user-friendly, this is what we can do. There are some disadvantages. The reproducibility will be not there in most of the times, this is a major disadvantage of 2D electrophoresis and again, softwares...they are very user-friendly, still there is lots to be need to be developed so that we can use very effectively.

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Professor: So as you rightly mentioned, so two-dimensional electrophoresis is still a very powerful tool to resolve thousands of proteins on a gel.

Expert: Yes

Professor: One can also analyze isoforms and post-translational modifications including phosphorylation and overall the process is very, very user-friendly. So still it is one of the very powerful technologies currently available for proteome analysis.

Expert: Yes

Professor: Now I also agree with your comment that there is some reproducibility issues because users have to run the gels from control and treatment separately. And then there will be lots 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 two-dimensional electrophoresis technology?

Expert: One should have to prepare very good sample preparation so that the reproducibility thing will overcome. The second point is, these days the New Generation 2D electrophoresis system, that is the DIGE technology is available where you can use 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 3 you can run in a single strip so that these kinds of reproducibility issues we can overcome as well as... there is a scanner, very powerful scanner is available in this technology, that is Typhoon Trio where you can have a.... this is based on the laser-based technology completely so that you will have very efficient gels in your hand. This is...

Professor: So as you mentioned that DIGE technology is one of the very powerful solution to overcome several challenges which people face in running traditional 2D gels, and so in that regard, I think if you can elaborate little bit on DIGE technology, that will be useful to the students.

Expert: Yes Sir. I have a small video regarding the DIGE technology. I will show you that video so that everyone can able to easily understand exactly what DIGE technology...

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Expert: Yes, as we were talking

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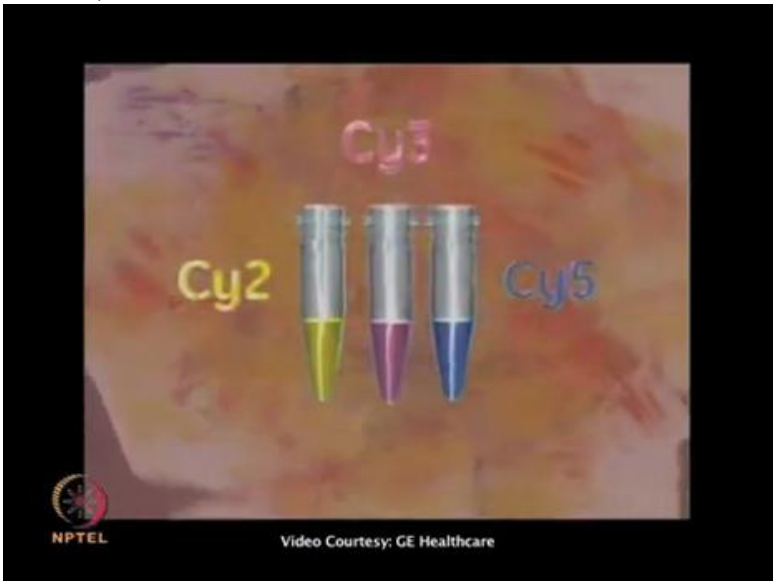

Expert: about the 3 samples we can load, label with Cy2, Cy3, Cy5,

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Expert: now we have 2 different samples from different source. Now you are taking these 3 samples into 3 different eppendorf tubes

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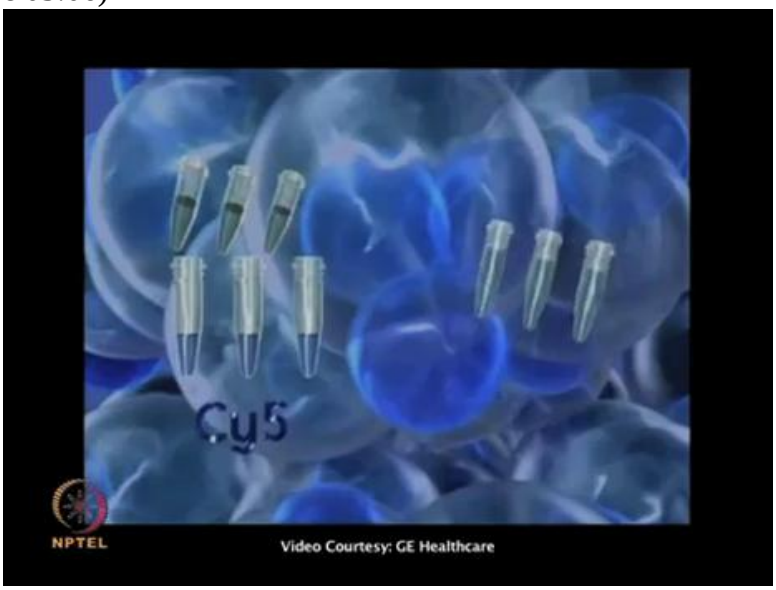

Expert: and you are labeling with Cy3 dye

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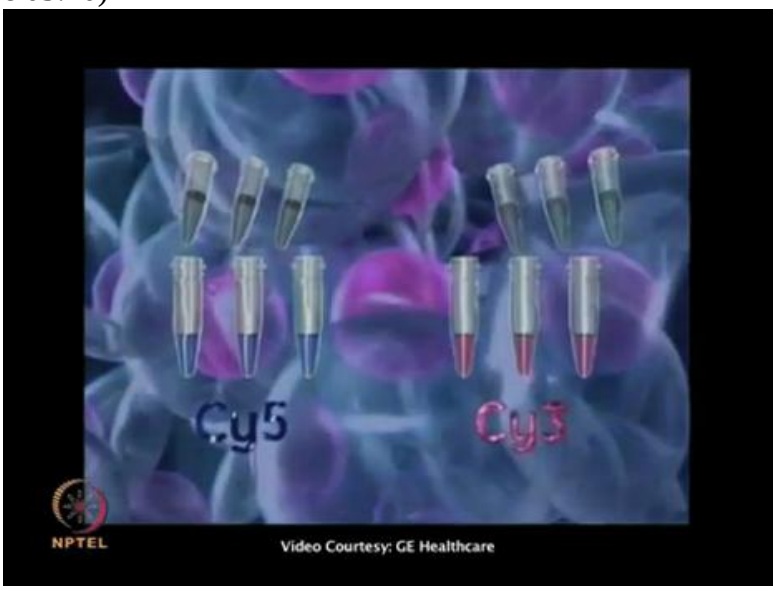

Expert: … and again another with Cy5 and you are taking the 2 samples and cooling them

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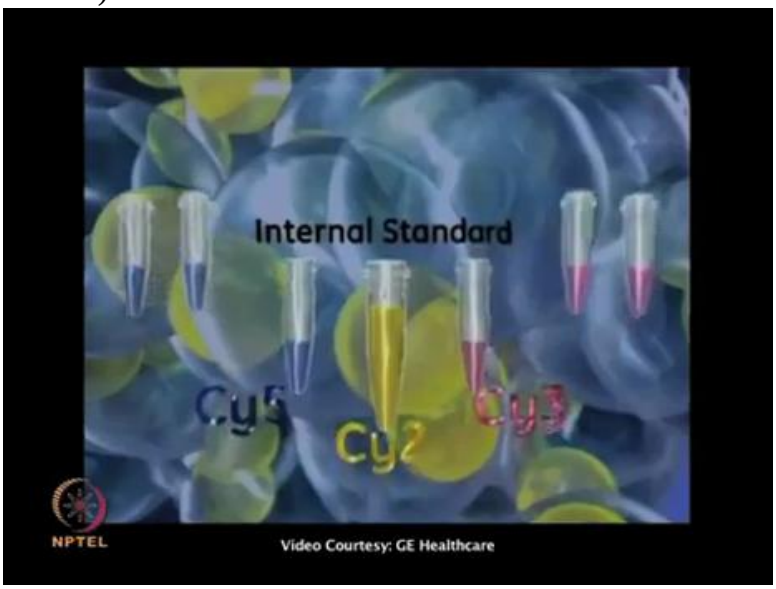

Expert: and labeling with Cy2, then

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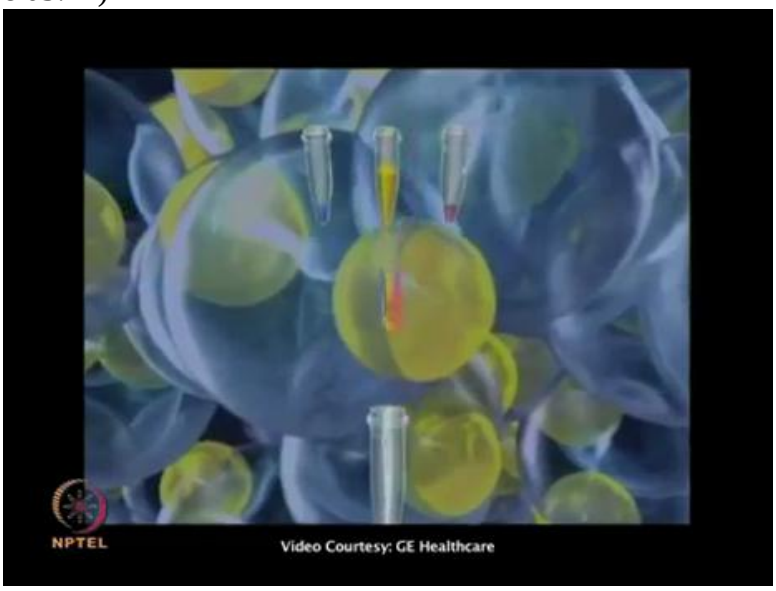

Expert: you are mixing those 3 into a single tube,

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Expert: then you are running in a single strip. There you will be analyzing

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Expert: first dimension, the IEF

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Expert: whereas the first dimension, after analyzing the IEF

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Expert: you are placing it to second dimension

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where you have completely analyzed the second dimension

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Expert: Now this gel can be scanned in the Typhoon TriPlus

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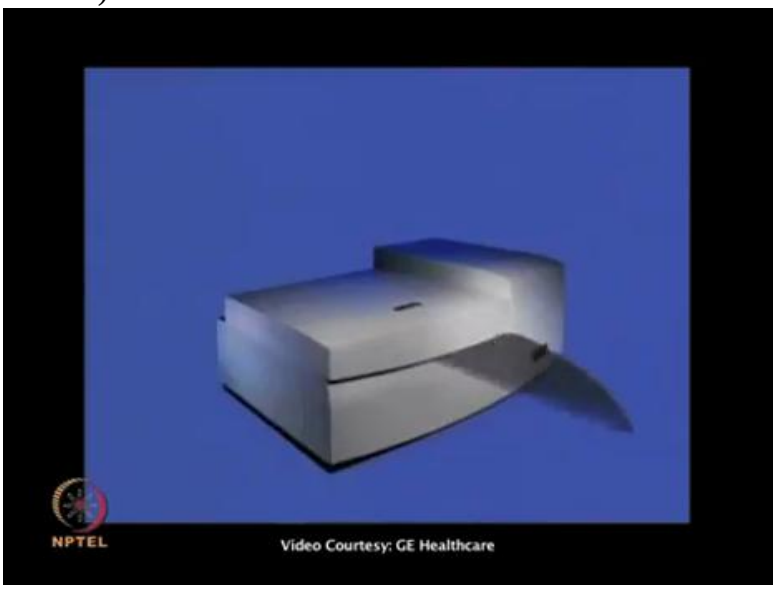

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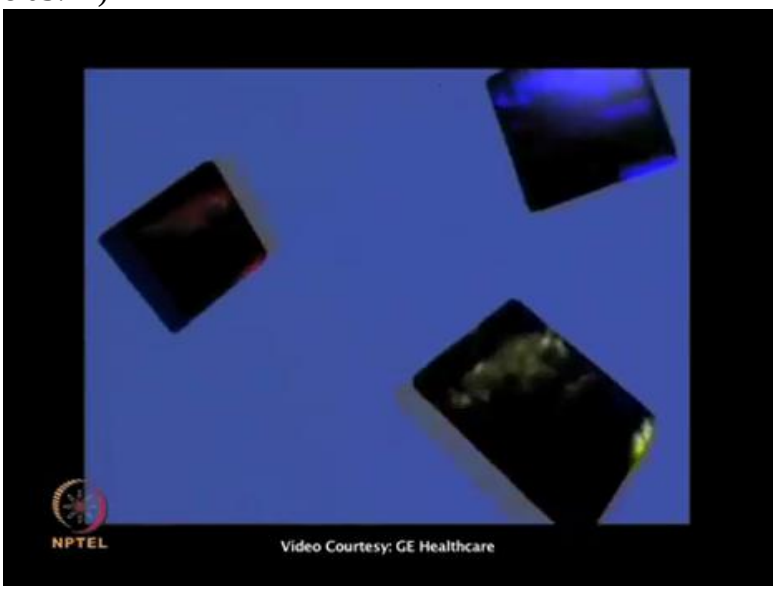

Expert: where you will have 3 images from a single gel. Then this can be analyzed…

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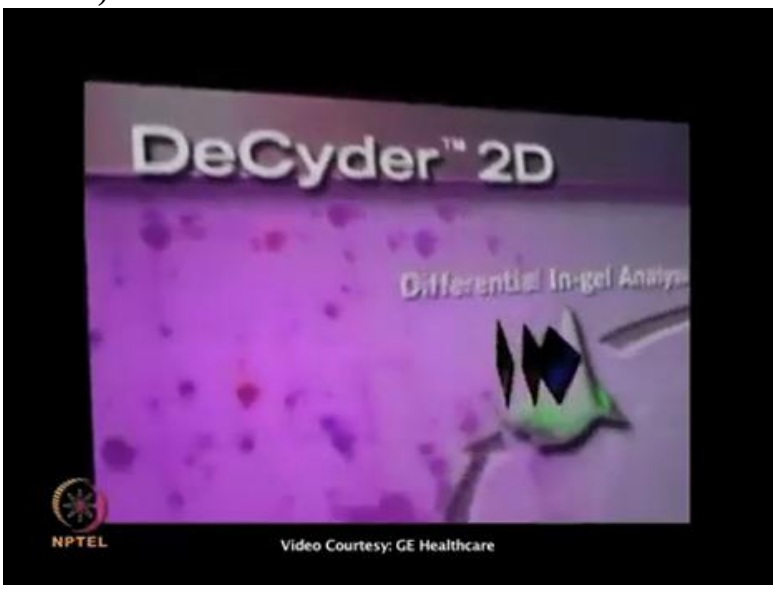

Expert: with the help of DeCyder software

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Expert: where the complete analysis can be done with the help of DeCyder.

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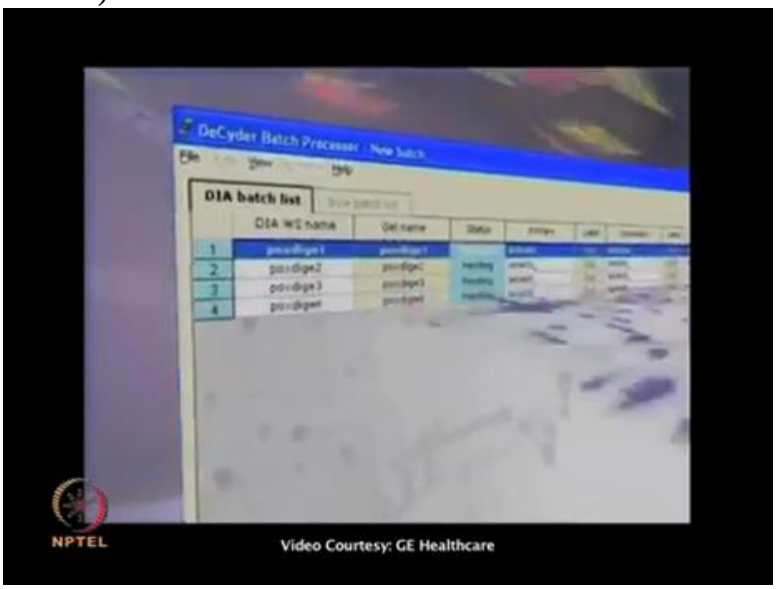

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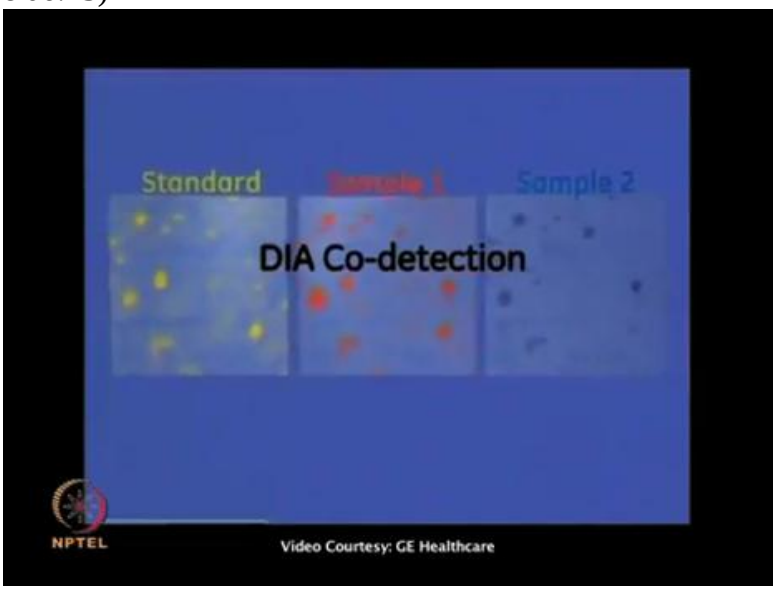

Expert: In DIA there will be a co-detection.

