Proteins and Gel-Based Proteomics
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Mod 05 Lecture Number 17

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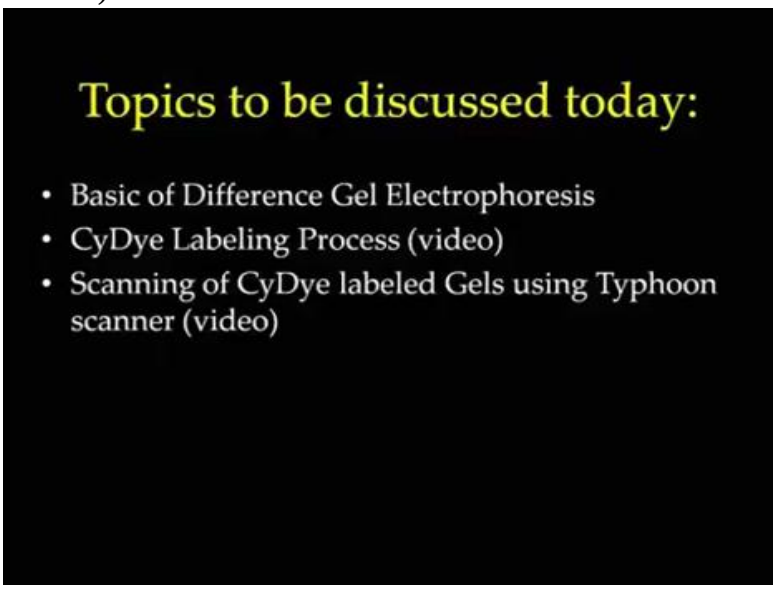

The 2D DIGE method was basically aimed to overcome the gel to gel variability…

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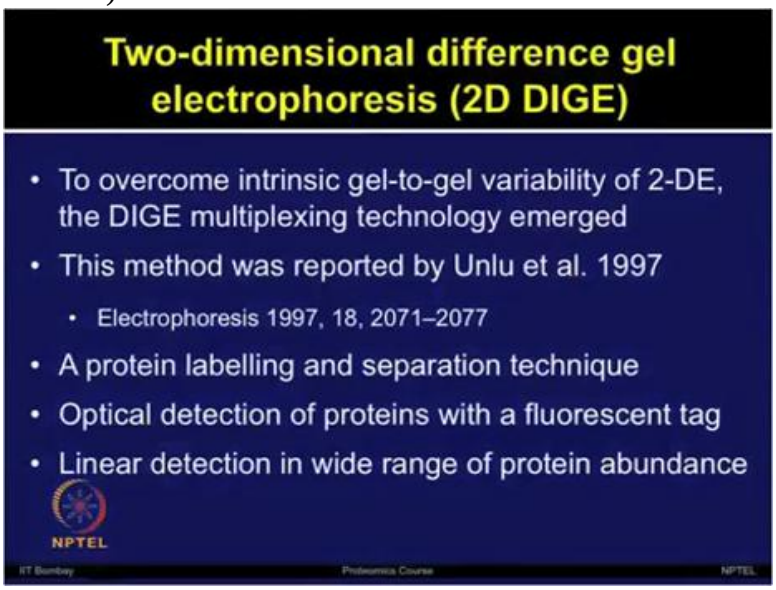

…which was observed in the two-dimensional electrophoresis and it allowed the multiplexing samples; this method was first reported by Unlu et al in 1997 and this method finally became very user-friendly and now thousands of laboratories worldwide are using this technology to overcome the gel to gel variations encountered by traditional or conventional two-dimensional electrophoresis.

So this method, the DIGE method involves protein labeling and separation techniques. The optical detection is done by using the fluorescent tags. And this method performs the linear detection in the wide range of the protein abundance. Overall one can expect a very sensitive method for giving rise to very, very large dynamic range of the protein detection by using the fluorescent tags.

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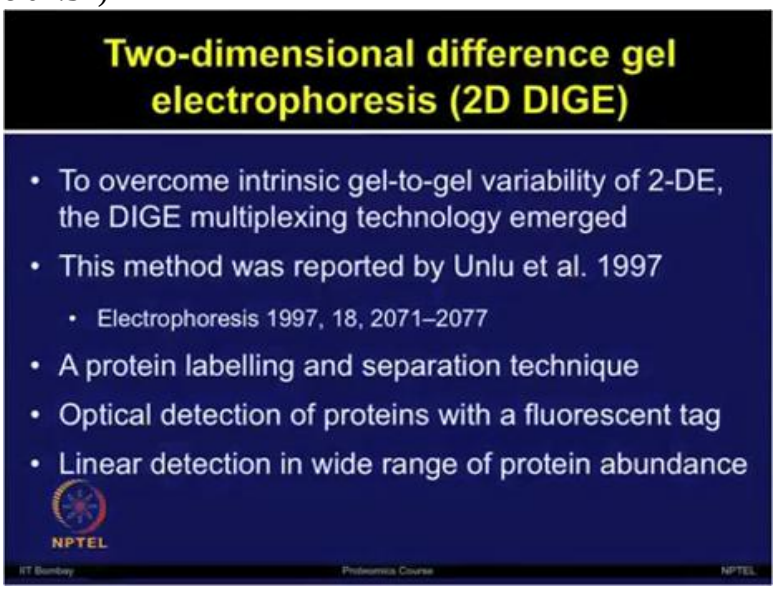

And this method is giving the multiplexing and separating proteins on one gel so it is expected that it will avoid lots of difficulties which are encountered in the traditional 2D gel.

So let's talk briefly about basic principle involved in DIGE technology. So we have to discuss how to do the protein labeling, then what is the need for doing the internal standards. So let's talk step by step.

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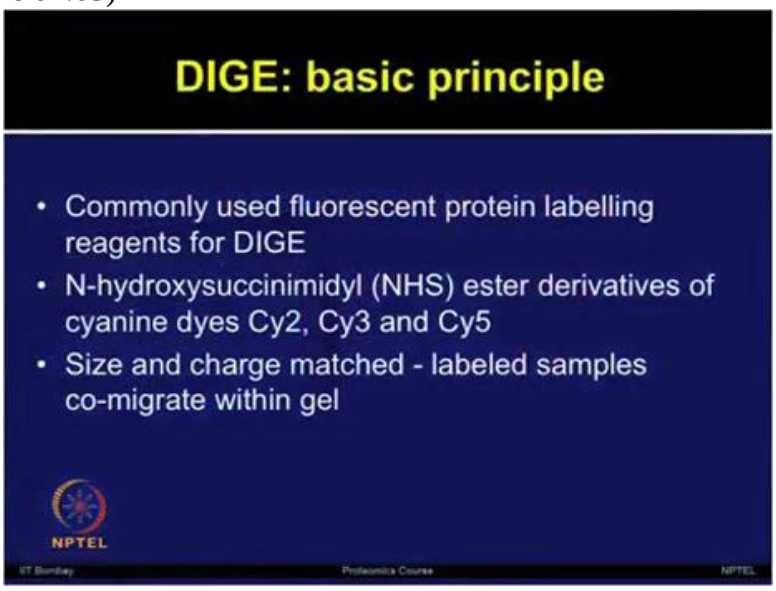

As we discussed, this is the commonly used fluorescent protein labeling reagent for the DIGE, N-Hydroxysuccinimide NHS ester derivatives of Cyanine dyes such as Cy2, Cy3 and Cy5 are used in this method. The Cy3 and Cy5 are used to label your control and your treatments. One should always try to do the dyes swapping so that there is no dye bias.

So if in one sample you are adding Cy3 in control and Cy5 in treatment, in other sample you should do the vice versa. So Cy2 remains consistent for doing the internal standards. Now forCy3 and Cy5, these dyes, the size and charge is matched so the label samples can co-migrate within the gel.

