Introduction to Proteomics
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Lecture - 16
2D DIGE: Basics

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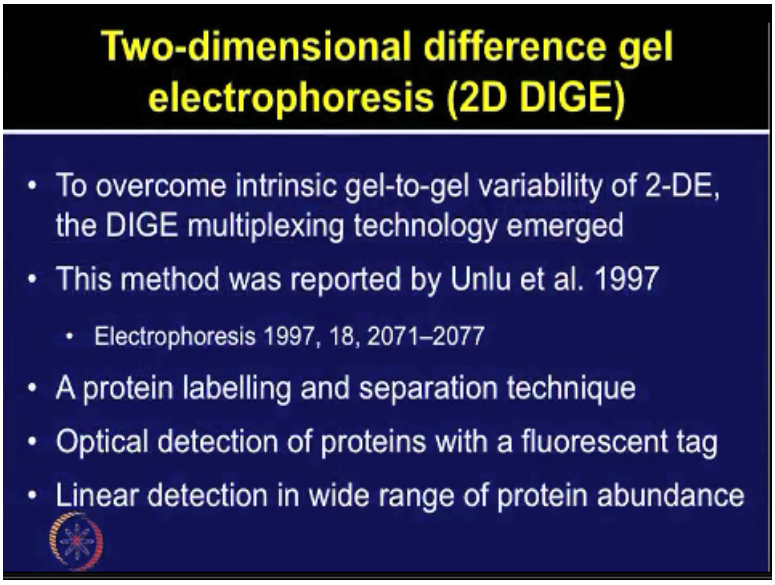

The 2D DIGE method was basically aim to overcome the gel-to-gel variability which was observed in the two-dimensional electrophoresis and it allowed the multiplexing examples. This method was first reported by Unlu et al in 1997 and this method finally became very user friendly and now thousands of laboratories 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 of the proteins is done by using the fluorescent tags and this method performs the linear detection in the wide range of the protein abundance. So overall one can expect a very sensitive method for giving to rise to very, very large dynamic range of the protein detection by using fluorescent tags.

And this method is giving the multiplexing and separating proteins on 1 gel. So it is expected that it will avoid lot of difficulties which are encountered in the traditional 2D gel.

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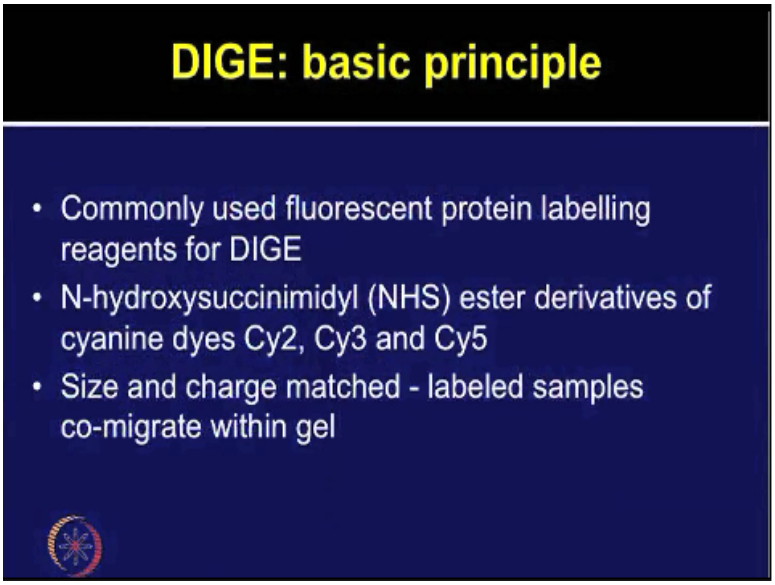

So let us talk briefly about basic principle involved in DIGE technology. So we have to discuss how to do the protein labeling then what is the need for doing the internal standards. So let us talk step by step as we discussed this is a commonly used fluorescent protein labeling reagents for the DIGE. The N-hydroxysuccinimidyl or NHS ester derivative of cyanine dyes such Cy2, C3 and C5 are used in this method.

The Cy3 and Cy5 are used to label your control and your treatments. One should always try to dyes swapping so that there is no dye bias. So if in 1 sample you are adding the Cy3 in control and Cy5 in treatment in other sample you should do the vice-versa. So the Cy2 remains consistent for doing the internal standard. Now for Cy3 and Cy5 these dyes the size and charge is matched so that the labeled samples can co-migrate within the gel.