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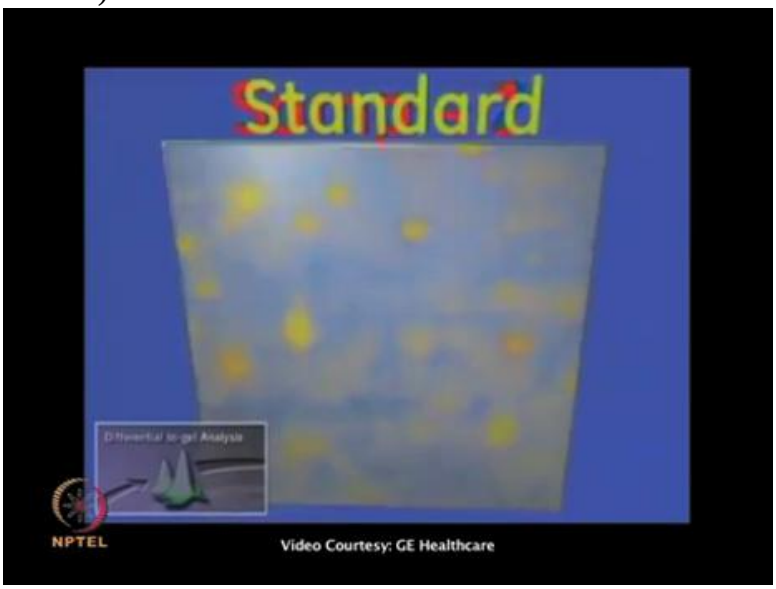

Expert: The co-detection is nothing but

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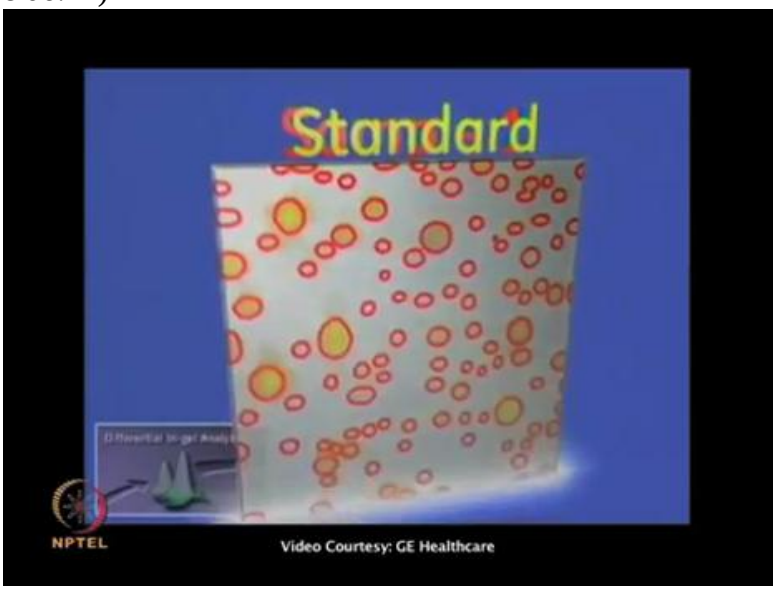

Expert: there is a standard gel labeled with Cy2 from the same area where

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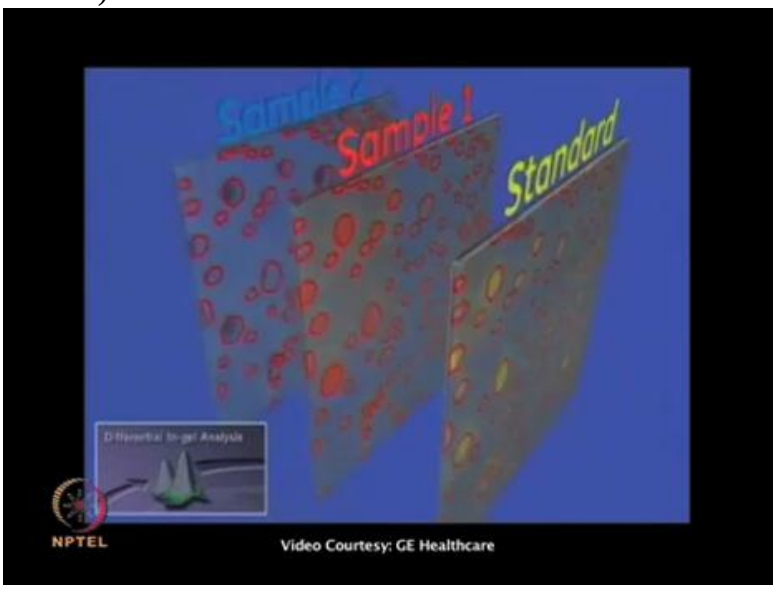

Expert: expanding remain 2 images also.

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Expert: So the artifacts can be minimized.

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Expert: Again it is fully automated analysis. There would be very less manual interference would be there

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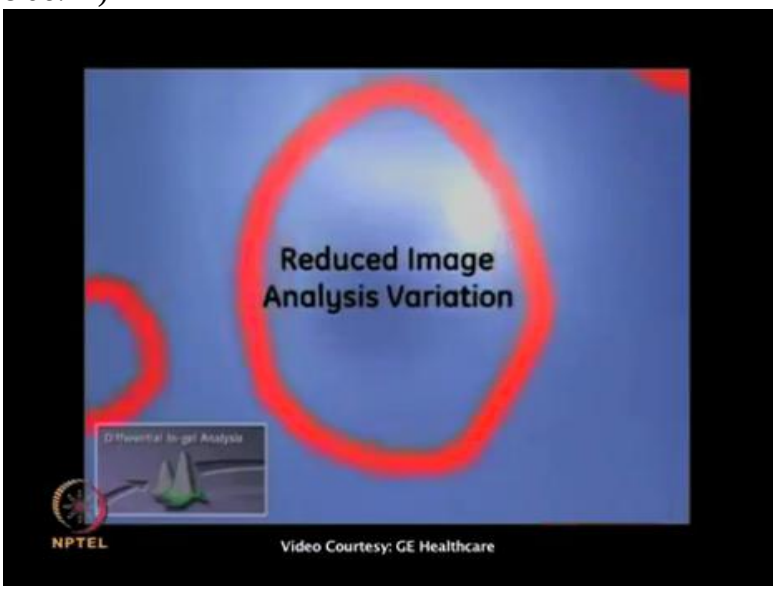

Expert: in this analysis and.

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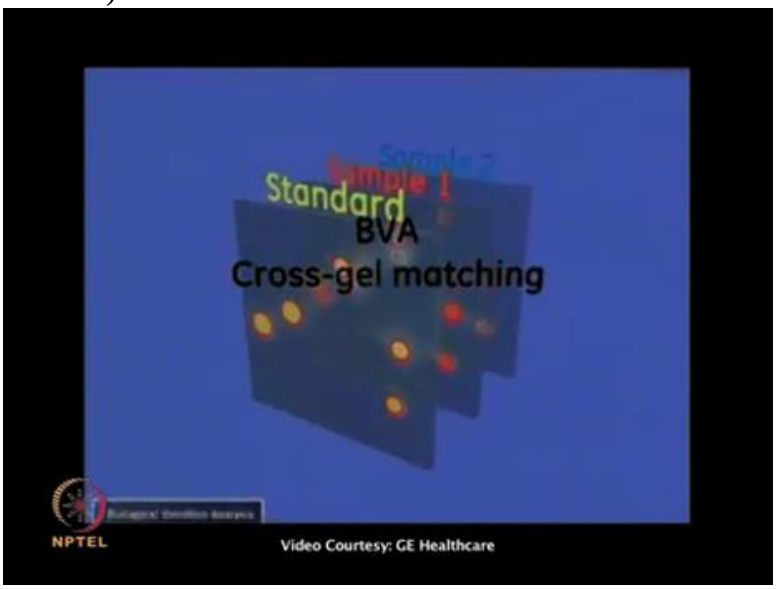

Expert: after finishing of DIA

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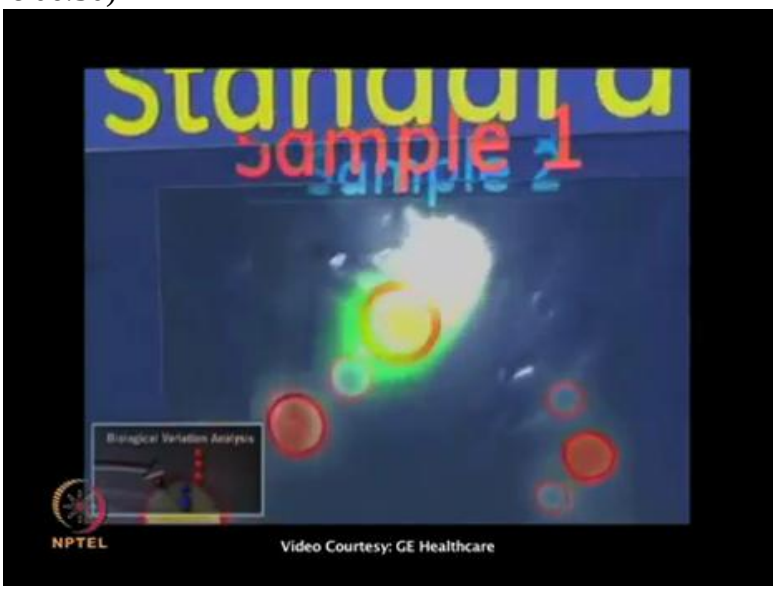

Expert: we can go to BVA.

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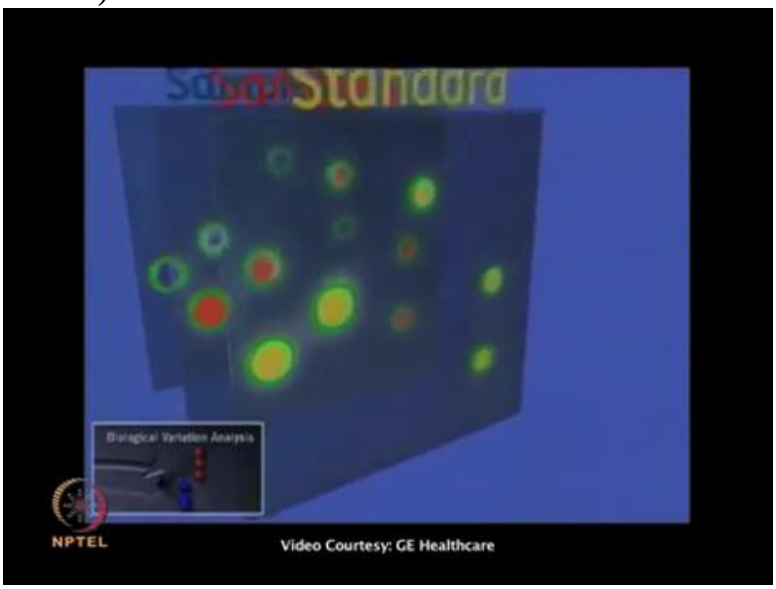

Expert: In BVA we can compare all DIS together like as you can see in this video, it is complete...first of all, it matches between gel and

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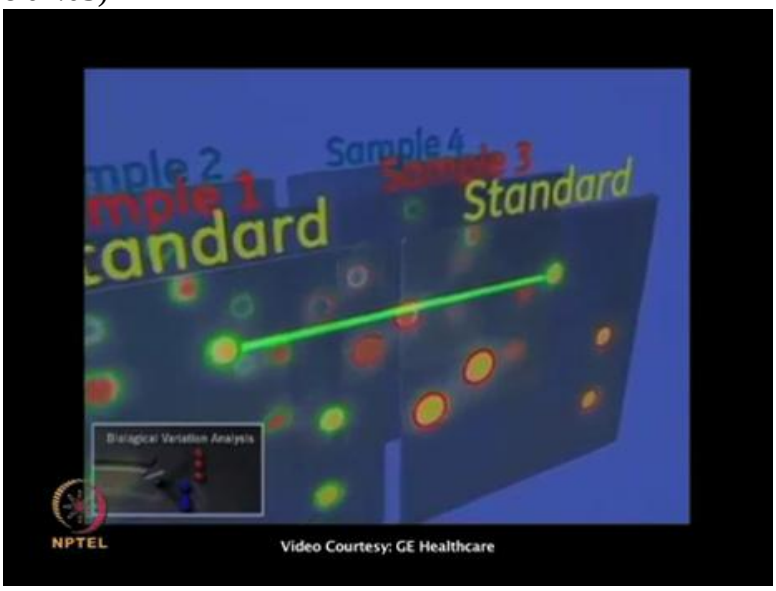

Expert: from standard gel to again corresponding DIA it matches

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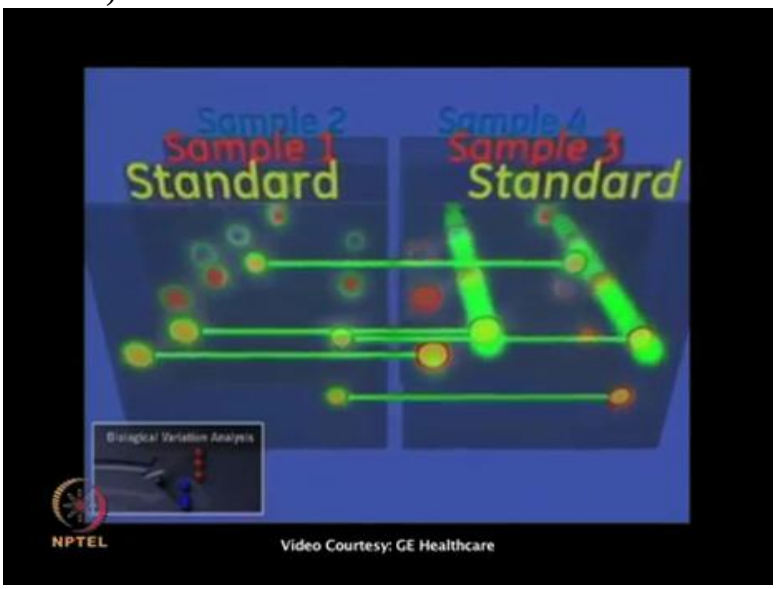

Expert: each and individual spot and

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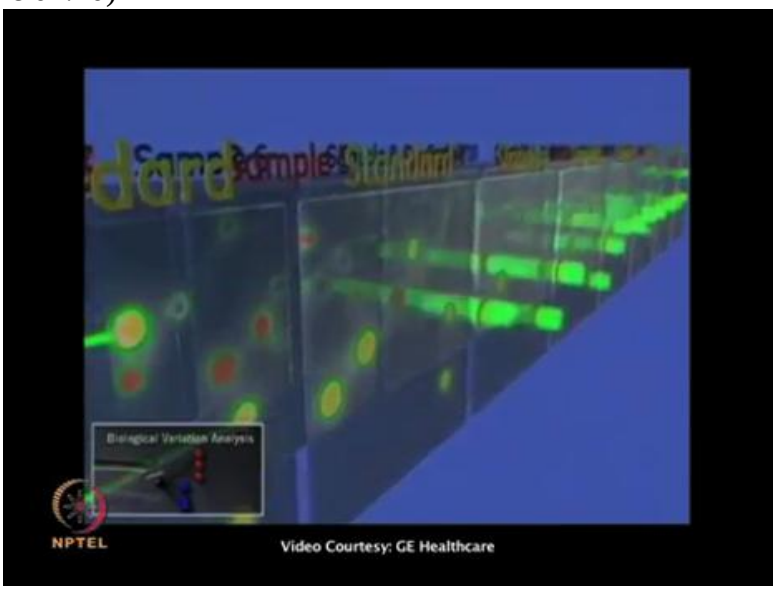

Expert: it will give you the final data. Then

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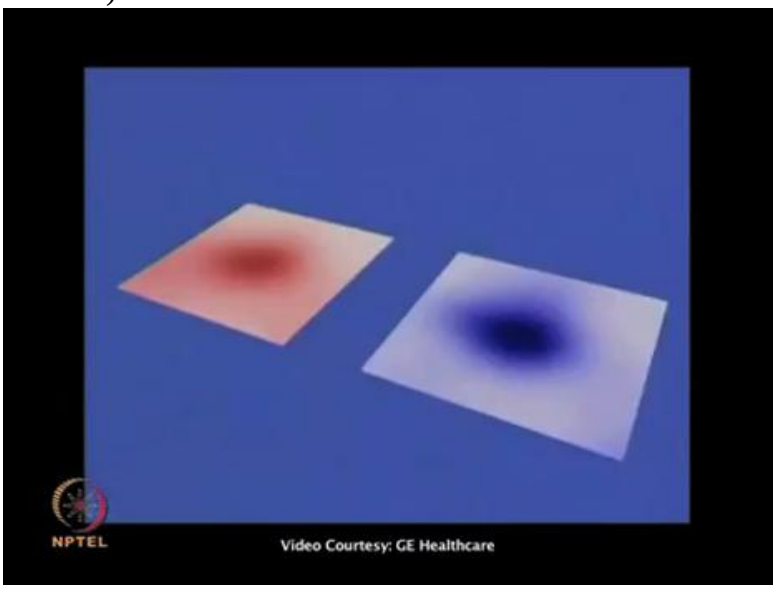