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Now I will talk about the labeling methods used in DIGE technology. So when we were talking about two-dimensional electrophoresis workflow in the last class, we had looked at different types of staining methods currently available, such as Coomassie, silver staining, SYPRO Ruby and in that context I briefly described about Cyanine dyes.

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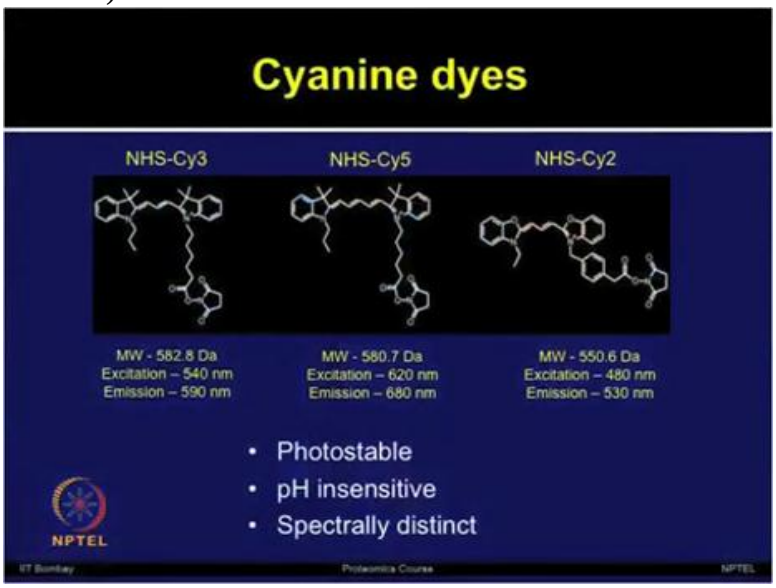

So now, in this technology the DIGE technology, Cyanine dyes are used. There are 3 different Cy dyes as I mentioned, the Cy3, Cy5 and Cy2. The excitation and emission wavelength of these three are defined.

The Cy3 excitation wavelength is at 540 nanometers; emission wavelength at 590 nanometers, the Cy5 excitation at 620 nanometers and emission at 680 nanometers; the Cy2 excitation at 480 nanometers and emission at 530 nanometers. So these dyes are photo-stable.

They are pH insensitive and spectrally distinct. By using these dyes, DIGE method can be used. Now let's talk about different types of labeling methods.

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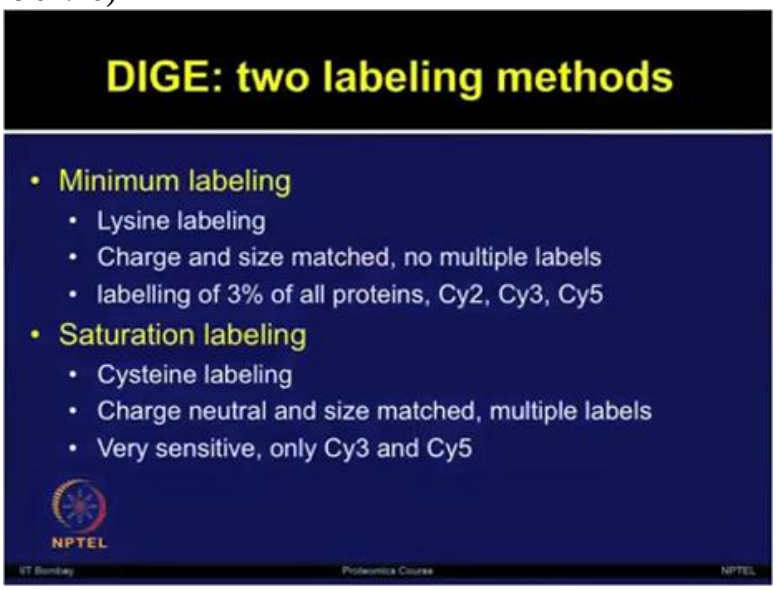

In DIGE, there are two different types of labeling methods which are possible. One is the most commonly used Lysine labeling or the other is Cysteine labeling. Lysine labeling method, also known as the Minimum Labeling method where the charge and size of dyes are matched

There are no multiple labels and it is known as Minimum Labeling because it is only labeling the 3% of all the proteins. I will give you the reason why that is, we are aiming for very, very small amount of proteome to be labeled, and that will be covered in the next slide.

The saturation labeling is done by using Cysteine tagging. So amino acid Cysteine is targeted to label, it is charge neutral and size matched. Multiple labels are used

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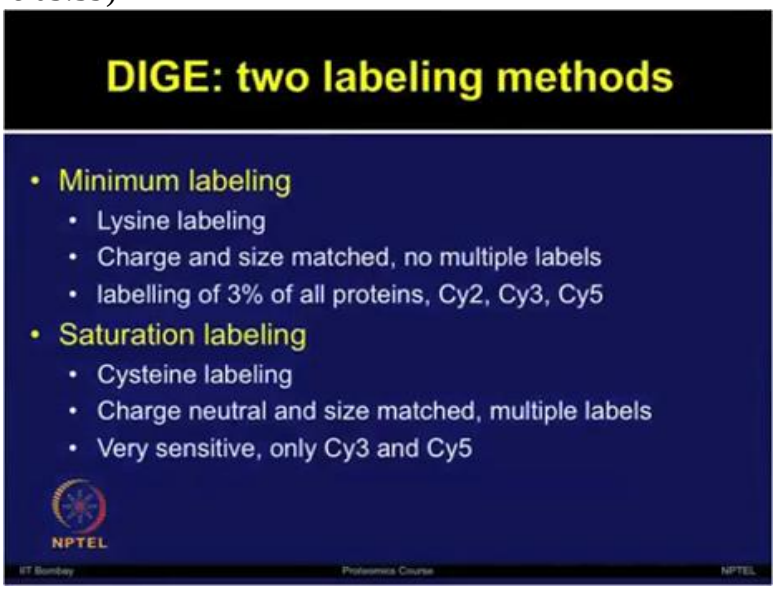

.It is very sensitive and used only for the Cy3 and Cy5 labeling. So saturation labeling is not so widely used but this is another method one can use for labeling the proteins and it can be used in DIGE methodology. So let's talk about the popular method of targeting Lysine. So why to label only Lysine amino acid?

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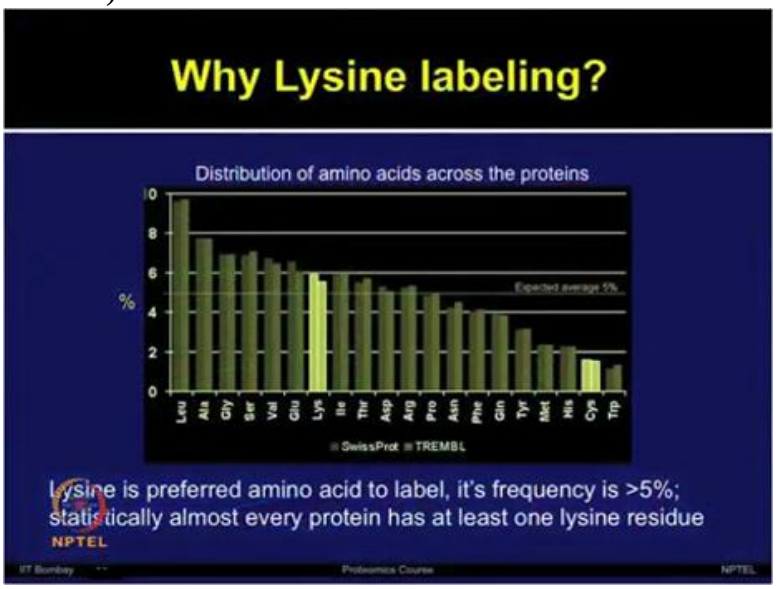

Lysine is preferred amino acid as I have shown you in this graph here the distribution of amino acids across the proteins and their percentage distribution. So Lysine is preferred amino acid to label. Its frequency is greater than 5%. So, statistically almost every protein has at least one Lysine residue

So there is a good probability that your protein will have Lysine residue and it will get labeled if you are targeting the Lysine. But if that is not the case one can always try the saturation labeling of Cysteines.