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

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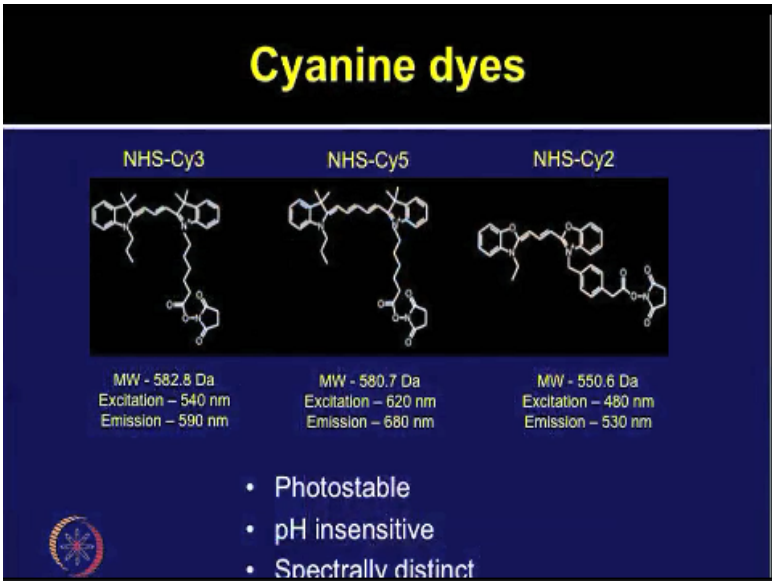

So now in this technology the DIGE technology those Cyanine dyes are used. There are 3 different Cy dyes as I mentioned the Cy3, the Cy5 and Cy2. The excitation and emission wave length of these 3 are defined. The Cy3 excitation wave length is at 540 nanometers, emission wave length at 590 nanometers, the Cy5 excitation at 620 nanometer and emission at 680 nanometers.

The Cy2 excitation at 480 nanometers and emission at 530 nanometer. So these dyes are Photostable they are pH insensitive and spectrally distinct. By using these dyes, the DIGE method can be used.

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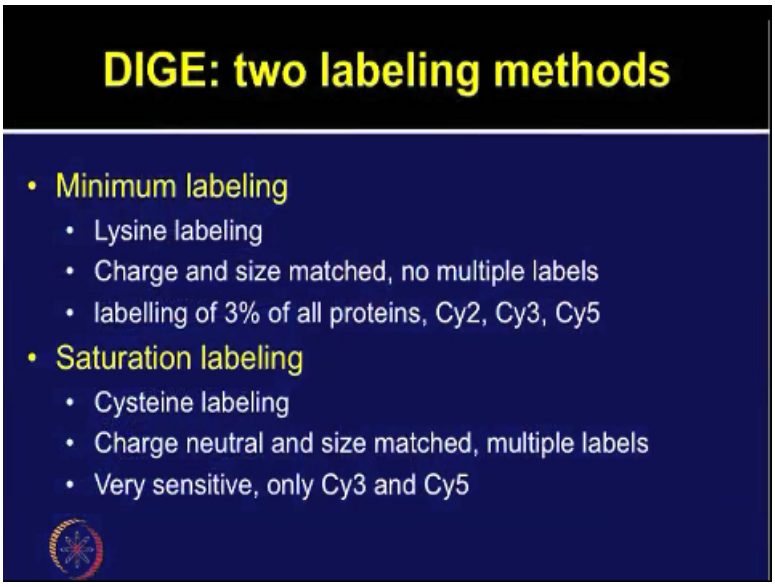

Now let us talk about different type of labeling methods. In DIGE there are 2 different type of labeling methods which are possible. One is most commonly used the Lysine labeling or other is cysteine labeling. Lysine labeling method also known as the minimum labeling method where the charge and size of dyes are matched there are no multiple labels and it is known as minimum labeling because it is only labeling a 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 that this charge neutral and sized matched multiple labels are used. It is very sensitive and used only for 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.

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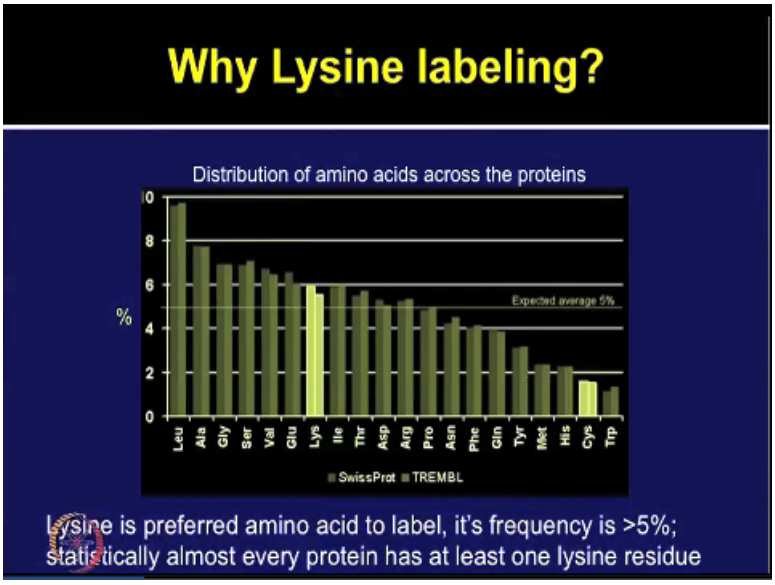

So let us talk about the popular of targeting Lysine. So why to label only Lysine amino acid. Lysine is preferred amino acid as I have shown you in this graph here the distribution of amino acids across the proteins and their percentage distribution. So Lysine is preferred amino acid to label its frequency is greater than 5%. So statistically almost every protein has at least 1 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 Cysteine.