Expert: these BVAs can be analyzed

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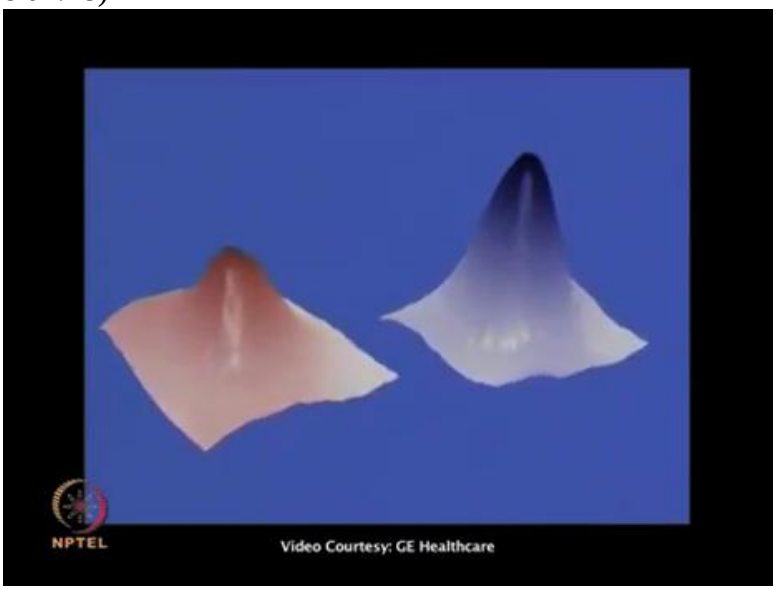

Expert: in the further in EDA

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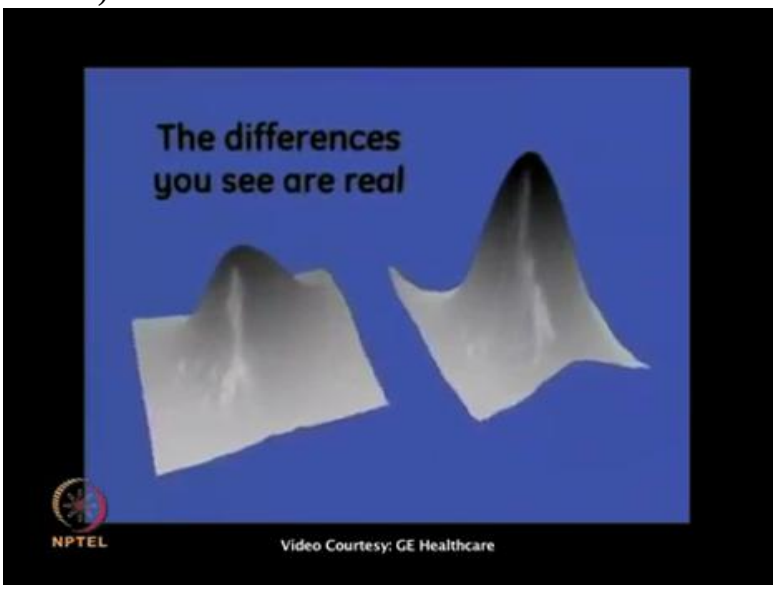

Expert: that is

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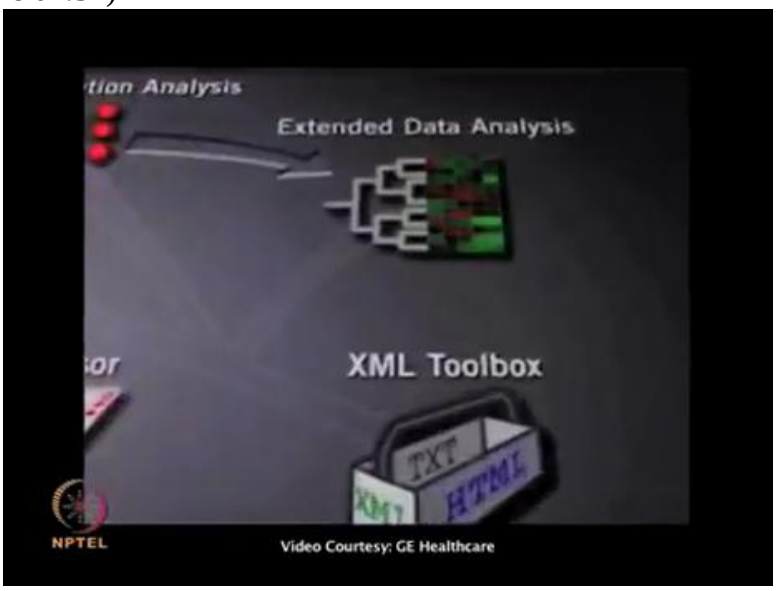

Expert: Extended Data Analysis.

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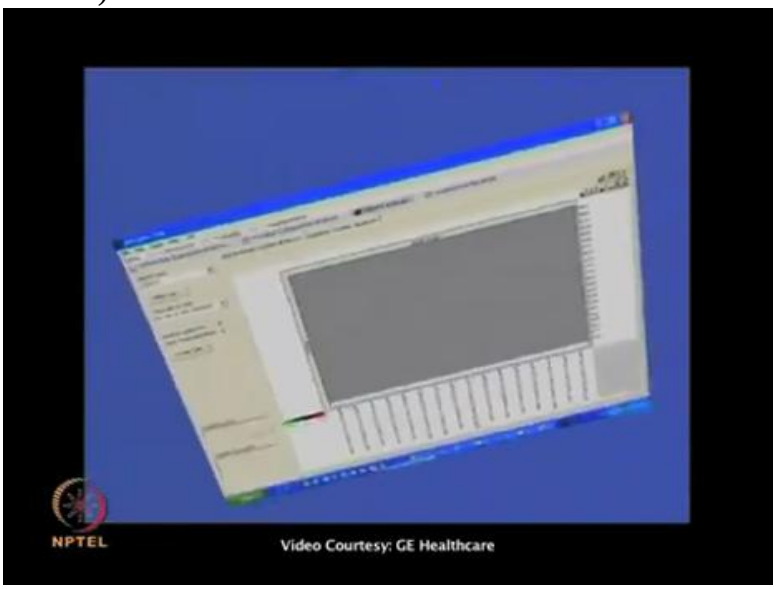

Expert: Here we will have

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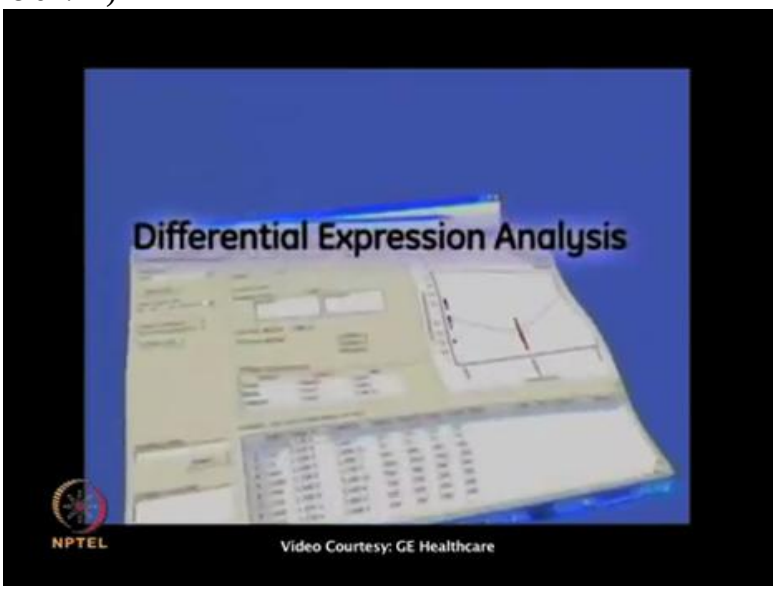

Expert: lots of stringent...this statistical data,

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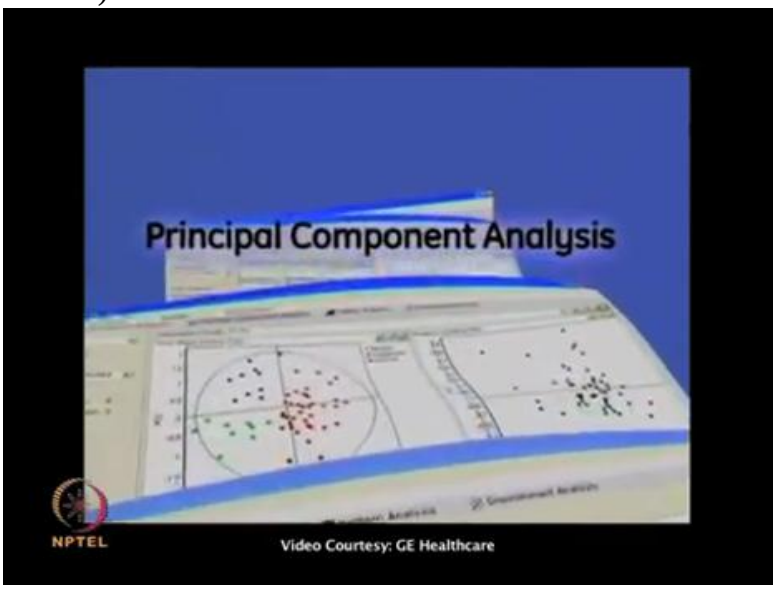

Expert: Differential Expression Analysis,

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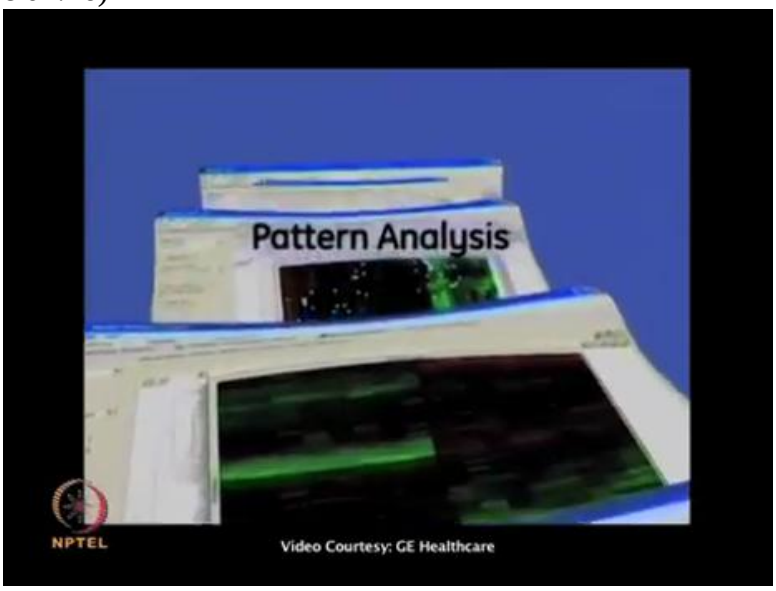

Expert: Pattern Analysis,

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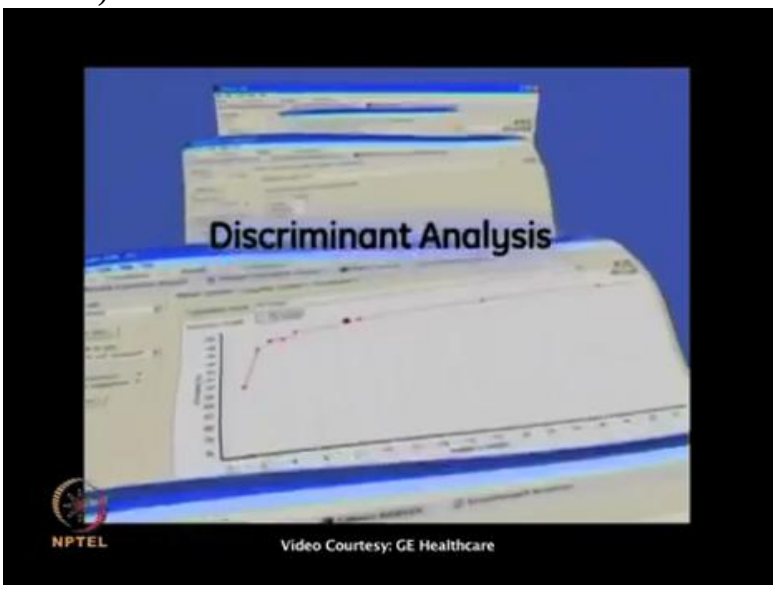

Expert: Discriminant Analysis

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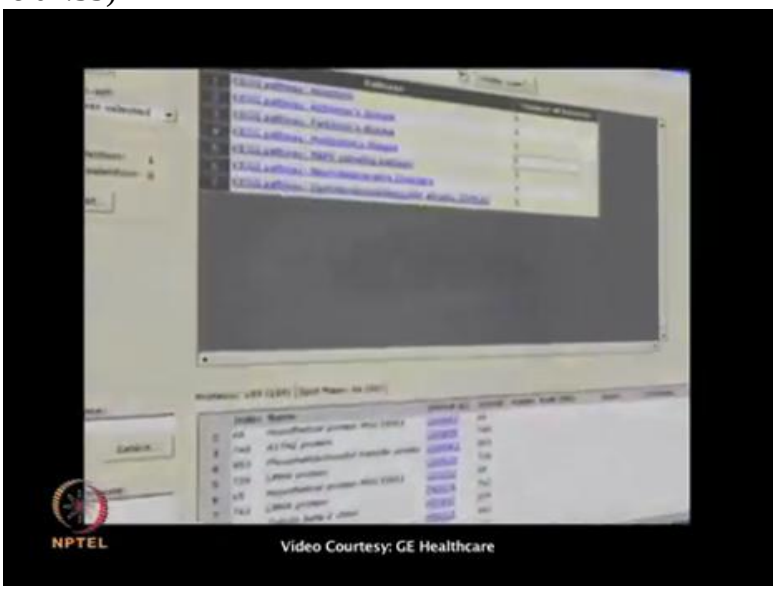

Expert: and PCA analysis

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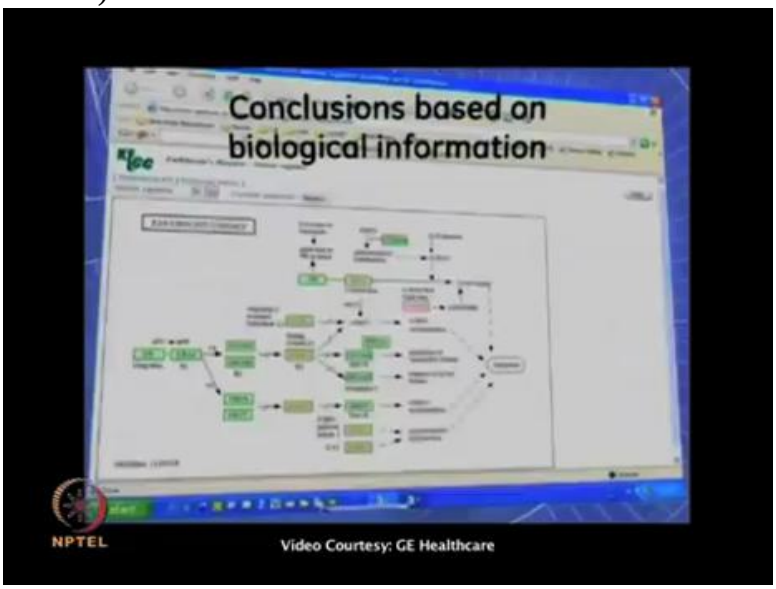

Professor - Expert conversation ends

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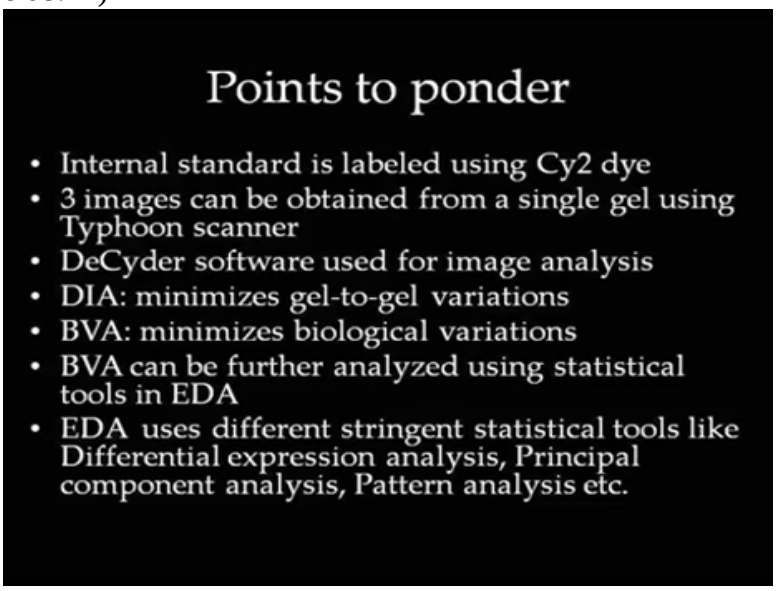

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Professor - Expert conversation starts

Professor: Doctor 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 details about steps involved in doing the labeling of this technology?