Now let's talk in more detail about Minimum labeling or Lysine labeling.

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Cy dyes, they react with the primary amino group of target proteins and the end terminal alpha amino group and the Lysine epsilon amino groups, they are labeled by the process of nucleophilic substitution. So all the available Lysine labeling would create a very hydrophobic environment of the proteins. That was the case observed by the original paper derived by Unlu et al.

So to avoid that hydrophobic protein atmosphere, it is recommended to do the minimum labeling of only 3% of the whole proteome so that overall it is not very hydrophobic. Now in this slide, I will describe you the labeling chemistry.

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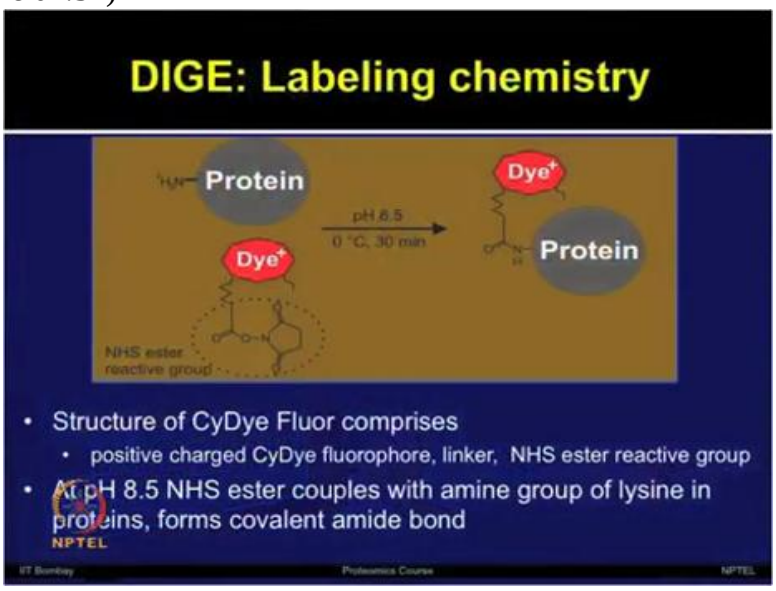

So as you can see in the structure here, the structure of Cy dye Fluor comprised of a positive charge Cy dye fluorophore, a linker and NHS ester reactive group. So at pH 8.5 the NHS ester couples with amino group of Lysine in the protein and it forms covalent bond.

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The amino group of Lysine residues, they are positively charged at neutral or acidic isoelectric points. Dye is also positively charged. So the net pI will be unchanged. Therefore the Cy3 and Cy5, these are not going to alter any mass as well as charge overall and they are very well spectrally resolved.

So people prefer doing Lysine labeling. It is not going to alter overall pI values. It is not going to alter overall mass because both Cy3 and Cy5 will carry the same mass of these

labels and there is a good probability that your protein will have Lysine residues and it will get labeled because of the Cyanine dyes. So, how to prepare the sample to perform the DIGE experiment?

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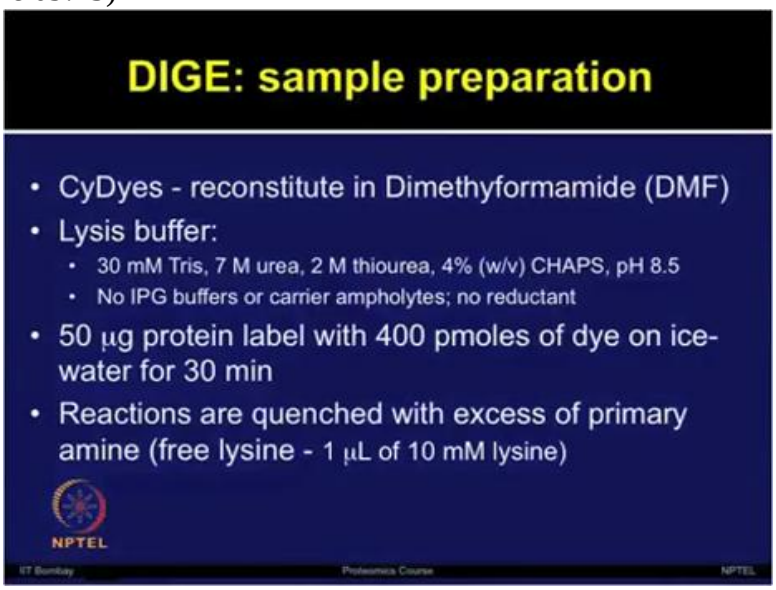

First of all you need to reconstitute your dye in dimethylformamide or DMF. The Lysine buffer that is, in which your protein sample is going to be dissolved consists of 30 milli Molar TRIS, 7 Molar Urea, 2 Molar of thiourea, 4% of CHAPS and you have to adjust pH at 8.5.

Please make sure there is no IPG buffer or carrier ampholyte and there is no reductant added in this Lysine buffer. Since you want to perform the Minimum Labeling, you need to add only 400 pico Mole of Cy Dyes in a small amount of protein, 50 micro grams of proteins and during that process; reaction should be performed on ice water for half an hour.

If you go back and remember about our previous discussion, to visualize a gel with the Coomassie stain, you need more protein. Even from silver and other stains, you need good amount of proteins, but these fluorescent dyes are very sensitive. So all you are looking for, a very small amount of protein, 50 microgram protein quantity is sufficient for performing this experiment.

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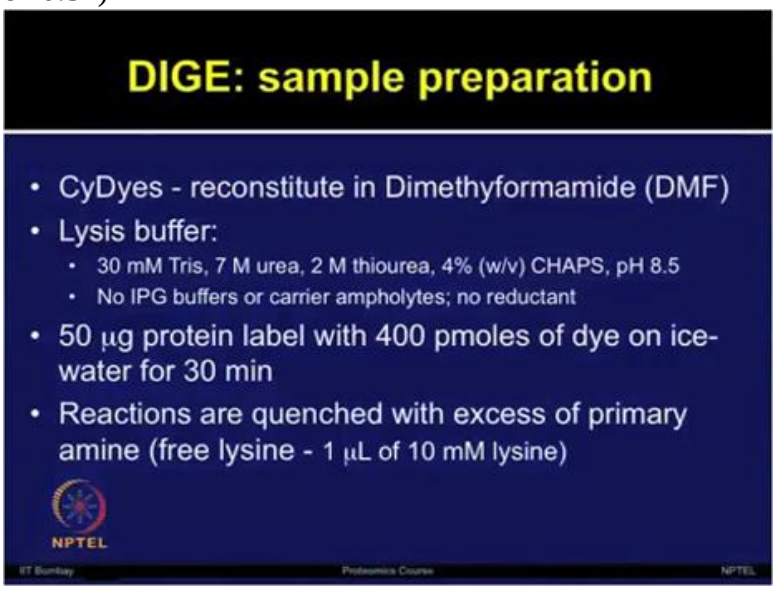

Now, if this reaction of half an hour is done, after that reaction should be quenched with excess of primary amine, the free Lysine by adding 1 micro liter of 10 milli molar of Lysine. So then reaction will be stopped and you can proceed with the next step.

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So while you are doing this reconstitution of dyes and labeling your protein samples with the Cy3 and Cy5 dyes, now let's think about our Cy2 dye and internal standard.