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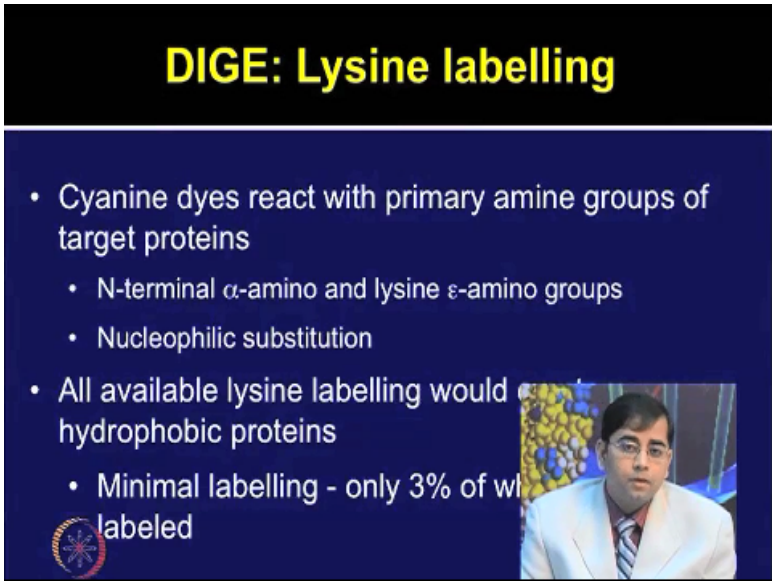

Now let us talk in more detail about the minimal labeling or Lysine labeling. So Cy dyes they react with the primary amino group of target proteins and the N-terminal alpha amino group

at 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 that is recommended to do the minimal labeling of only 3% of the whole proteome so that overall it is not very hydrophobic.

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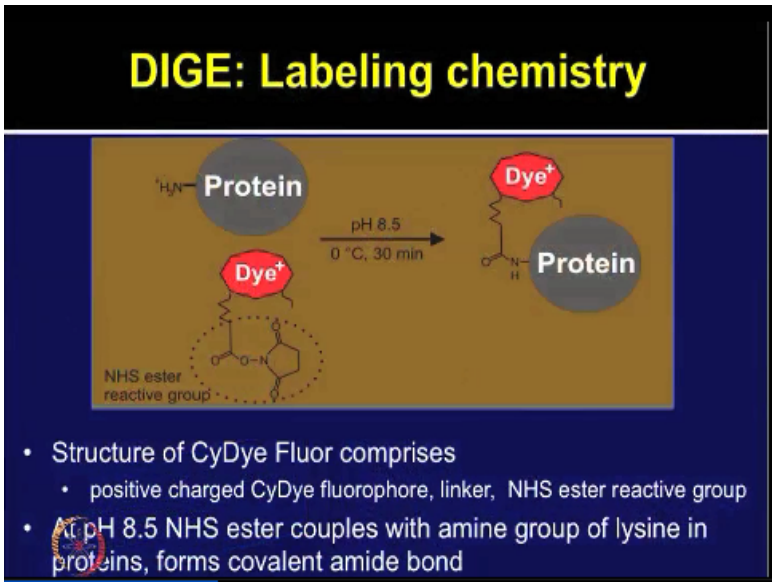

Now in this slide, I will describe you the Labeling chemistry. So as you can see in the structure here the structure of Cydye Flour comprised of a positive charged Cydye fluorophore, a linker and NHS ester reactive group. At pH 8.5 the NHS ester couples with amino group of lysine in the proteins and it forms the covalent bonds.

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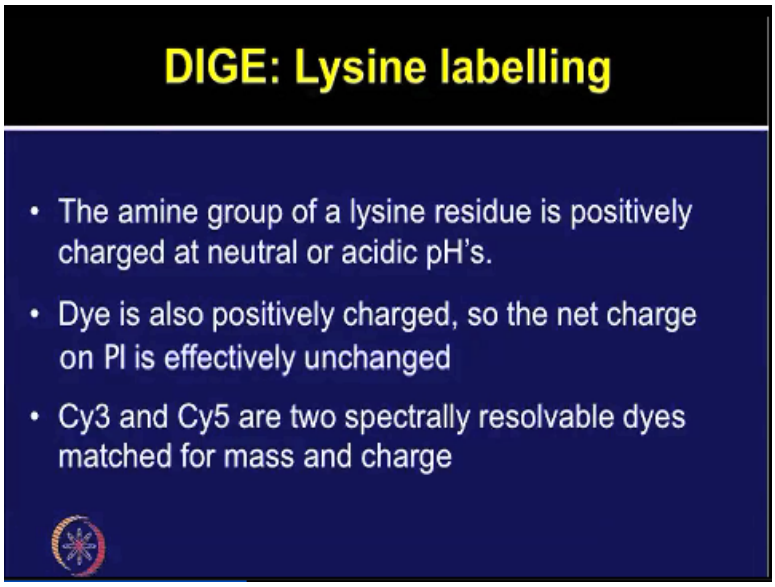

The amine group of lysine residue they are positively charged at neutral or acidic isoelectirc points. Dye is also positively charged so the net PI will be unchanged. Therefore, the Cy3 and Cy5 these are not going to alter any mass as well as charge overall and they are very well spectrally resolved. 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 residue and it will get labeled because of the cyanine dyes.