Expert: Yes, there is another video where you can see now. This is the second video which you can see more elaboratively

Professor - Expert conversation ends

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Expert: …it is particularly a protocol which is developed for membrane protein analysis. Now we can see this video.

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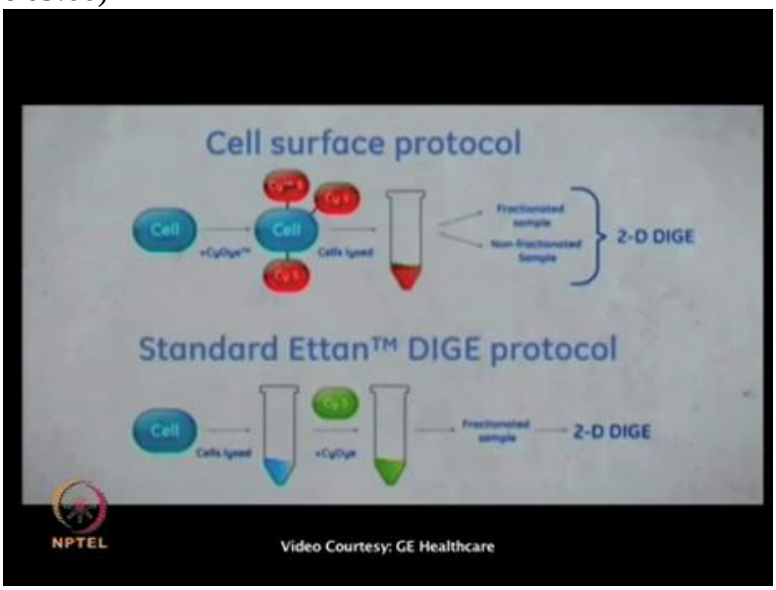

Video Narration starts
In the cells surface Labeling protocol, say non-TOF

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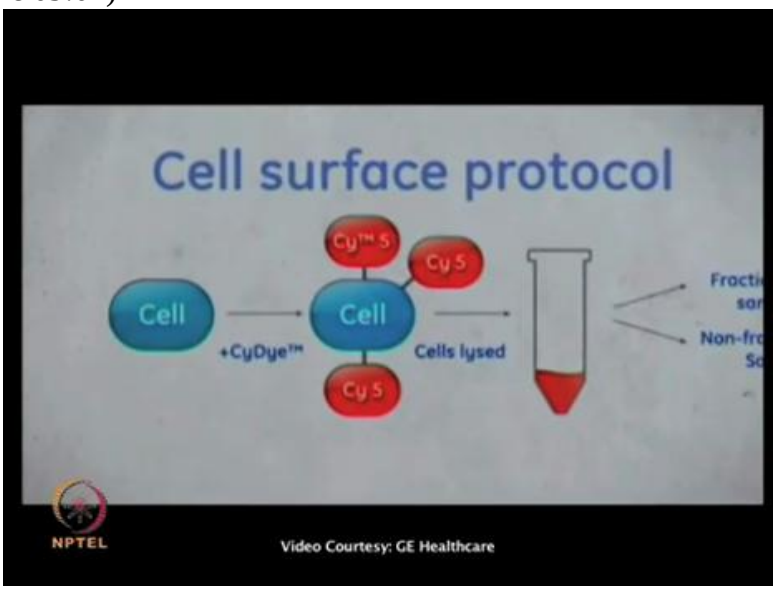

you label the cells while they are still intact. During the labeling process, the dye will only have access to the cell surface proteins. After the labeling step, the cells were lysed. Verify cell surface specific labeling.

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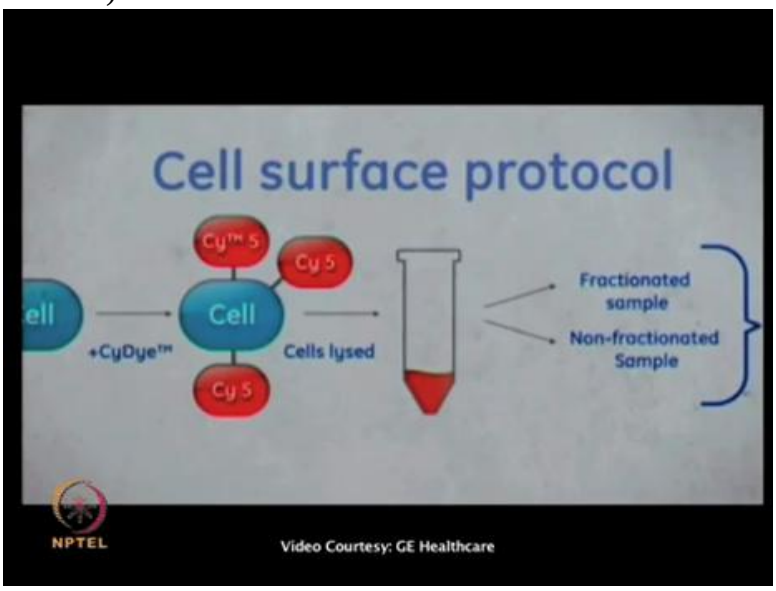

Label sample was fractionated into membrane and cytosolic proteins. A non-fractionated sample was prepared in parallel for comparison.

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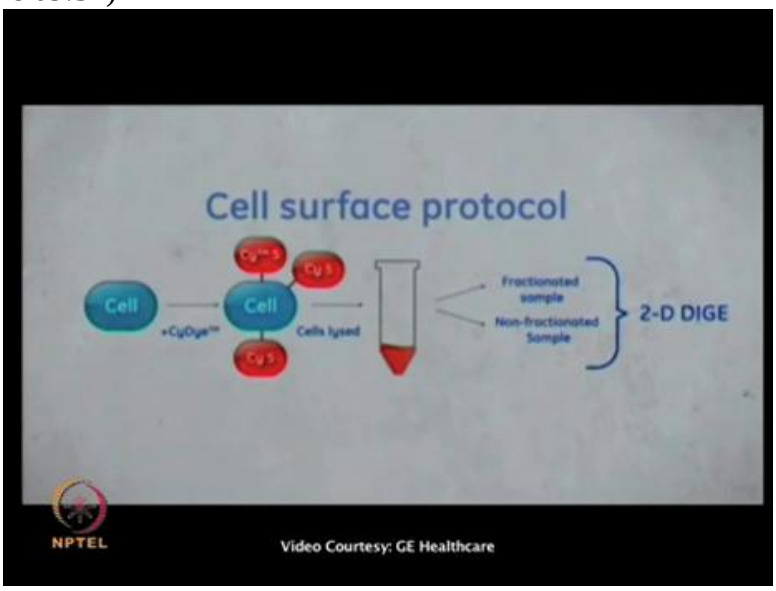

This fractionation analysis is not necessary but was done here just to show that the cell surface protocol is specific for cells of the proteins.

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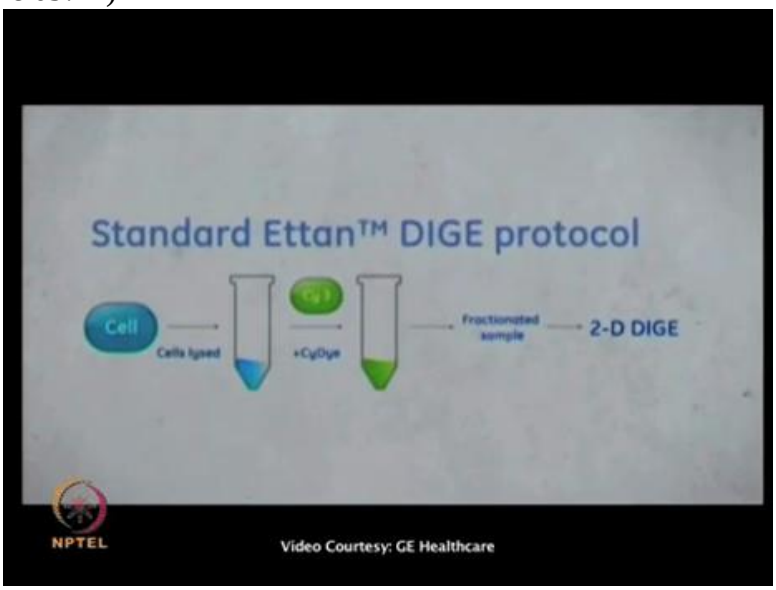

We also performed the standard Ettan DIGE Labeling Protocol shown below.

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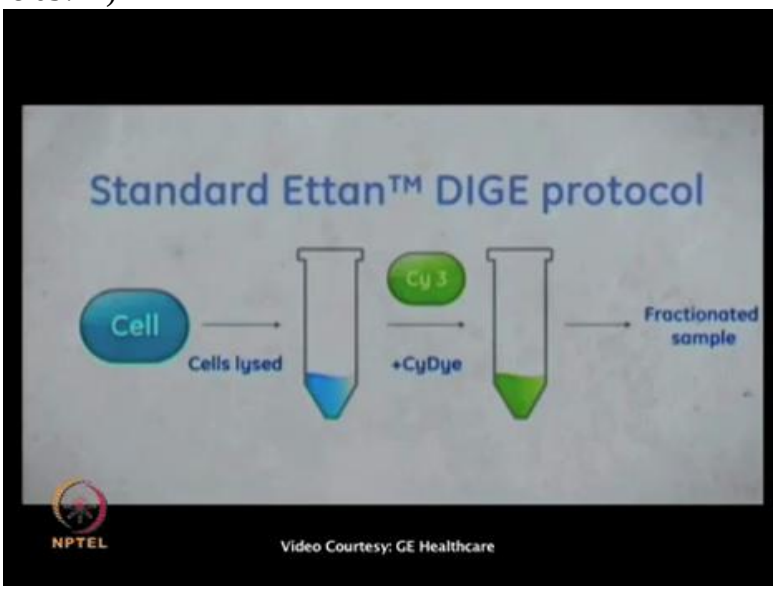

The cells were lysed before labeling and in this way all cellular proteins are accessible for labeling. After the labeling step

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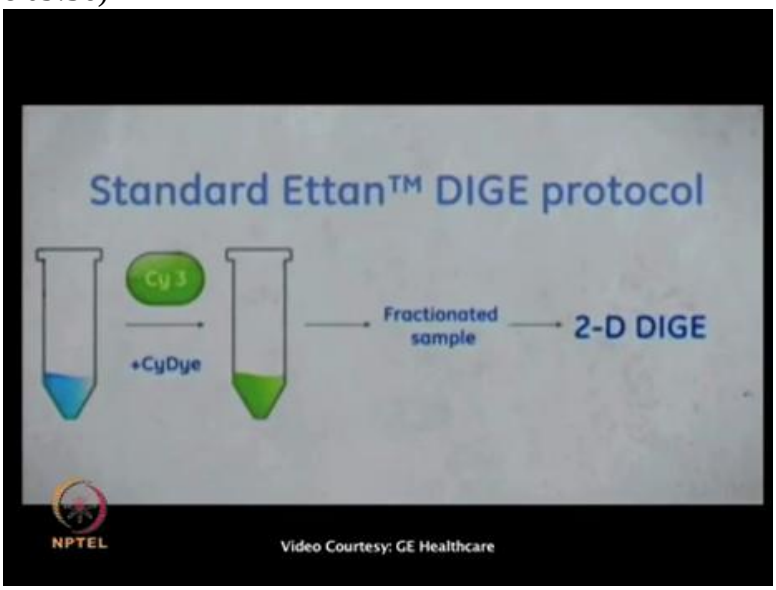

the samples are subjected to 2D electrophoresis...

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Adherent cells are detached using non-enzymatic procedure to avoid digestion of cells of these proteins targeted in this protocol.

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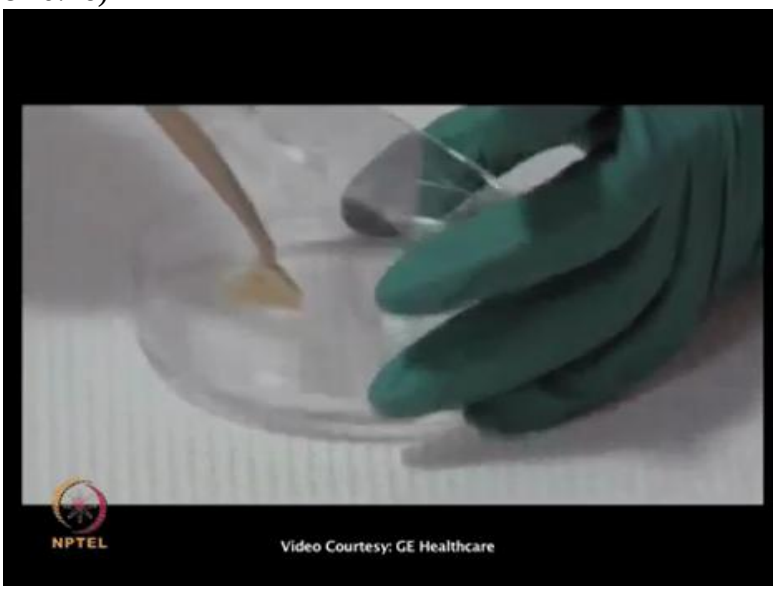

We used rubber policeman but using enzyme free cell dissociation media is also an option.

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Count the divide this cell suspensions into aliquots

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…of 5 to 10 million cells per tube

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The cells are then pelleted and washed in HBSS pH 7.4 to remove traces of cell culture media. Contamination from serum proteins

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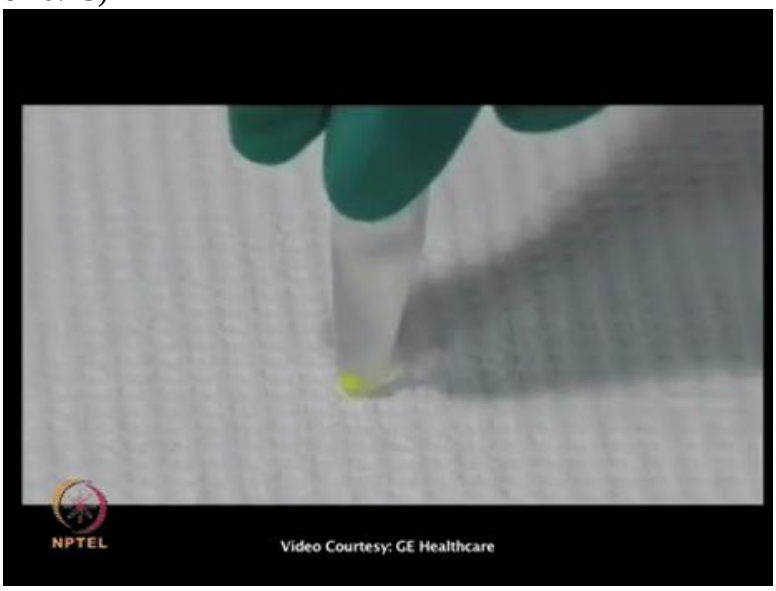

and fluorescent components can interfere with labeling and detection Cells grown in suspension are directly pelleted and washed before the labeling step.

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After the wash, the cell pellet is re-suspended in 200 micro liters ice cold labeling buffer containing HBSS pH 8.5 and 1 molar urea for optimal labeling conditions of cell surface proteins.

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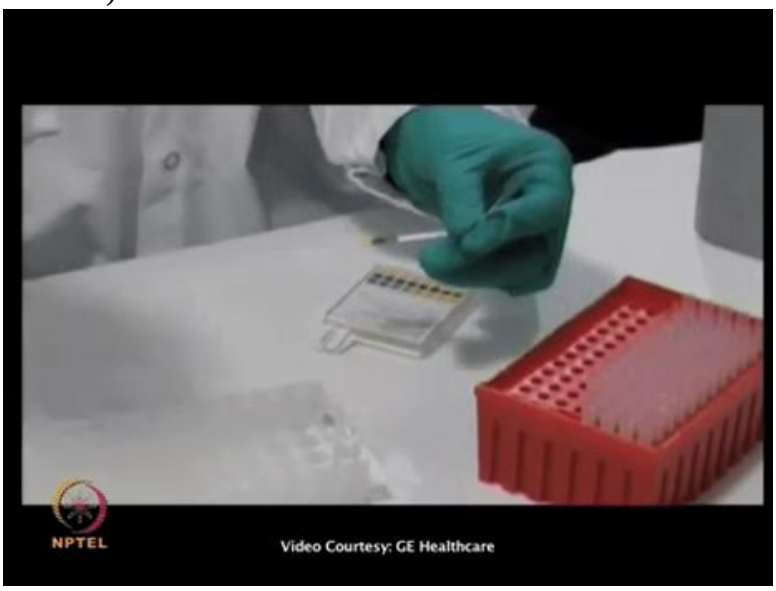

Always check pH before labeling. We used

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600 pica mole CyDye for 10 million CHO Cells

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The optimal ratio of CyDye to cell number will vary

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depending on the cell type Since we do not know the exact protein concentrations on the surface, how to determine the optimal conditions for Cye dye labeling of proteins

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… is described in the 2D Electrophoresis Principles Handbook.