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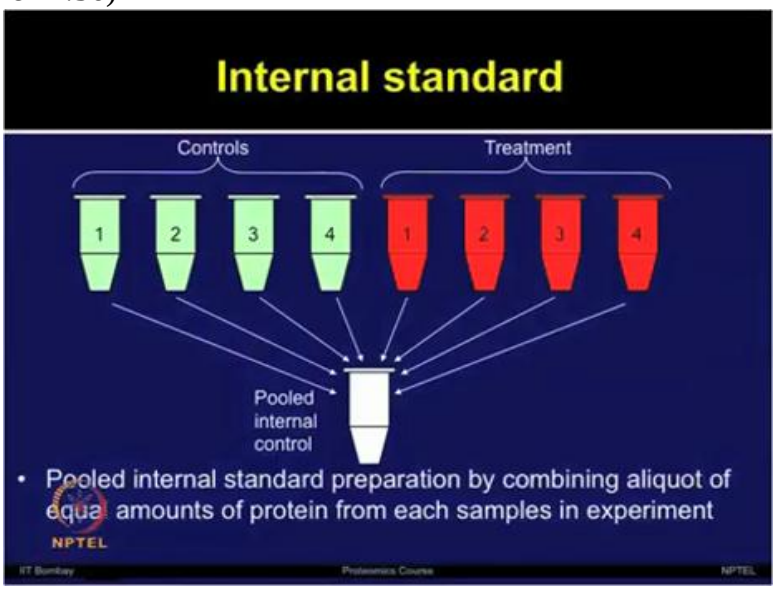

So I am taking a very simple case here where you have 4 healthy controls and 4 patients suffering from a disease. So to represent all the proteins which are present in each of these samples in healthy individuals and diseased patients, first you need to make an internal pool which is representative of all of these 8 samples.

So you can take 25 micro liters from each of these 8 samples and make it to 200 micro liters of one pooled sample which is your internal pooled standard. So this pooled standard preparation can be performed by combining aliquots of equal amount of protein from each samples in the given experiment.

Now the Cy2 dye we are going to label with the pooled sample so that can be used as internal standard. This internal standard has many advantages and this is actually one of the major success that how 2D limitations have been overcome.

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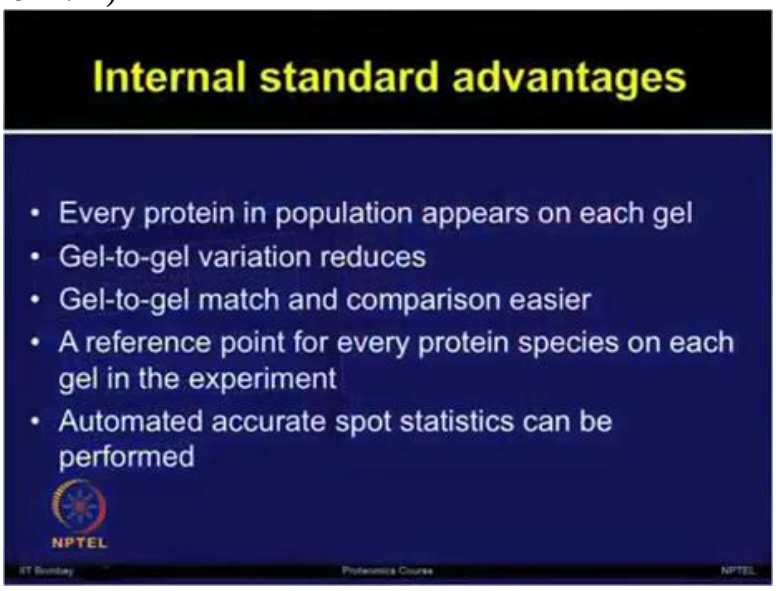

Since every protein is present in the internal pool population, so that is going to be appearing on your gel. So there is a reference point for each gel already present on your gel, whether it is from any patient or any controls. So the gel to gel variation will be reduced. Now gel-to-gel matching and comparison will be much efficient and easier.

Now this internal standard is creating a reference point for every protein on each gel in the experiment. Later on, when software performs some data analysis, this results into accurate spot statistics and more meaningful biological data can be interpreted from this analysis.

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So now we have talked about the DIGE principle, how to label the sample with different Cy Dyes, why there is a need for making the Internal Standard, and how to label the internal standard with the Cy2 dyes. So let's go to the step by step procedure of DIGE method.

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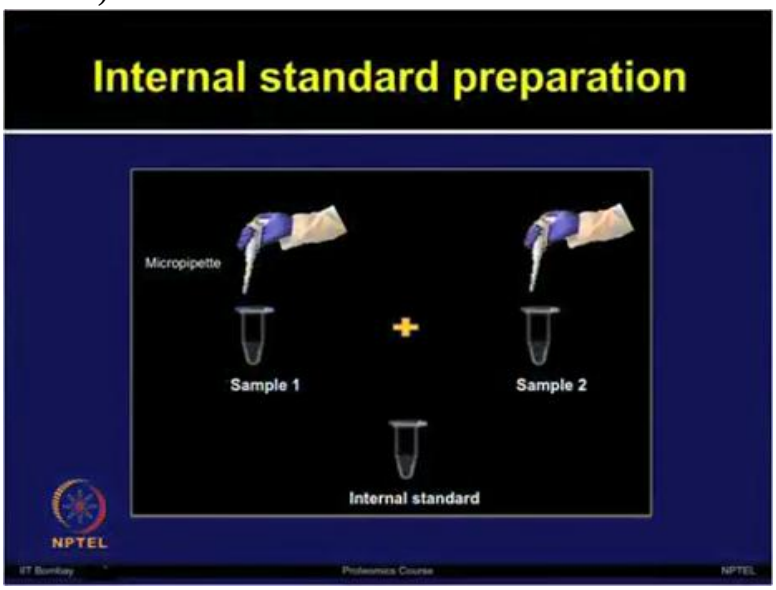

So very first thing is you have extracted your protein samples and you need to make the Internal Standard.

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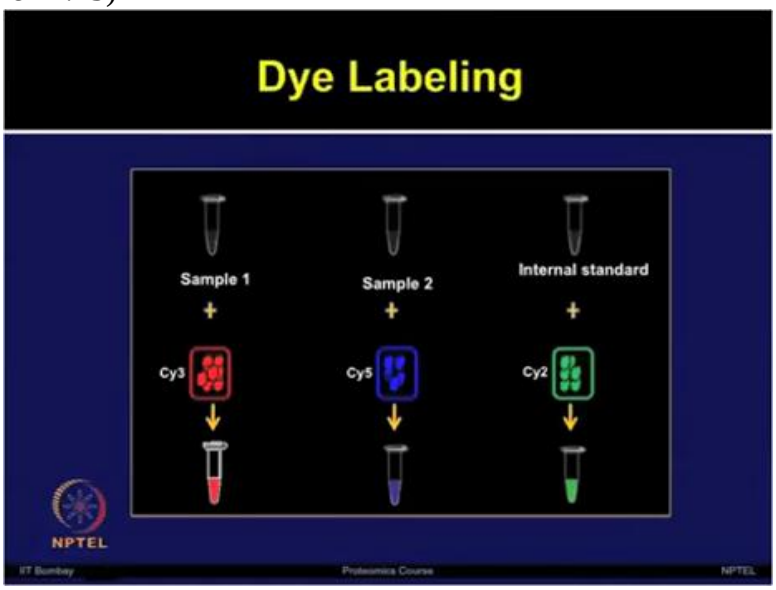

Now your control sample, treatment sample and Internal Standards should be labeled with different Cyanine dyes. Internal Standards are always fixed with Cy2 dyes but your control and treatment should be dye-swapped, so sometimes it should be Cy3 and then few samples should be Cy5 and vice versa