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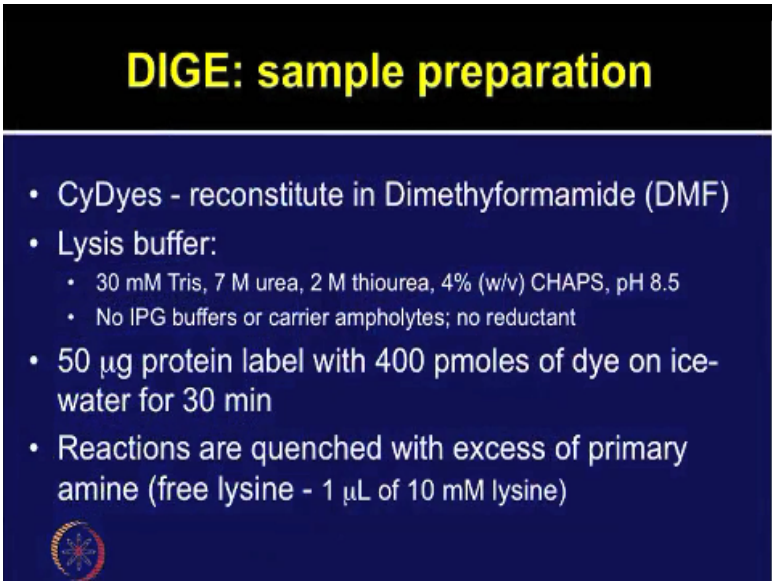

So how to prepare the sample to perform the DIGE experiment. First of all you need to reconstitute your dye in Dimethyformamide or DMF. The Lysis buffer in which your protein

sample is going to be resolved consist of 30 millimolar Tris, 7 molar urea, 2 molar of thiourea, 4% of CHAPS and you have to adjust the pH at 8.5. Please make sure there is no IPG buffers or carrier ampholytes and there is no reductant added in this Lysis buffer.

Since you want to perform the minimal labeling only 400 picomole of Cy dyes in small amount of protein 50 microgram of protein and during that process the 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 a Coomassie Cystine you need more protein even from silver and other stains you need good amount of protein.

But this fluorescent dyes is very sensitive. So all you are looking for a very small amount of protein 50 microgram protein quantity is sufficient for performing these experiments. 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 millimolar of lysine so then reaction will be stopped and you can proceed with the next step.

So while you are doing this reconstitution of dyes and labeling your protein samples with the Cy3 and Cy5 dyes.

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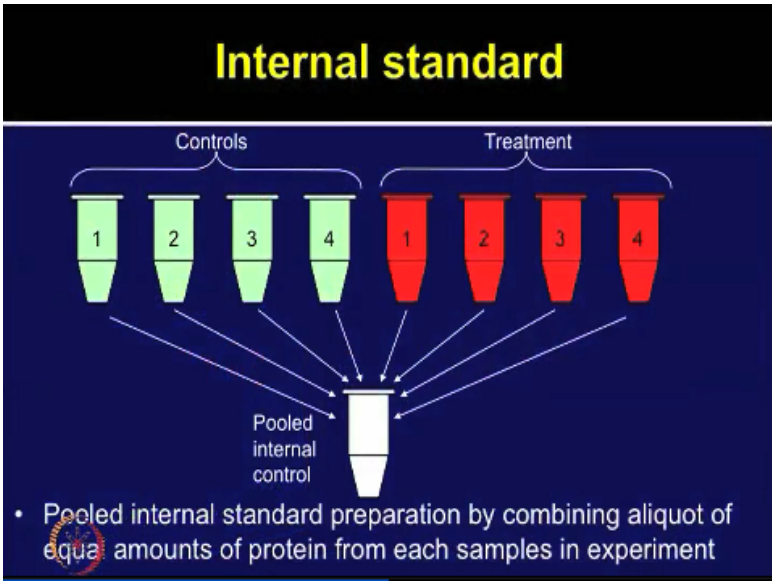

Now let us think about our Cy2 dye and internal standard. So I am taking a very simple case here where you have 4 healthy controls and 4 patients suffering from a disease. So to represent all the proteins which are present in each of these samples in healthy individual and diseased patients first you need to make an internal pool which is representative of all of the

(()) (11:54) samples.

So you can take 25 micro liter from each of these 8 samples and make it to 200 micro liter of 1 pool sample which is your internal pooled standard. So this pooled standard preparation can be performed by combining the aliquots of equal amount of protein from each samples in the given experiment. Now the Cy2 dye we are going to label with this pooled samples so that can be used as an internal standard.