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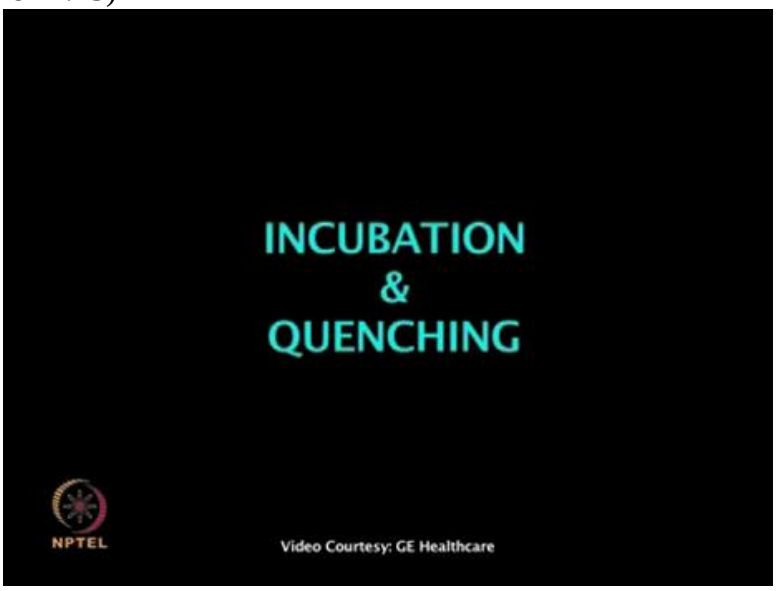

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The cells are incubated with CyDye DIGE Flour Minimum DIGE

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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 milli molar Lysine...

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. The labeled cells are now washed twice in cold HBSS pH 7.4 buffer to remove the excess CyDye.

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There will therefore be no free dye left for unwanted intra-cellular labeling of proteins in the next step which is cell lysis.

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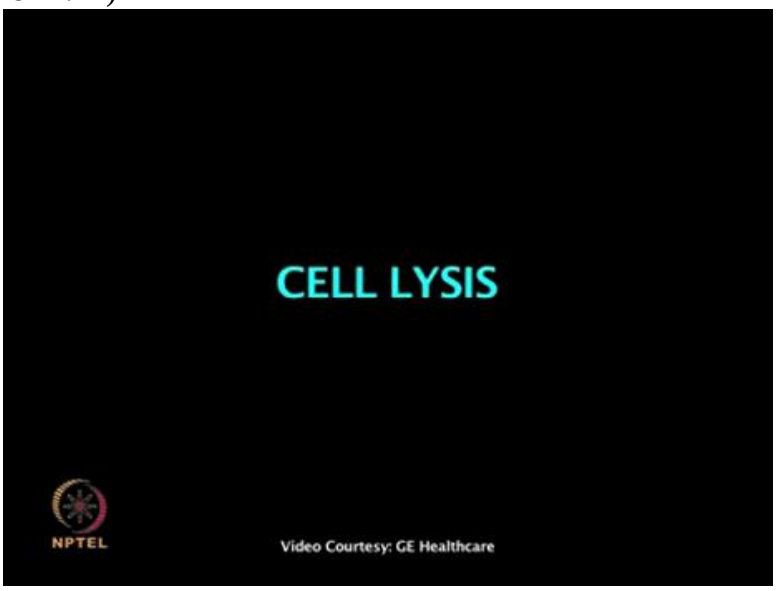

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The proteins on the cell surface are now labeled and cells are washed and ready to be lysed.

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The pellet from the last washing step is

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re-suspended in 150 micro liter cold lysis buffer containing 7 molar urea, 2 molar thiourea, 4% CHAPS, 30 milli molar trace

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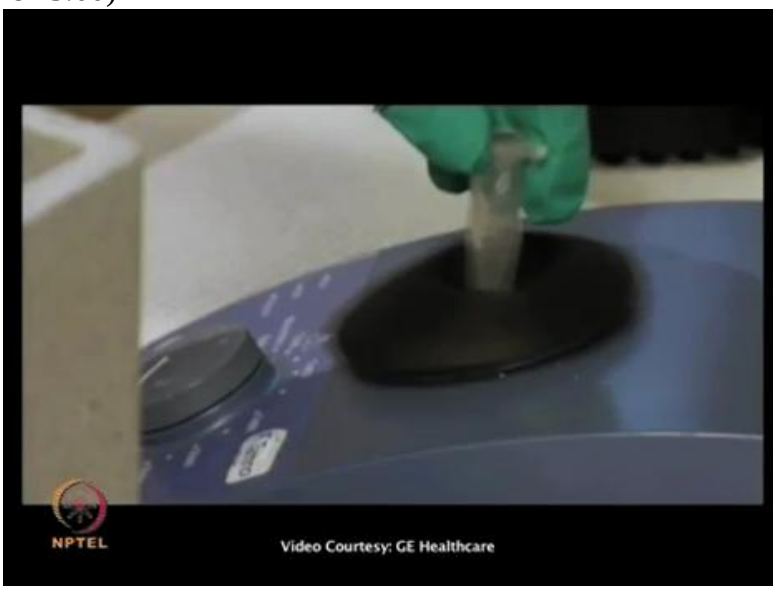

, 5 milli molar magnesium acetate pH 8.5, and

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left on ice for at least 1 hour with occasional vortexing

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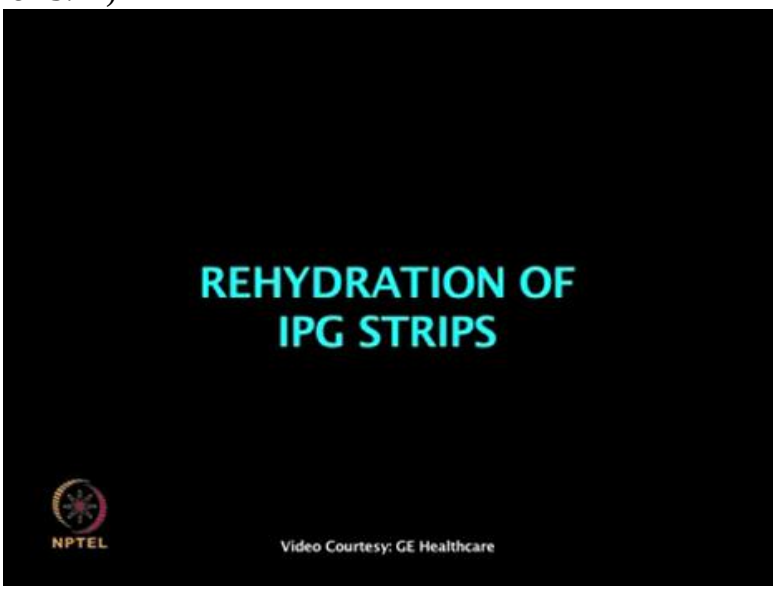

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The samples are now ready for 2D gel separation.

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The first step in 2D electrophoresis is to prepare IPG strips for rehydration.

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Prepare the strip rehydration solution by adding IPG buffer

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corresponding to the pH interval of the strips you use and

(Refer Slide Time 13:37)

add the solution in the lanes of rehydration tray.

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Remove the protective film of the IPG strip

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and place the strips carefully

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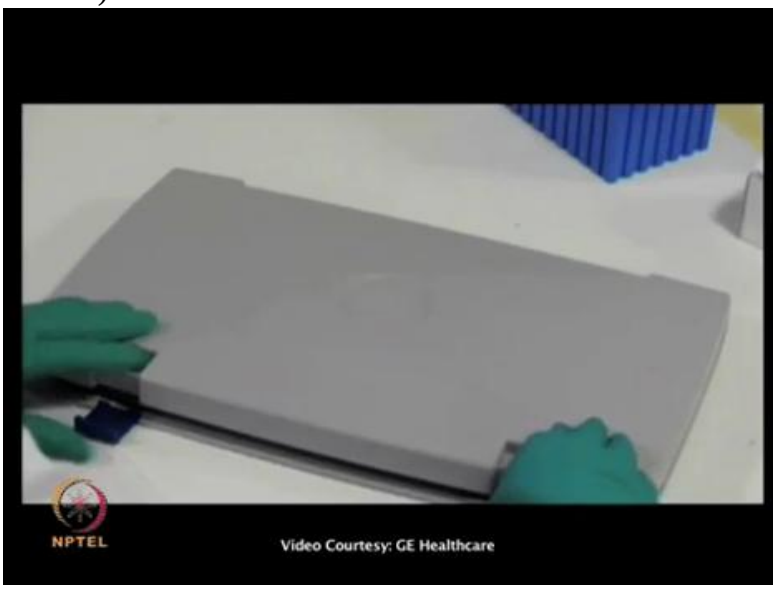
… with the dried gel facing down in the rehydration tray containing the rehydration solution

(Refer Slide Time 14:10)



Close the lid of IPG box and rehydrate the strips overnight.

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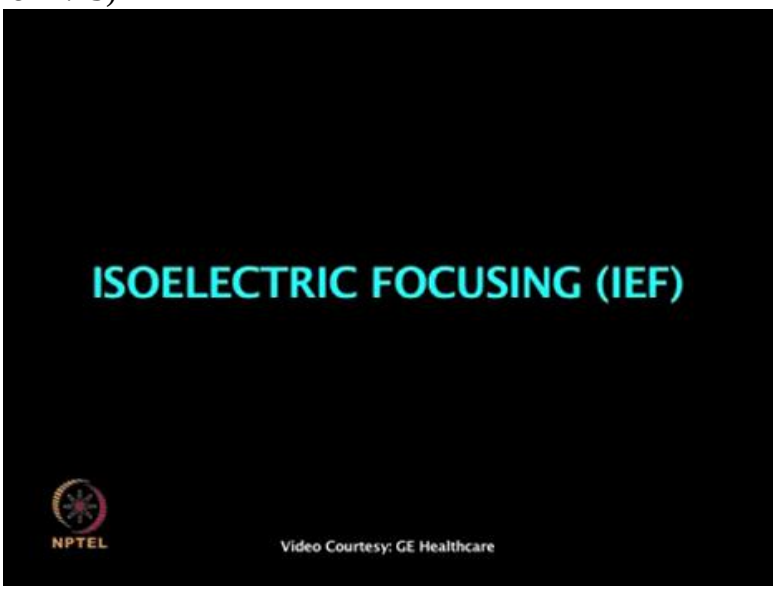

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In the first dimension isoelectric focusing, the proteins are separated according to their pI

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This is performed using the IPG 43.

The rehydrated strips is placed in the manifold

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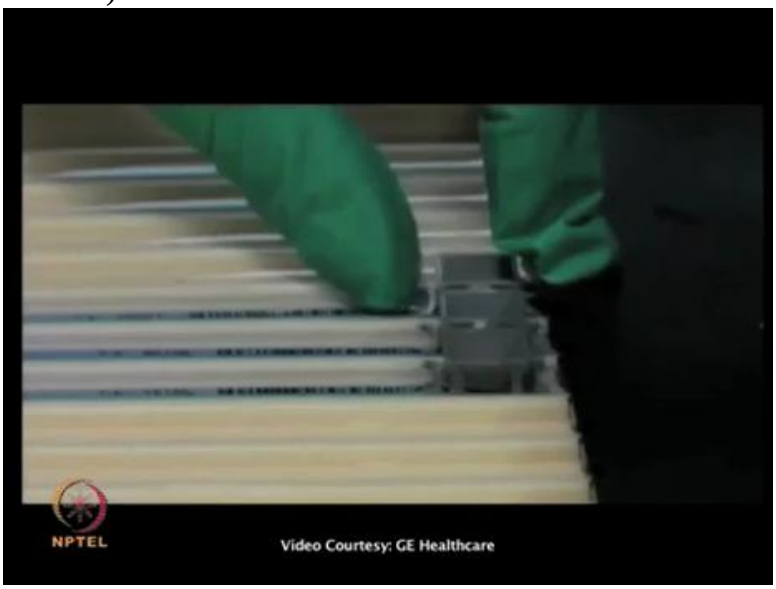

and the electrode is mounted on top.

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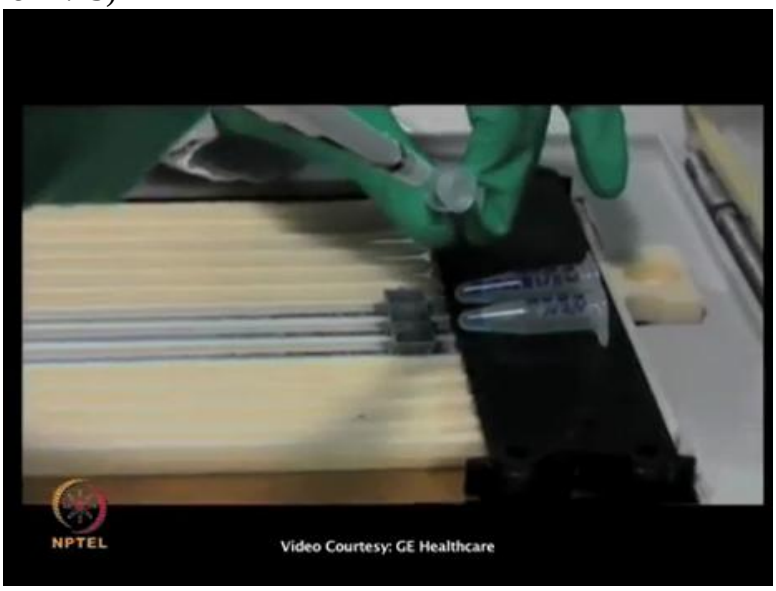

50 micrograms from each sample were applied using sample application caps.

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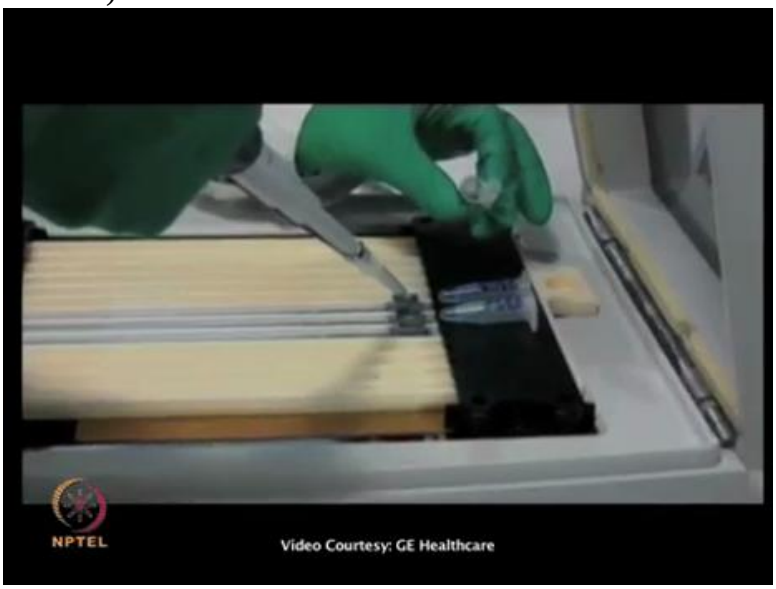

We have here directly applied non-fractionated samples without prior fractionation, all fractionated samples into membrane and cytosolic fractions before they were applied.

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The lid is closed to protect the fluorescent samples from light. The instrument was programmed according to recommendation and run overnight.

(Refer Slide Time 15:09)

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Large 12% Laemmli gels were cast using a dual 12 gel caster.