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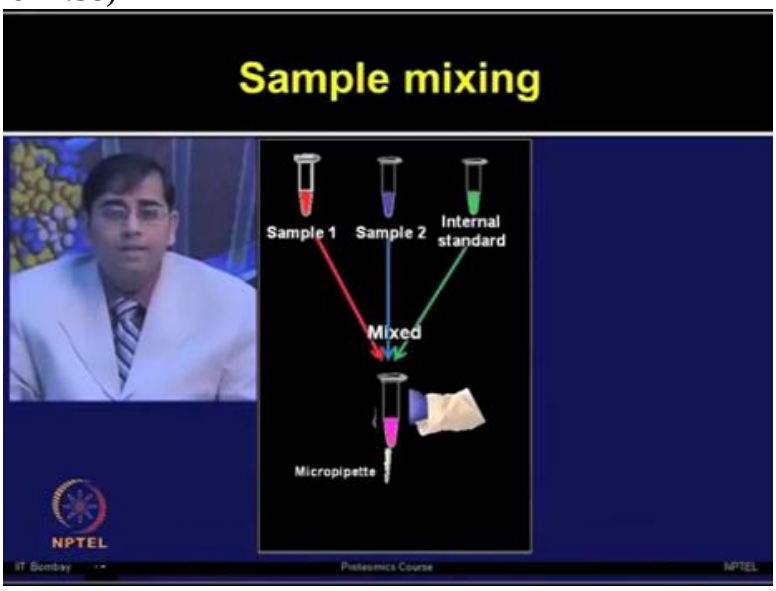

.Once labeling is done then samples should be mixed. So all the 3 samples, Cy3, Cy5 and Cy2, all 3 labeled samples should be mixed in one combined tube. After that process is done…

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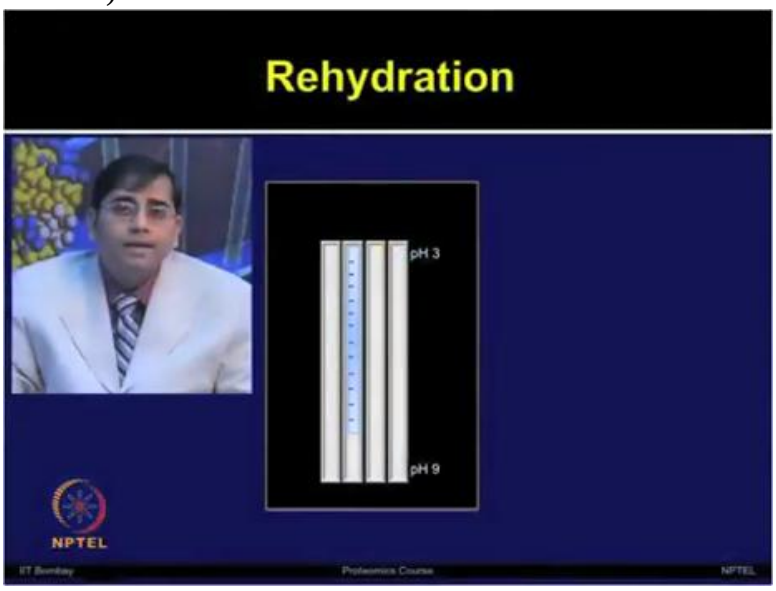

… you can take that sample and do the rehydration. We have talked about rehydration and the need for it in the previous lecture in the 2D workflow.

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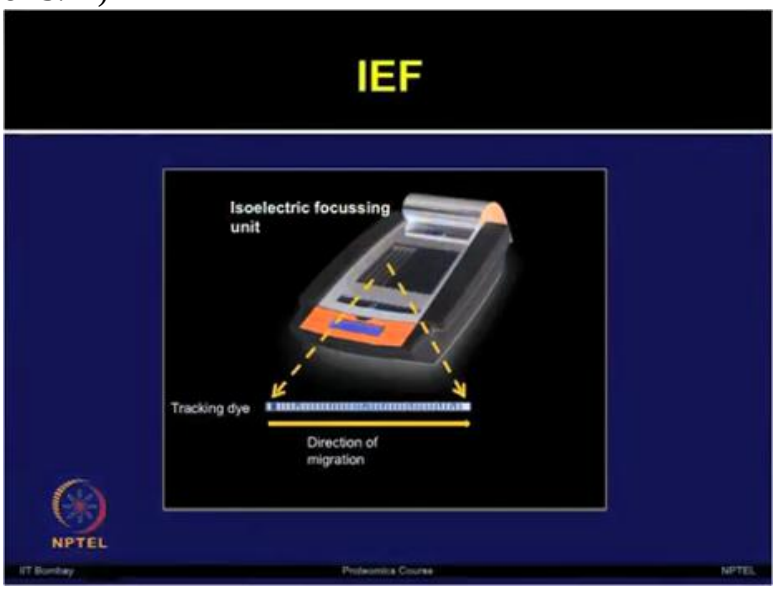

Now this rehydrated strip can be focused in Isoelectric Focusing instrument. It is good idea to avoid any light exposure during rehydration, dye reconstitution process as well as during the isoelectric run is in process.

Once IEF is done, again you have to do the equilibration, first equilibration and second equilibration followed by SDS PAGE. So overall step as you can see, it is exactly same what one is doing in the two-dimensional electrophoresis.

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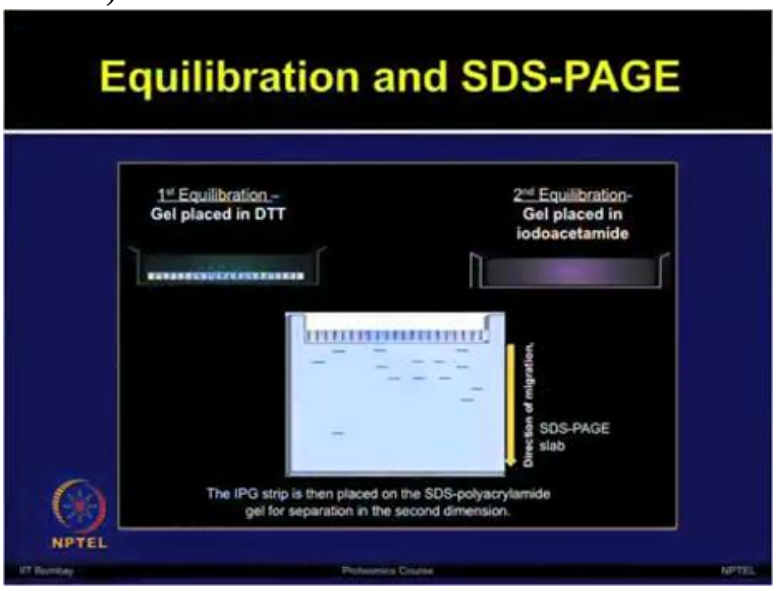

Only variation here is that all your control and treatment samples have been mixed together and they have been separated on one IPG strip. So this strip is placed on one SDS PAGE gel

and all the proteins will be separated in one gel itself, whether it is coming from the control or the treatments.

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The pH of the sample will be adjusted 8.5 using 100 milli molar NaOH.

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Take out the Cy Dye tubes which are stored in a dark container.

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These dyes using mini centrifuge.

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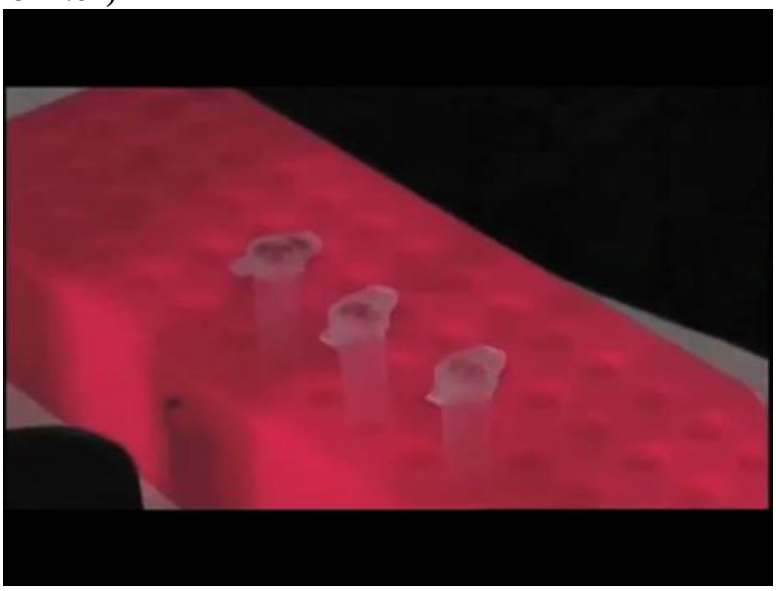

Now label the tubes properly.