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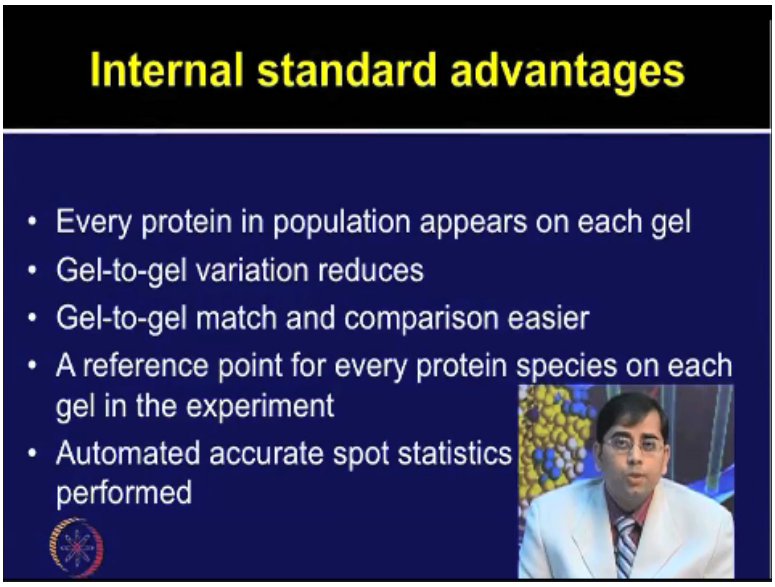

This internal standard has many advantages and this is actually one of the major success that how 2D limitation have been overcome. Since every protein is present in the internal pool population so that is going to be appearing on your gel. So there is a reference point for each gel already present on your gel whether it is from any patient or any control. 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. So now we have talked the DIGE principle how to label the samples with different Cydyes why there is a need for making the internal standard and how to label the internal standard with the Cy2 dyes.

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So let us go to the step-by-step procedure of DIGE method. So very first thing is you have extracted your protein samples and you need to make the internal standard.

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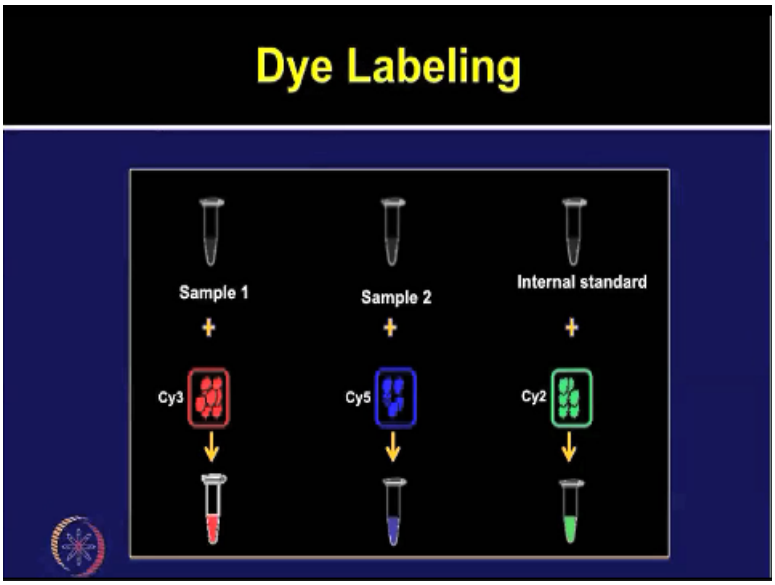

Now your control samples, treatment samples and internal standards should be labeled with different Cyanine dyes. Internal standard is always fixed with Cy2 dyes, but your control and treatment should be dye swapped sometimes it should be Cy3 and then few samples should be Cy5 and the vice-versa.

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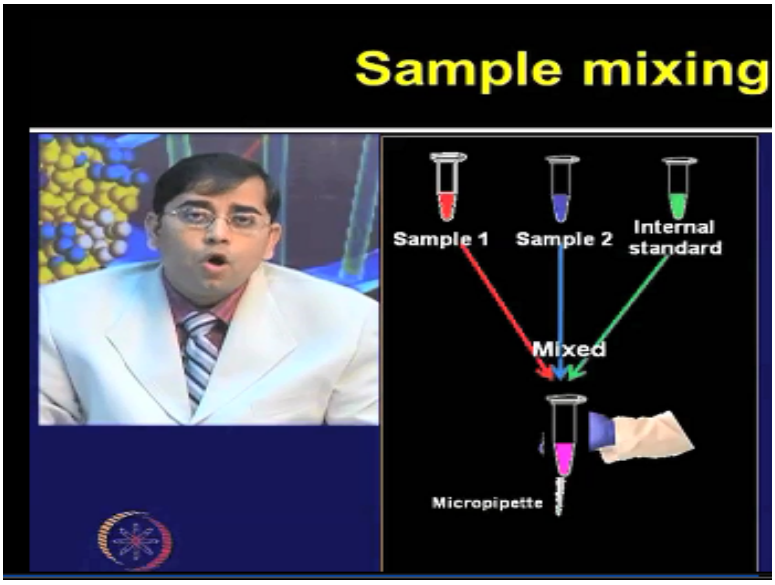

Once labeling is done then sample should be mixed so all the 3 samples Cy3, Cy5 even Cy2 all 3 labeled samples should be mixed in one combined tube.