(Refer Slide Time 15:24)

Displacing solution was added to avoid polymerized acrylamide gels in the tubings. The gels were

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… allowed to polymerize overnight at room temperature prior to use

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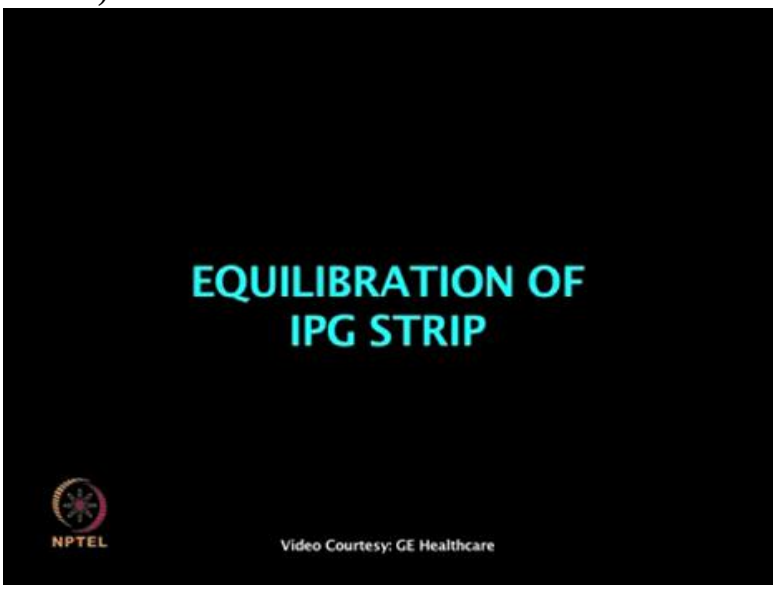

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After the isoelectric focusing the strips are removed and

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equilibrated in SDS containing buffer in 2 steps; using DTT to reduce the disulfide bonds of Cystine residues

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followed by

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alkylation with iodoacetamide to avoid modification by acrylamide

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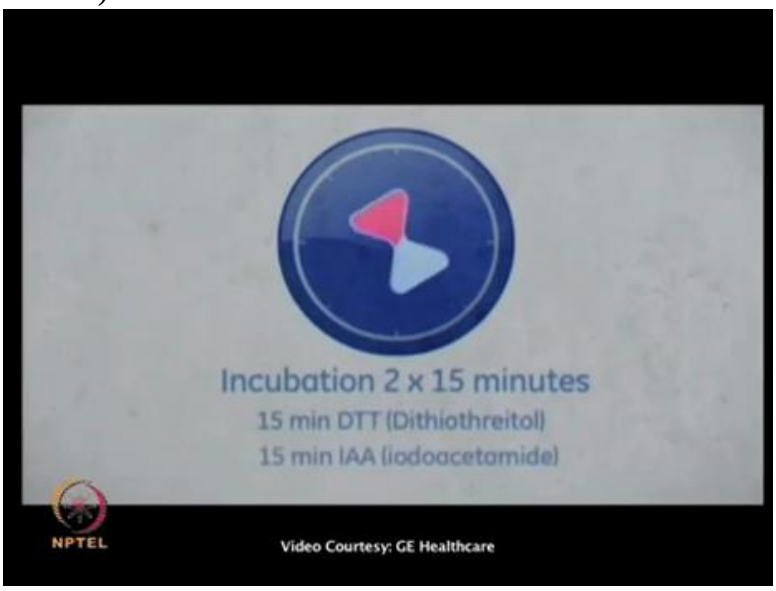

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The IPG strips are dipped in running buffer and

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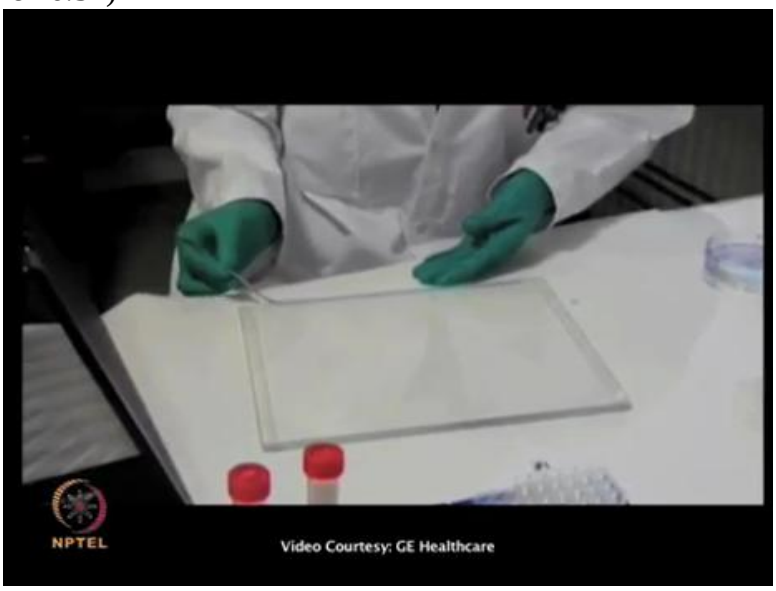

carefully placed on the top of large 2D gels

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Avoid trapping air between the strips and the gel.

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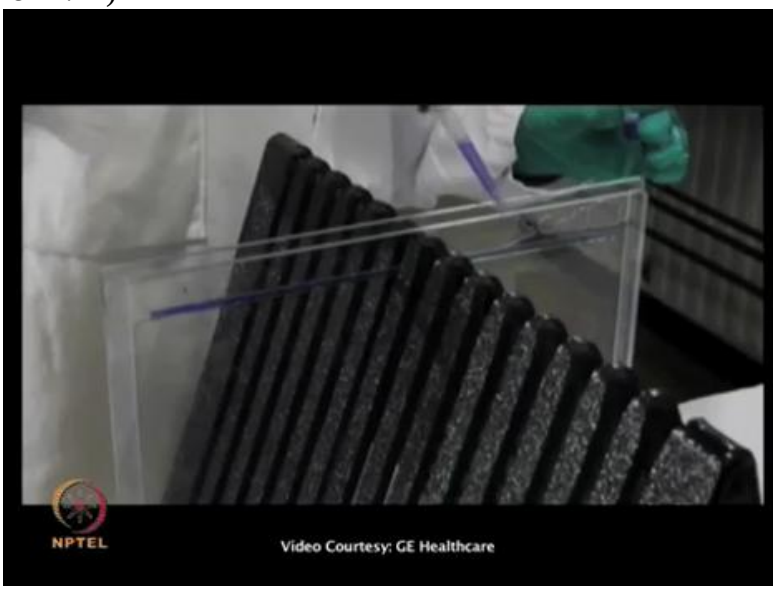

Seal by adding melted 2% agarose solution with bromophenol blue on top.

The gels are now ready for second dimension SDS PAGE separation.

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In the second dimension SDS PAGE the proteins will be separated according to molecular weight and this is performed with Ettan DALTsix system.

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Fill the electrophoresis chamber with anodic running buffer. Insert the gels and

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fill the top compartment with cathodic running buffer. Program the power supply according to recommendations and run second dimension. Protect it from light for approximately 4 to 5 hours or until the dye front reaches the bottom of the gel.

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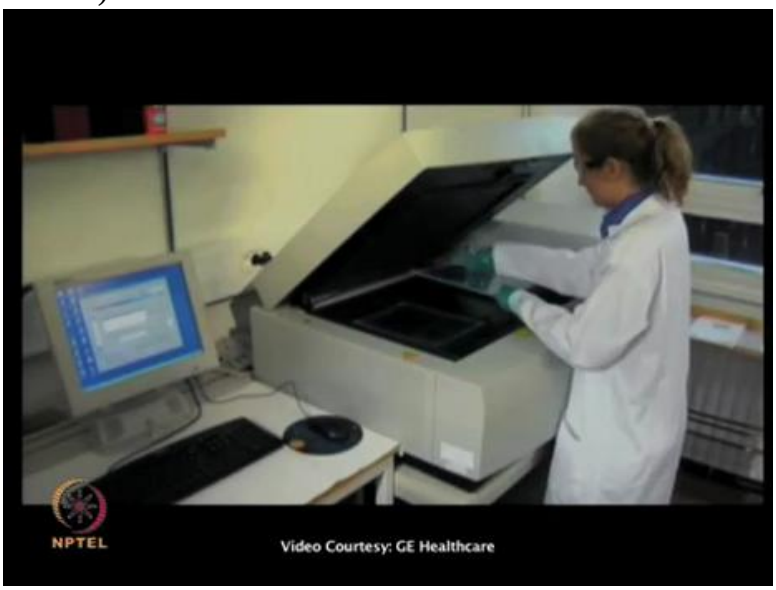

After the second dimension electrophoresis, the gel cassettes are placed by using the grippers in the Typhoon FLA gel imager.

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2gels and 3 channels can be scanned simultaneously.

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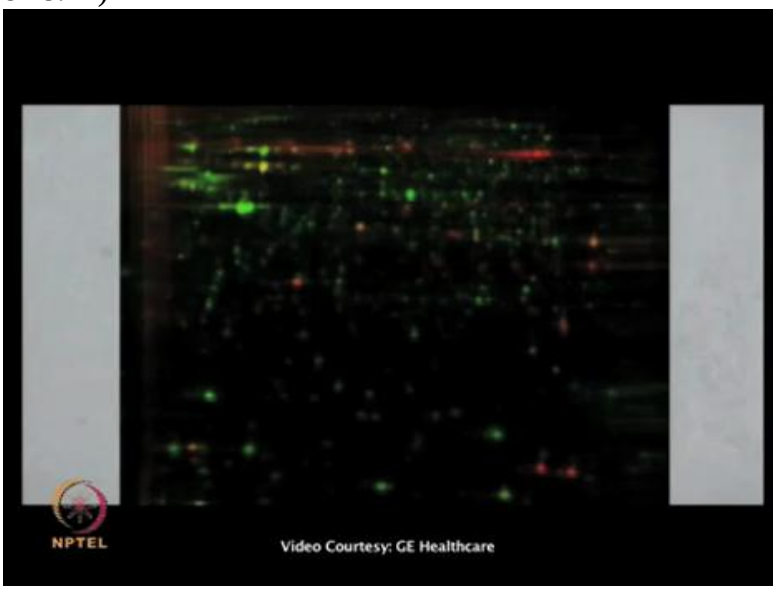
(Refer Slide Time 18:42)



The result from this 2D gel shows high resolution of membrane proteins in the sample. Even if there are some known restrictions for hydrophobic proteins to be detected in a 2D gel; the results show many new cell surface label spots shown here in red, that are not detected using the standard labeling protocol shown here in green.

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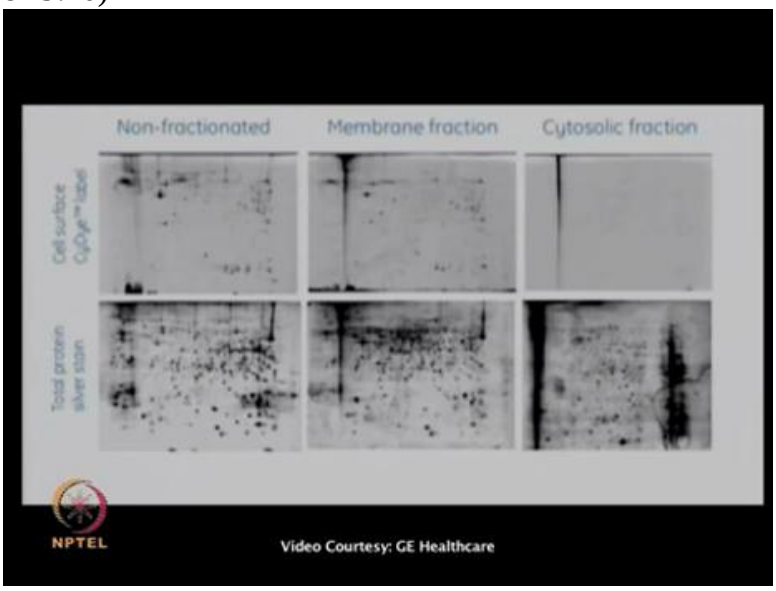

These results show that the cell surface labeling protocol is highly specific for labeling cell surface proteins. Since cell surface proteins are exclusively labeled, they are more easily visualized and attenuation by abundance cytosolic proteins is avoided.

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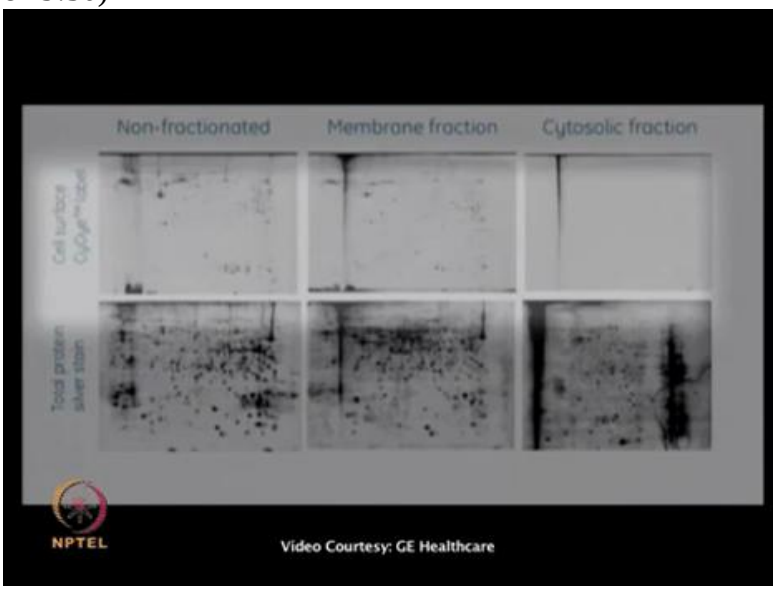

The fluorescent image of gels with non fractionated membrane fraction or cytosolic fraction is shown on top.

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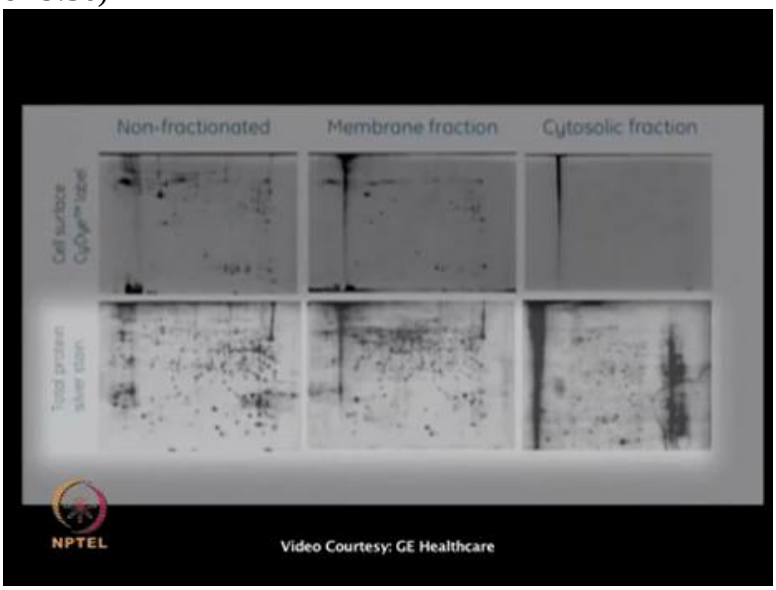

Below is an image of the same gel co-stained with silver.

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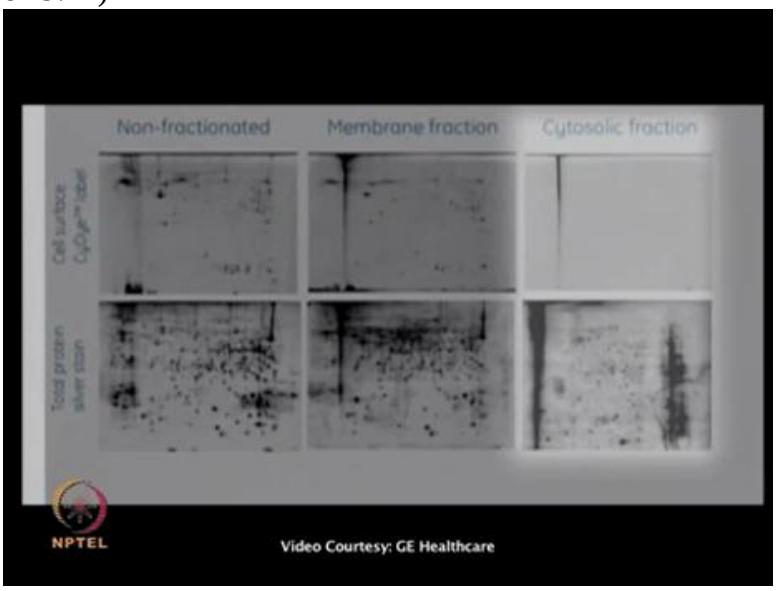

The result showed no fluorescent labeling of cytosolic proteins but the silver staining shows there are proteins in the gels.