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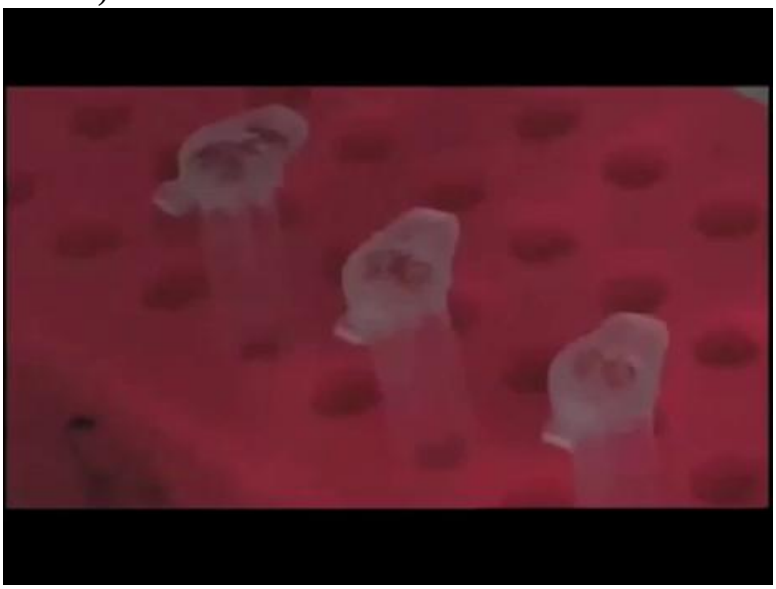

50 micrograms of each sample will be separately labeled with 400 pico Molar of Cy dye.

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Add 1 micro liter of Cy dye in each tube.

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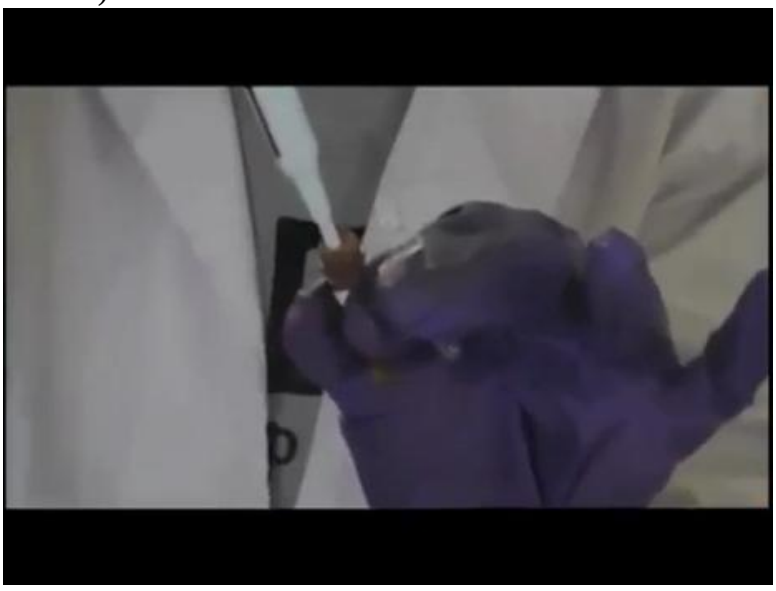

Add 1 micro liter of each Cy dye in the respective tubes.

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Here you can see all the 3 Cy dyes in different tubes.

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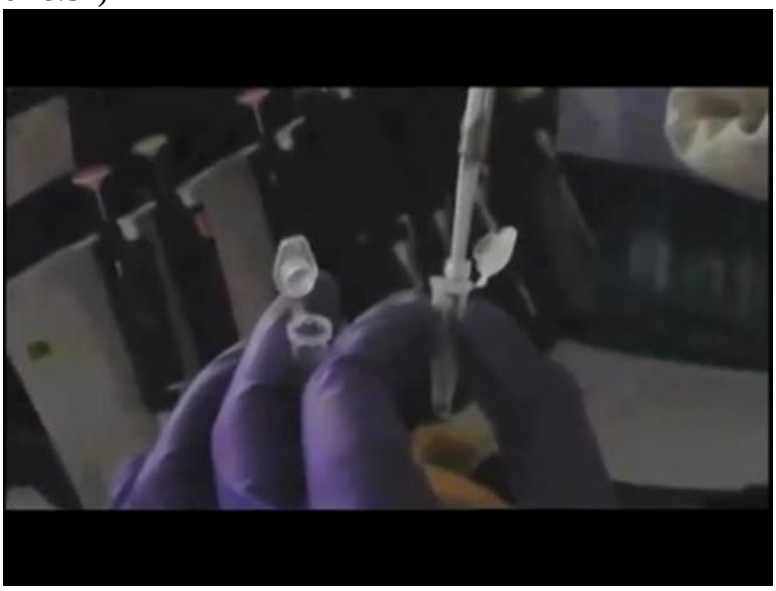

After addition of Cy dye, samples will be incubated on ice for 30 minutes in the dark.

Labeling reaction will be stopped by addition of 10 milli molar Lysine followed by incubation on ice for additional 10 minutes.

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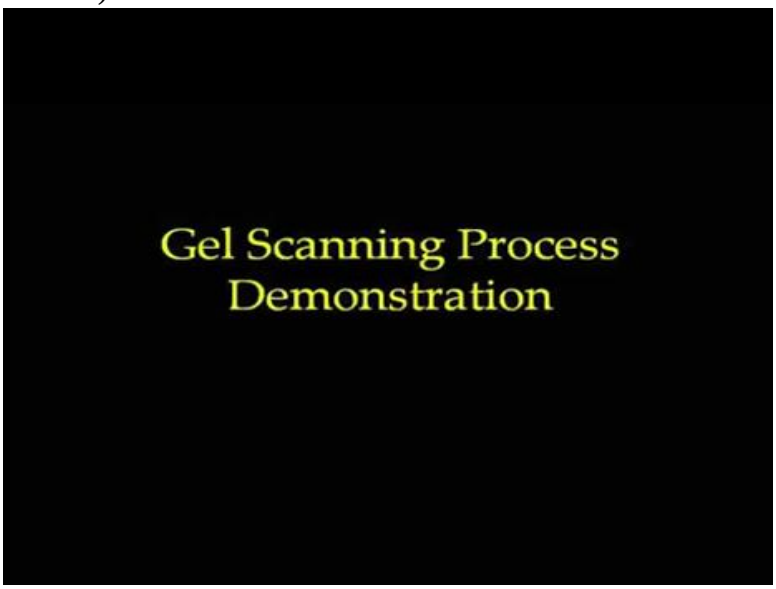

Dye gels scanning.

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2D dyed gels will be scanned using Typhoon Variable Mode Imager at a 100 micro Molar resolution employing suitable excitation emission wavelength. Switch on the Typhoon scanner

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Place the gel plates on the tray provided as shown and insert it in Typhoon scanner.