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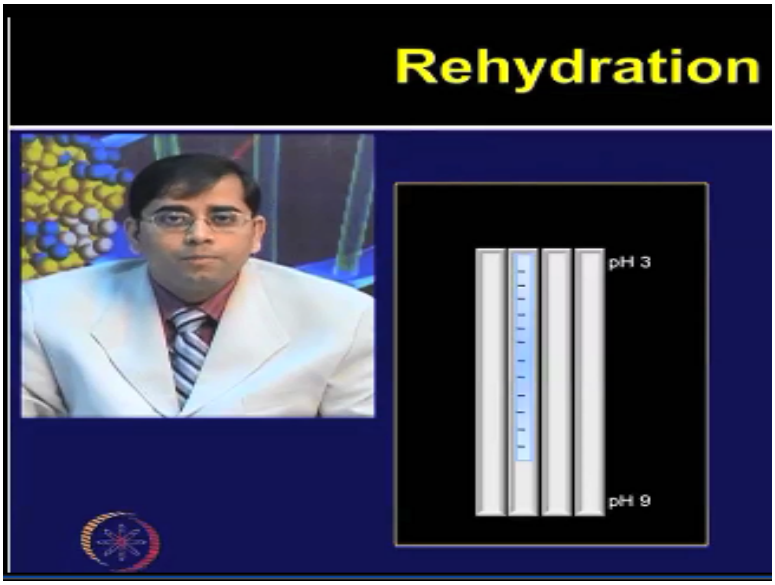

After that process is done you can take that sample and do the rehydration. We have talked about the rehydration step and the need for it in the previous lecture in the 2D workflow.

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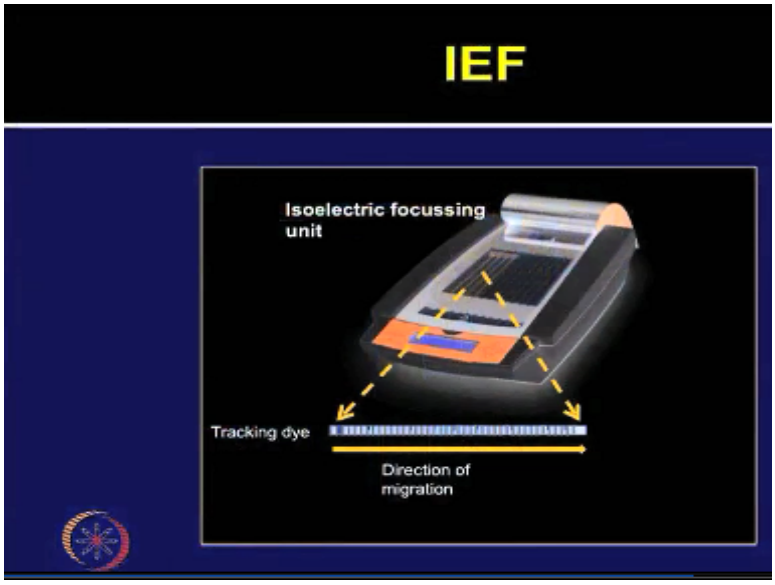

Now this rehydrated strip can be focused in an isoelectric focusing instrument. It is good idea to avoid any light exposure during the rehydration dye reconstitution process as well as during the isoelectric focusing run is in progress.

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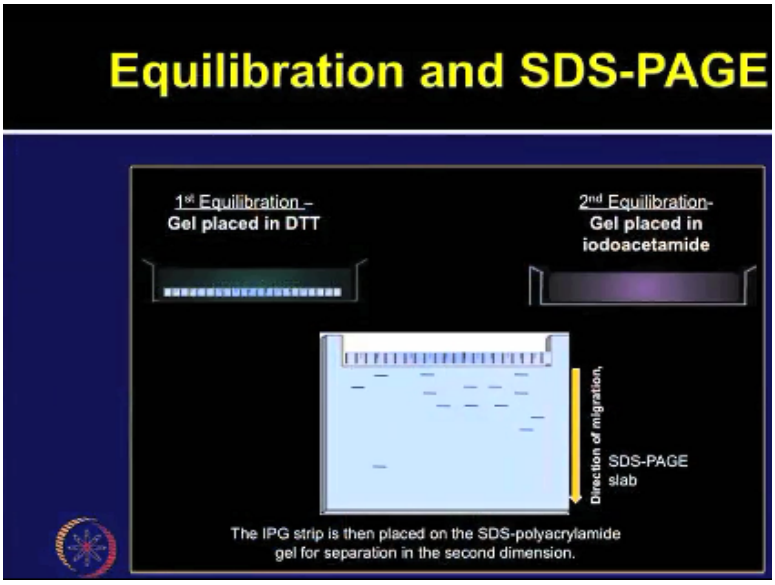

Once IEF is done again you have to do the equilibration first equilibration and second equilibration followed by SDS-PAGE. So overall step as you can see it is exactly same what one is doing in the two-dimensional electrophoresis only variation here is that all the control and treatment samples have been mixed together and now they are separated on one IPG strip.

Now 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 treatments.