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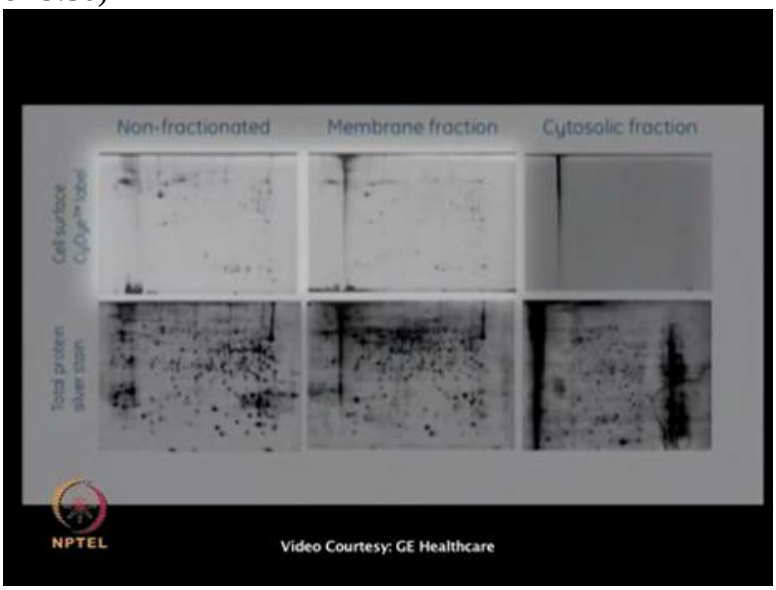

The result also shows singular spot map patterns from non-fractionated and membrane fractions demonstrating there is no need for fractionation prior to 2D electrophoresis which makes this protocol both simple and convenient.

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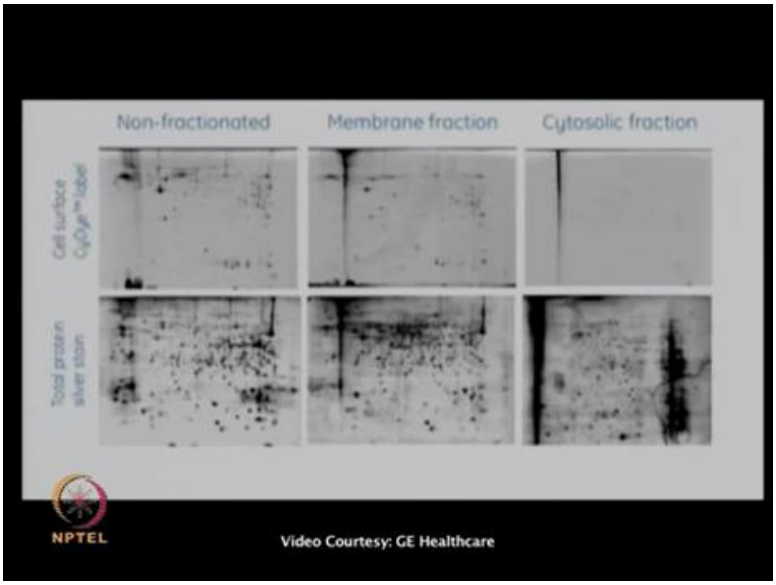

Cy2, Cy3 and Cy5 show similar labeling patterns and are all compatible with the cells surface protocol.

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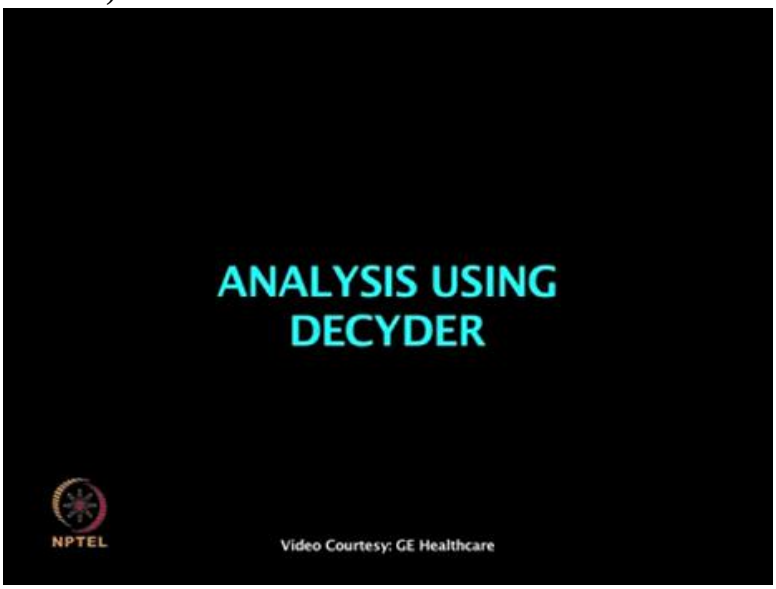

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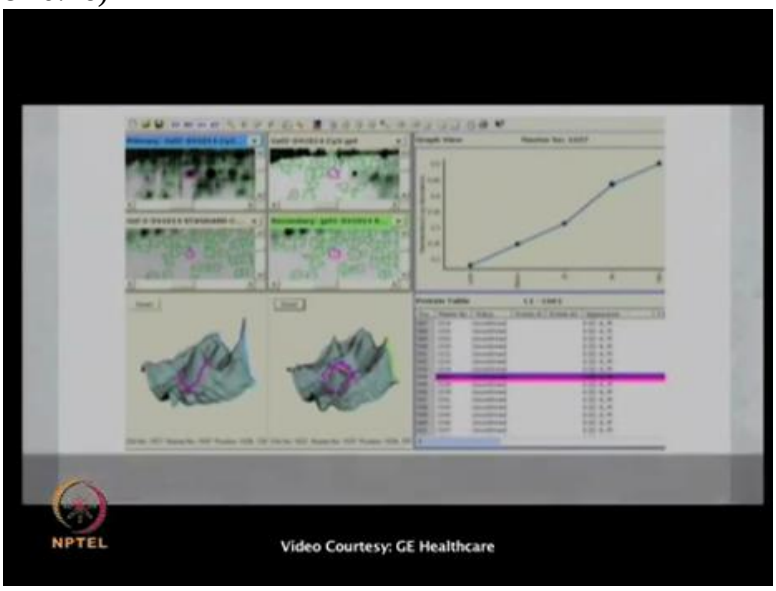

DIGE experiment was performed using all 3 CyDye DIGE Fluor minimum dyes for studying differential expression of cell surface proteins in CHO cells of the serum starvation for different lengths of time.

Cy2 cell surface samples from all surface from the experiment were pooled and used as an Internal Standard. The differential changes of several cells of these proteins could be followed during the starvation period.

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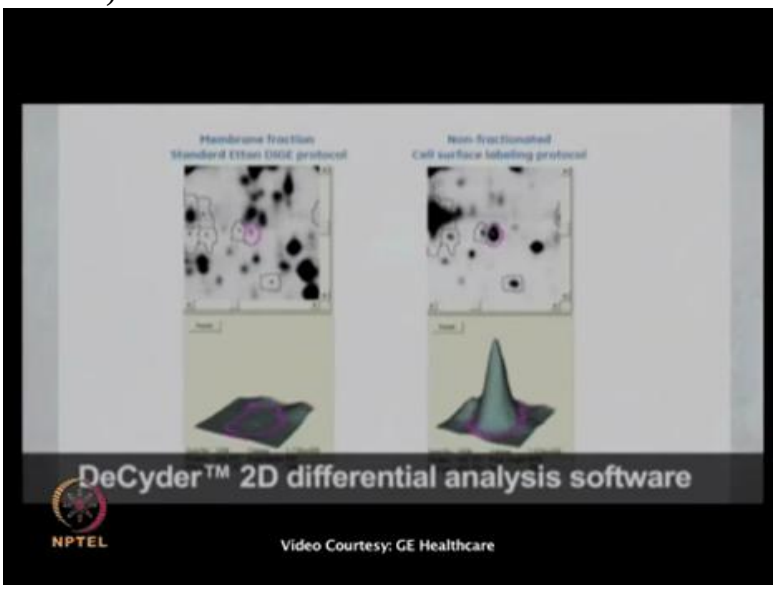

Over 18 novel membrane proteins were detected using the cells of this protocol that were not detected with the standard Ettan DIGE protocol. For finding the identity of the proteins in the preparative gel it was necessary to spike with the cells of this sample to facilitate matching back to the spots on the analytical data set.

Video Narration ends

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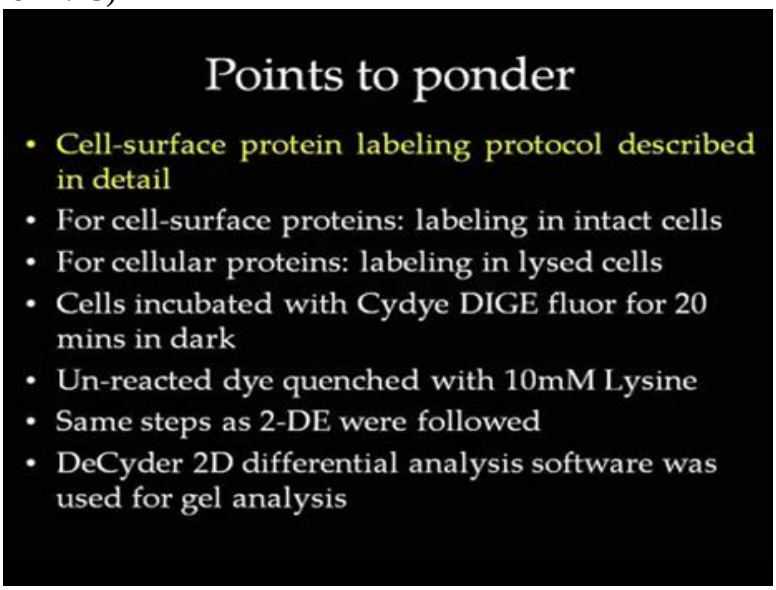

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Professor - Expert conversation starts

Professor: Doctor Srinivas, it was a very useful to see all these steps which are involved in doing this DIGE technology. There is a discussion going on in the proteomic field that due to advancement in mass spectrometry and Microarrays, how do you see, foresee the future of two-dimensional electrophoresis and DIGE technology? Do you think it is going to be simultaneously working along with mass spec and Microarrays? Or do you think it is going to be behind because now more advanced approaches have been emerged. So what is your suggestion on that?

Expert: Yes, the MS and Microarrays developed differently. They have their own advantages as well as disadvantages. Like disadvantages, like very high instrumentation cost and protein requirement as well as technical variance would be there. That is why still 2D DIGE will be core technology to analyze proteomics. One can easily analyze...

Professor: So you are still very convinced that by using 2D DIGE approach that will still remain one of the core proteomic technology for the proteomic analysis...

Expert: Yes, definitely

Professor: And it will be used simultaneously along with mass spectrometry and Microarrays.

Expert: Yes

Professor: So at the end I would like to request you to give a brief overview of the DeCyder Software which is being used for analysis of DIGE gel.

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Expert: Sure. DeCyder is the software to analyze DIGE gels where we can analyze DIGE gels n different stages. In first stage we have to upload our gels into our software

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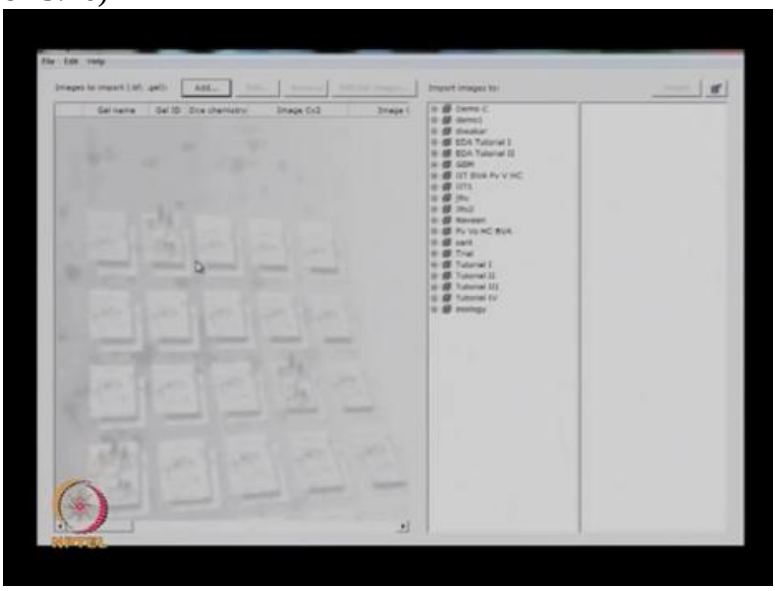

Expert: This basically works on the Oracle database where we are incorporating our gels into database.

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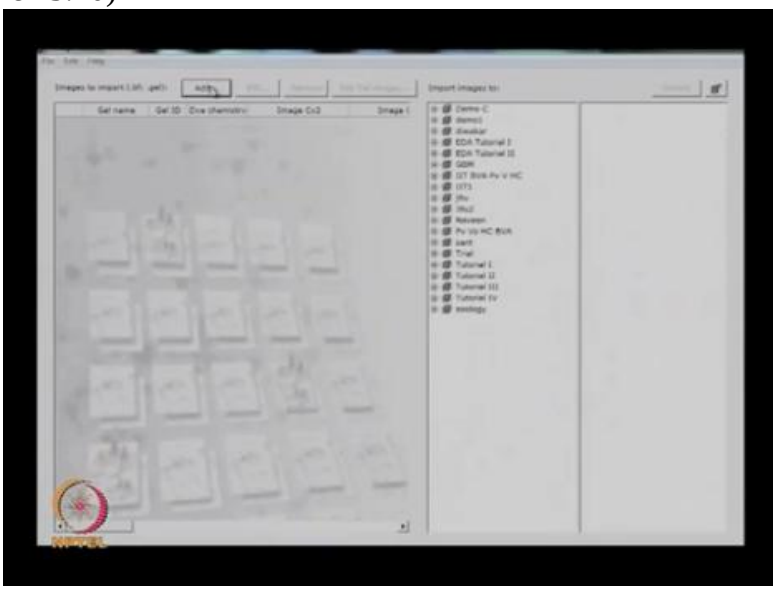

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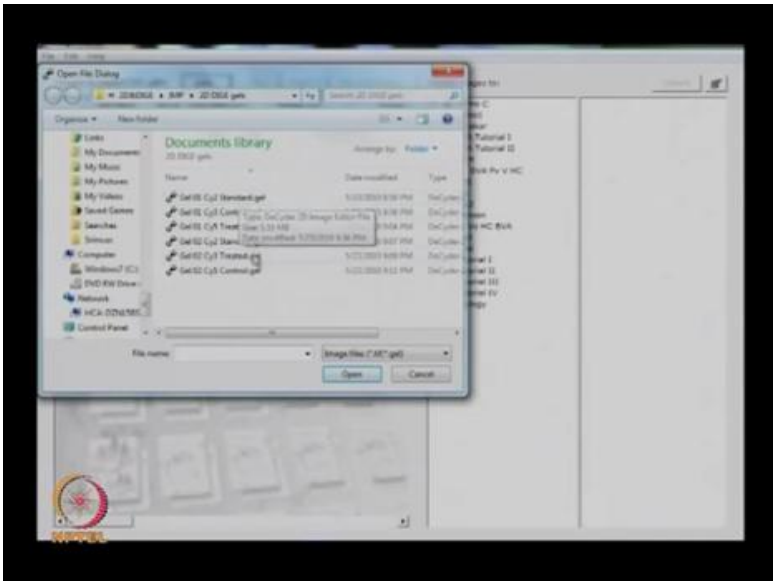

Expert: There you can add these gels into software by this way and

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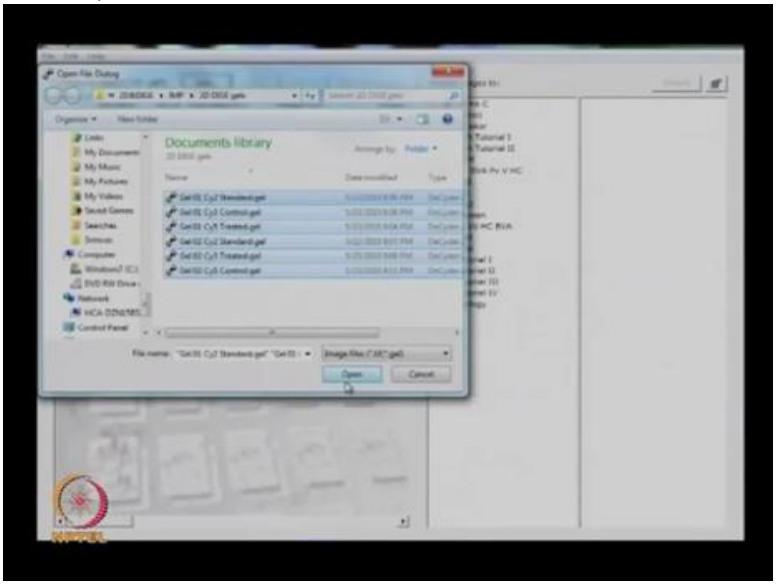