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Click on the Typhoon Fla 1900 icon. Click on 2D DIGE

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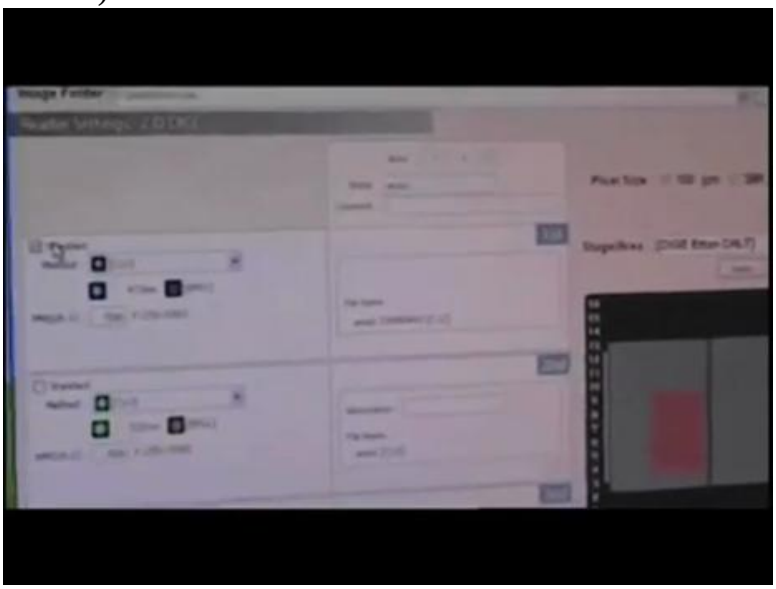

You can see a dye window open.

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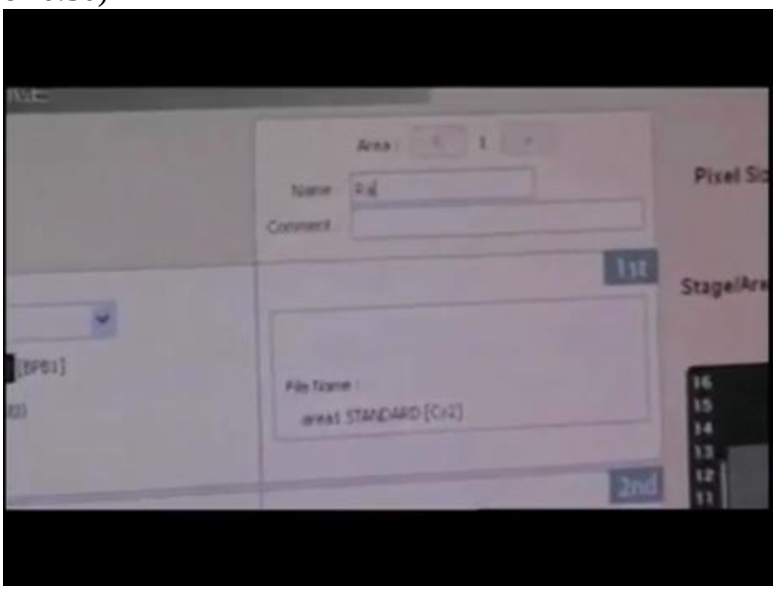

Before starting the scanning, name the file properly

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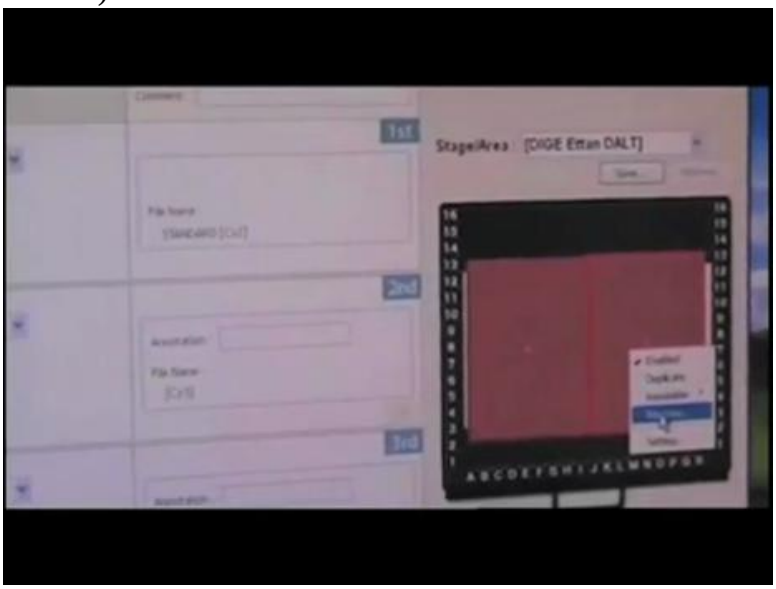

Set area as DIGE Ettan DALT

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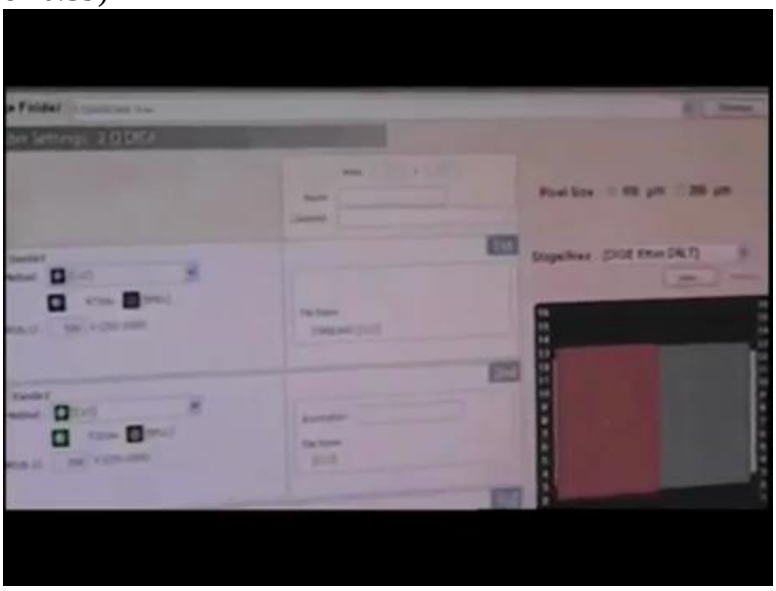

Resolution should be 100 micro Molars.

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Set Standard at Cy2

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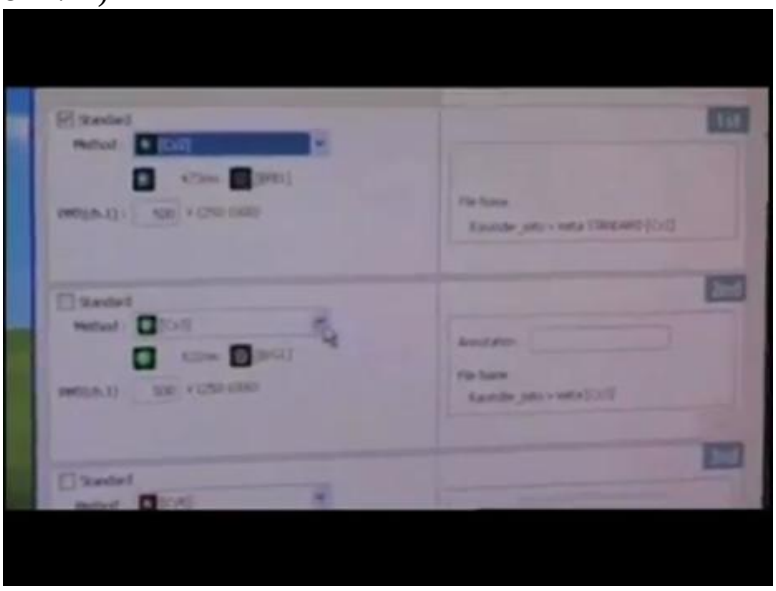

Set the wavelength for each of the Cy Dye

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Excitation wavelength for the Cy 3 is 523 nanometers, Cy 5 is 633 nanometers and Cy2 is 488 nanometers.

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Now by clicking on Start Scan button we can initiate the scanning process.