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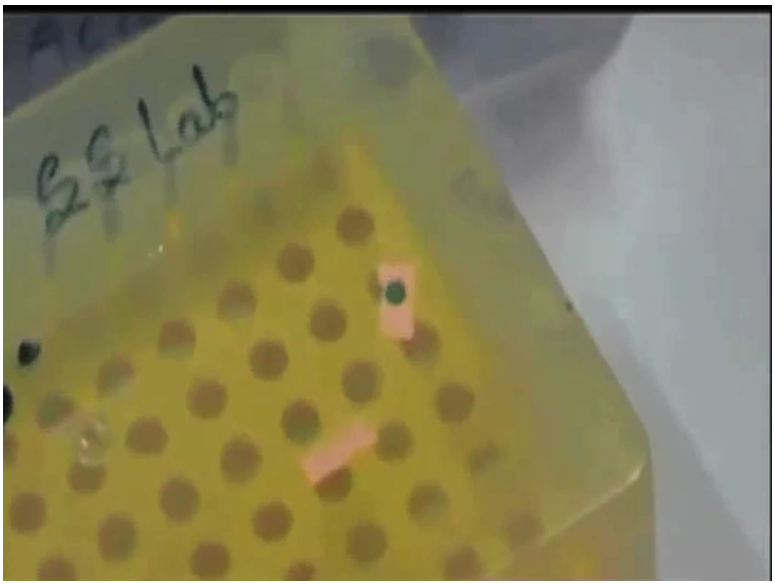

The pH of the sample will be adjusted to 8.5 using 100 millimolar.

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Take out the Cydye tubes which are stored in a dark container or these dyes using mini centrifuges.

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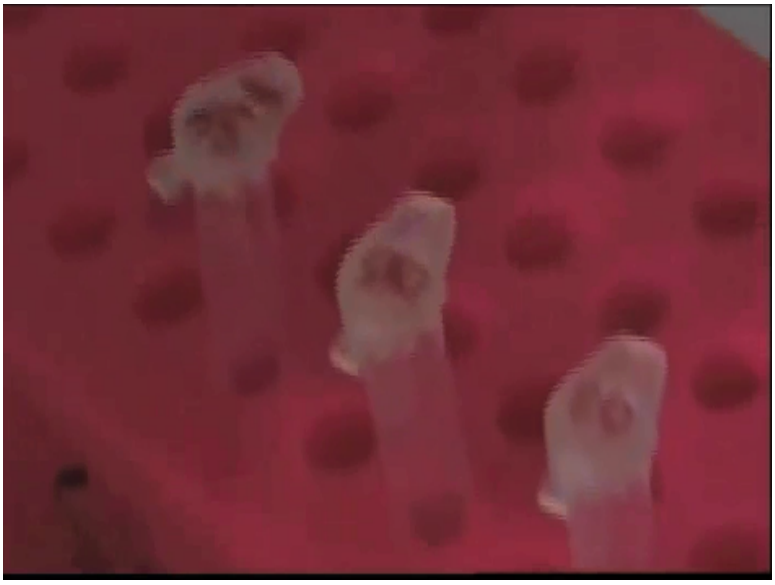

Now label the tubes properly 50 microgram of each sample will be separately labeled with 400 picomolar of Cydyes. Add one micro liter of Cydye in each tube.

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Add one microliter of each Cydye in their respective tubes.

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Here you can see all the 3 Cyedyes in different tubes.

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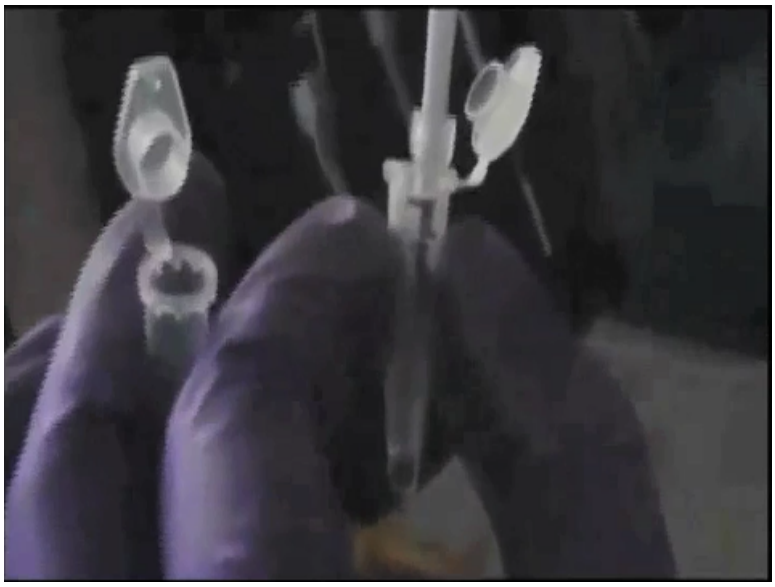

After addition of Cyedyes sample will be incubated on ice for 30 minutes in the dark. Labeling reaction will be stopped by addition of 10 millimolar lysine followed by incubation on ice for additional 10 minutes.

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DIGE gels scanning 2D DIGE gels will be scanned using typhoon variable mode imager at the 100 micromolar resolution and drawing suitable excitation, emission wavelength.

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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 9500 icon, click on 2D DIGE you can see a DIGE window open.