Expert: you can select whatever the gels you want to upload

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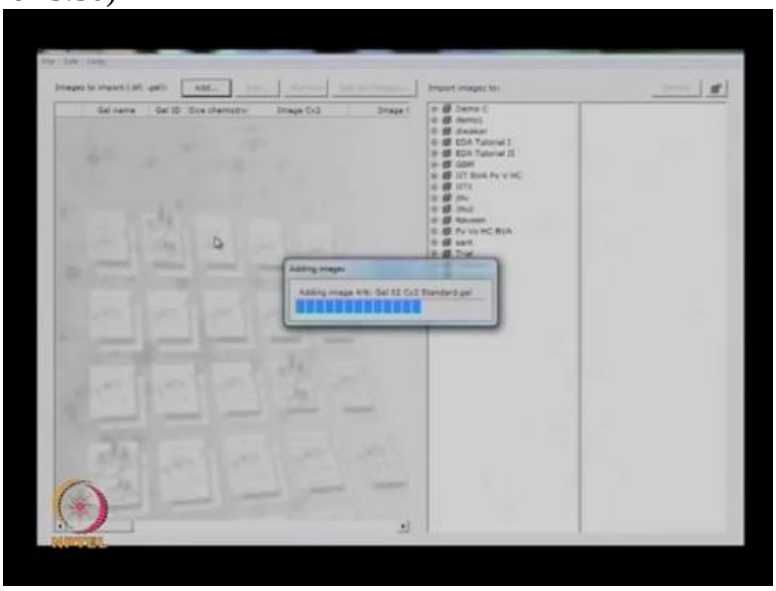

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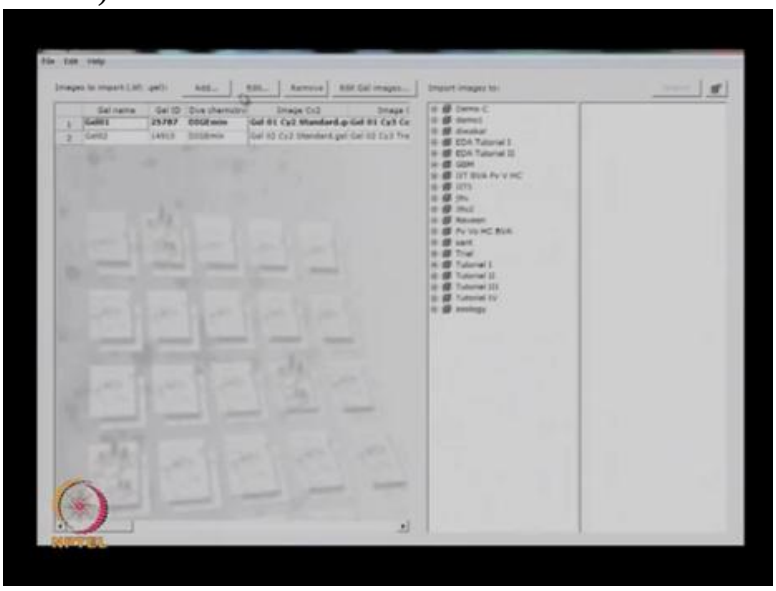

Expert: and once you have upload, then

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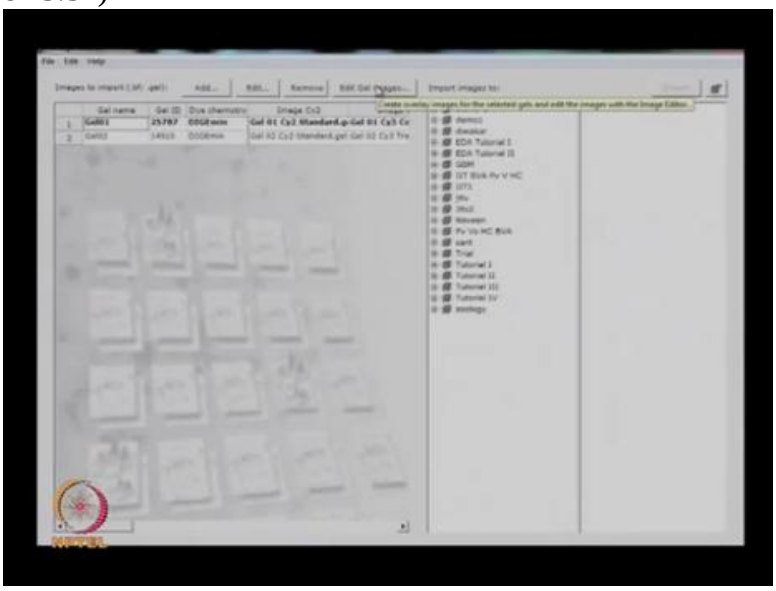

Expert: you can able to edit these gels here itself. Here you want to any crop, or edit, any sort of things you can do here. It will allow you

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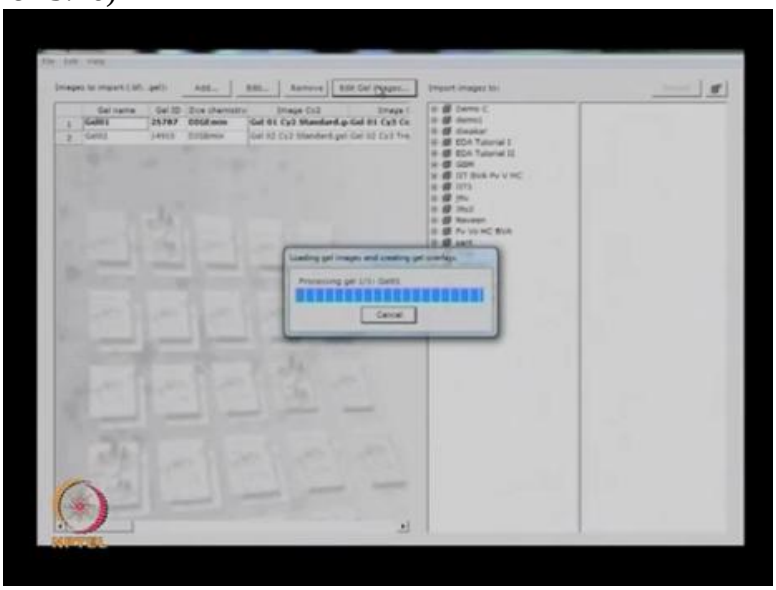

Expert: to edit these.

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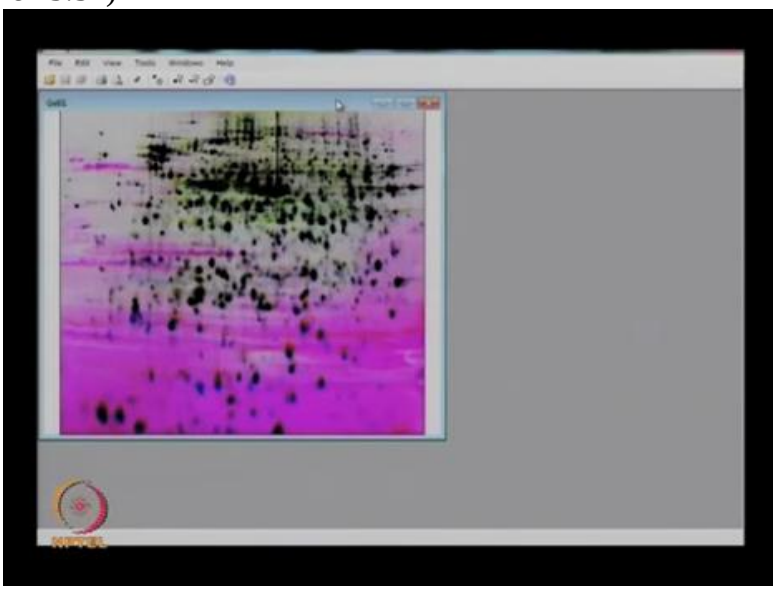

Expert: So now you can see this, the overlay of 3 images. Now you can crop all these images like whatever the portion you want to take, any area which is not your interest of, then you can directly crop those images. Save once you can directly editing, you can save it,

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Expert: yes save it

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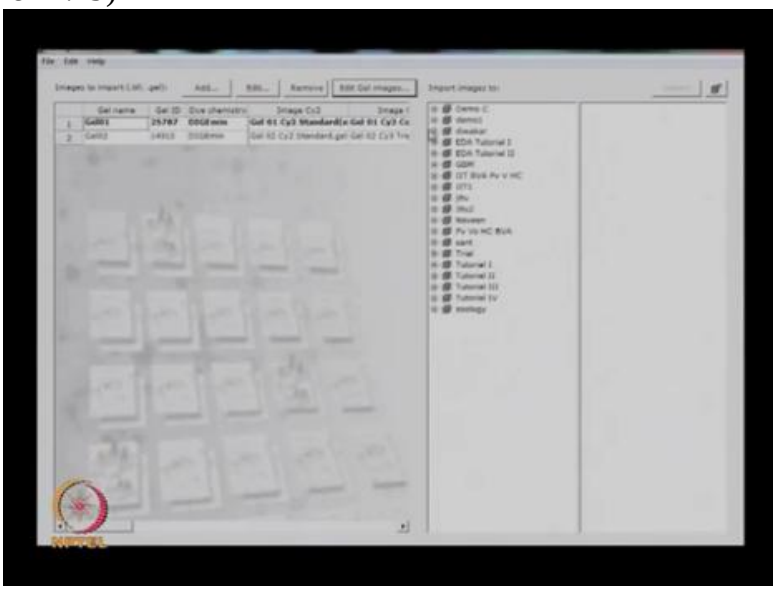

Expert: and close it. This is what; you can edit these images,

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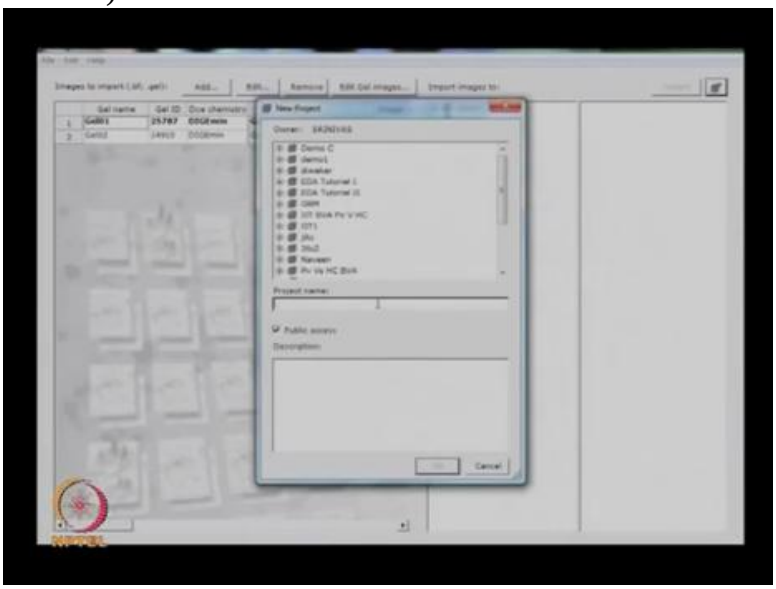

Expert: Then you have to create a new project. Suppose your project name would be according to your project name, this is Demo, Ok.

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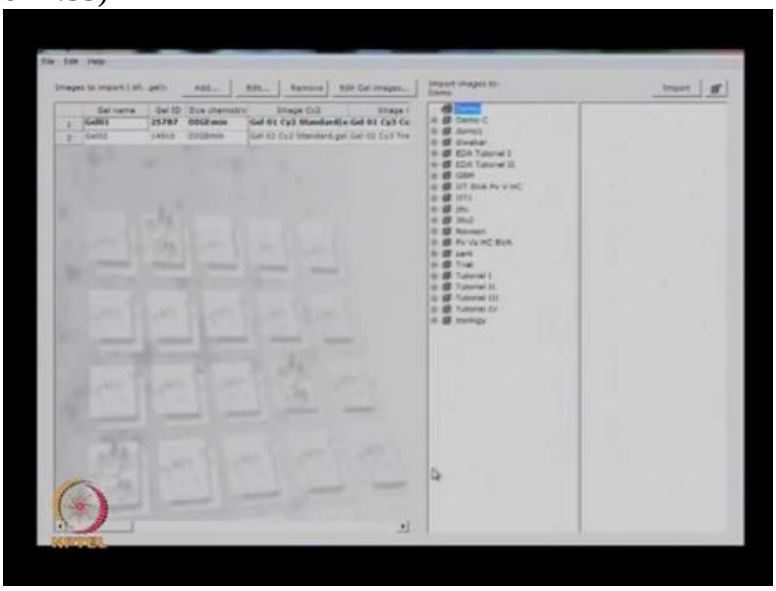

Expert: Now we have the project name of Demo. Now we can able to import these 2 gel images into the new project where we created, just click on import.

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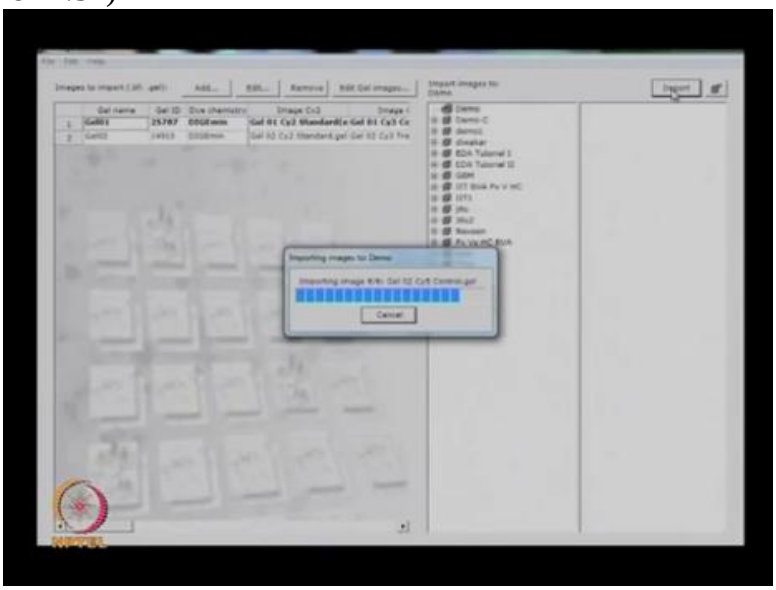
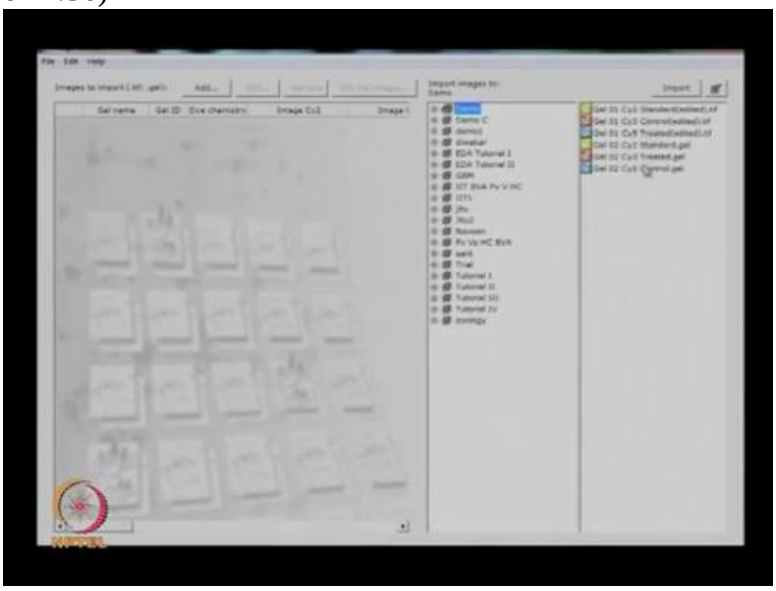


Expert: These gels will be imported into this particular project.

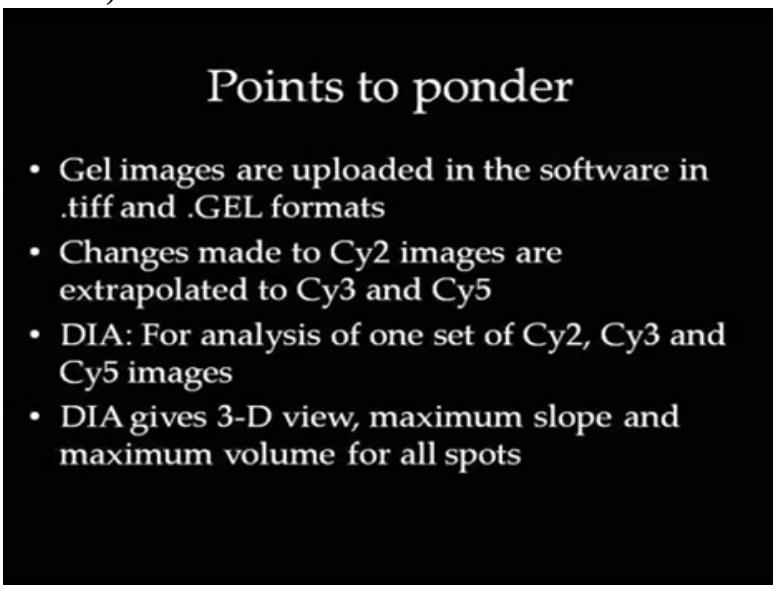
(Refer Slide Time 24:56)



Expert: Now here you can see all 6 images These are baically2 gels, to these 2 gels we have Cy2, Cy3 and Cy5 kind, total 6 gel images. Ok this is the image editor and image loader.

Professor - Expert conversation ends

(Refer Slide Time 25:21)



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