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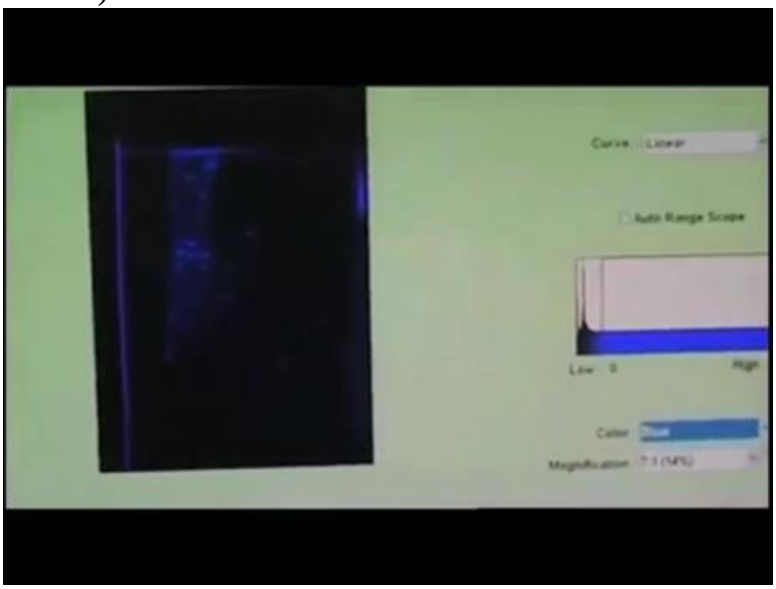

The scanning will initiate with Cy2, proceed with Cy3 and we will end up with Cy5.You can adjust the contrast and see the gels.

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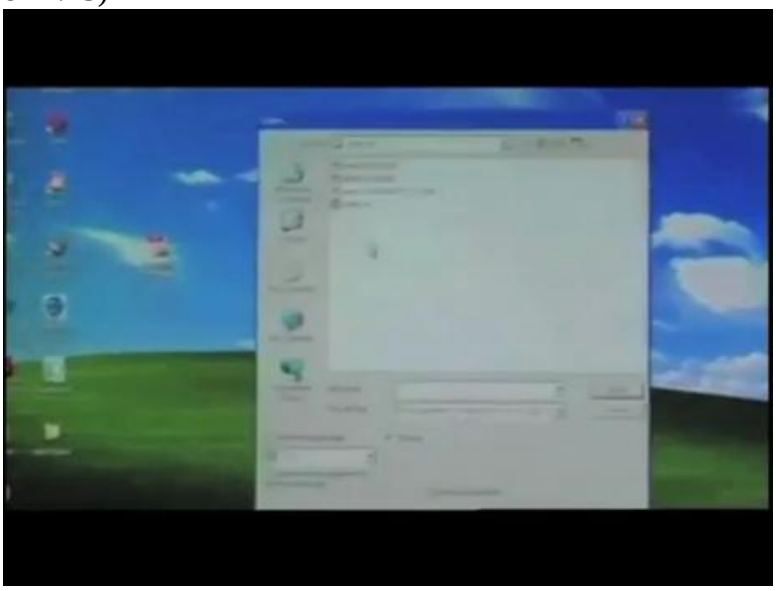

After scanning, gel images will be cropped properly using the image forms of your version 5.0

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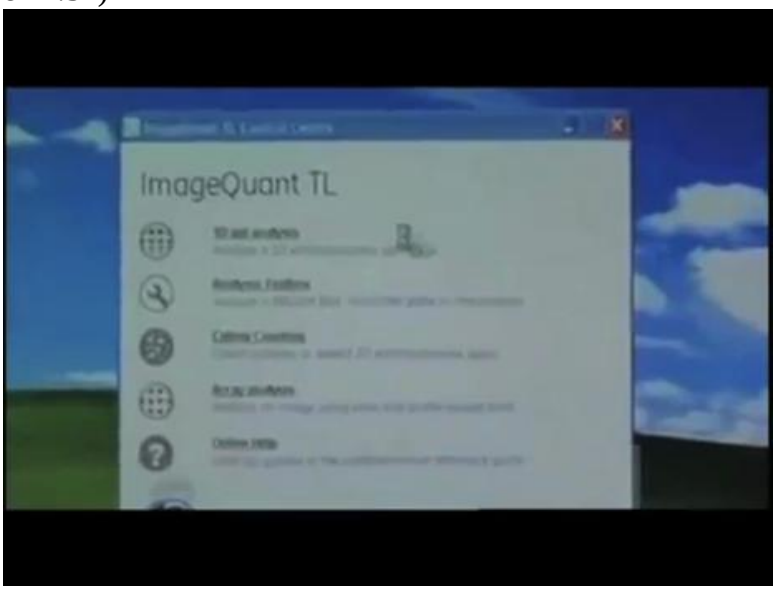

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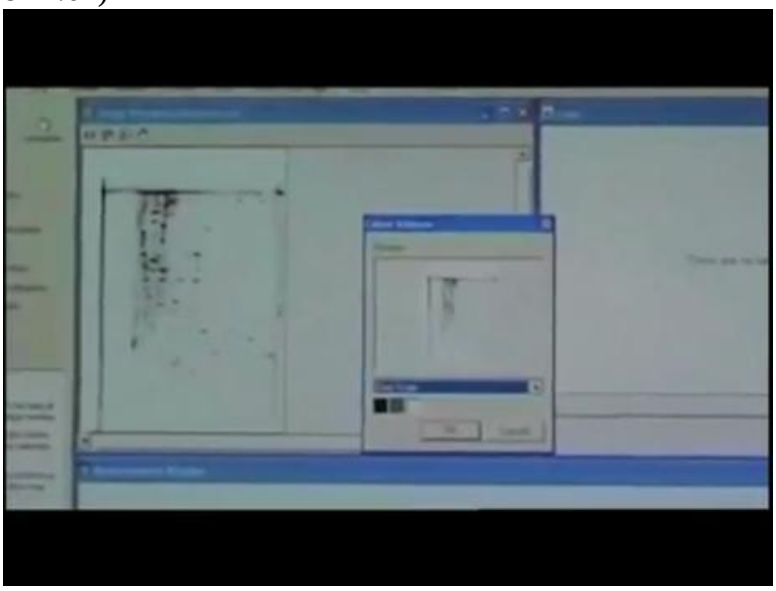

…prior to importing the DeCyder 2D software version 7.0 for comparative analysis, for relative protein quantification across disease and control samples

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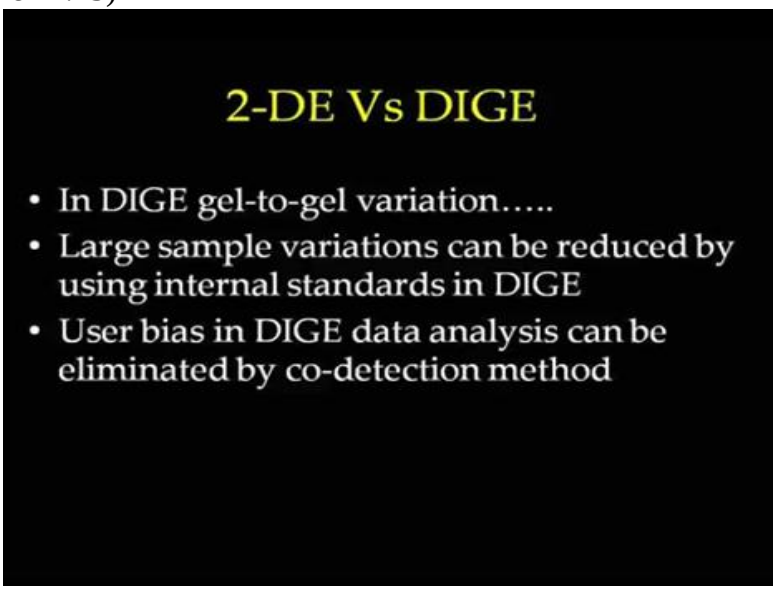

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