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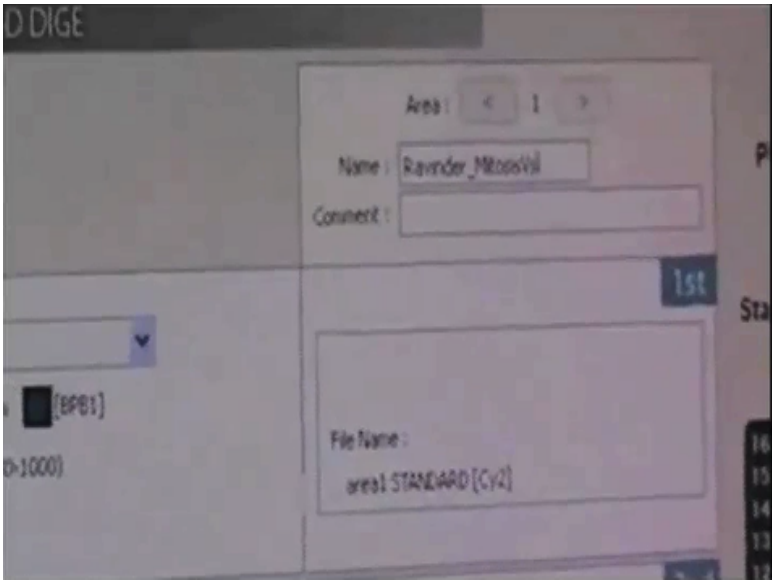

Before starting the scanning name, the file properly.

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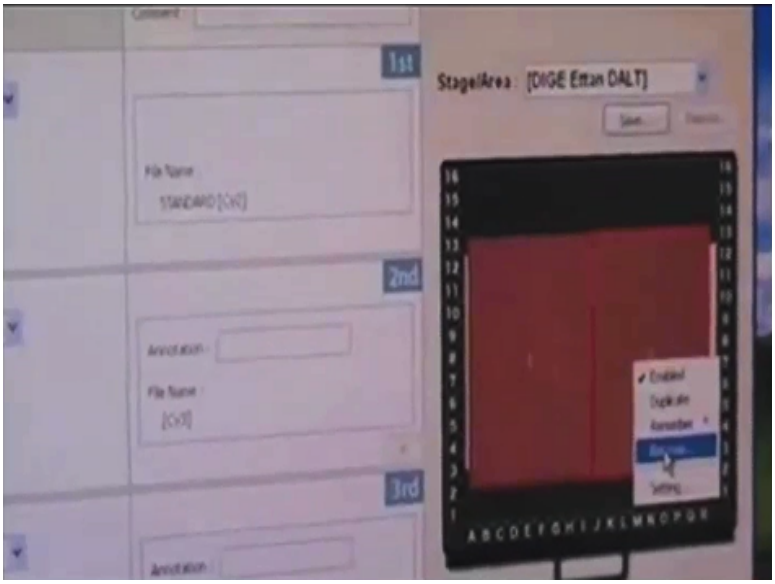

Set area as DIGE Ettan DALT.

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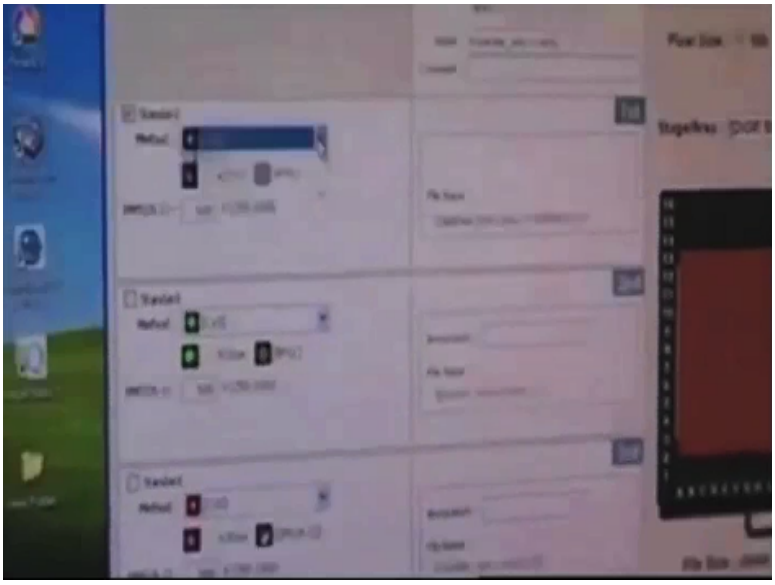

Resolution should be 100 micromolars set standard at Cy2, set the wavelength for each of the Cydye. Excitation wavelength for Cy3 is 523 nanometer Cy5 is 633 nanometer and Cy2 is 488 nanometer. Now by clicking on start scan button we can initiate the scanning process. The scanning will initiate with Cy2 proceeds with Cy3 and will end up with Cy5. You can adjust the contrast and see the gel.

After scanning gel images will be cropped properly using image point software version 5.0. Prior to importing the Decyder the 2D software version 7.0 for comparative analysis for relative protein quantification across disease and control samples.