Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 04 Lecture Number 12

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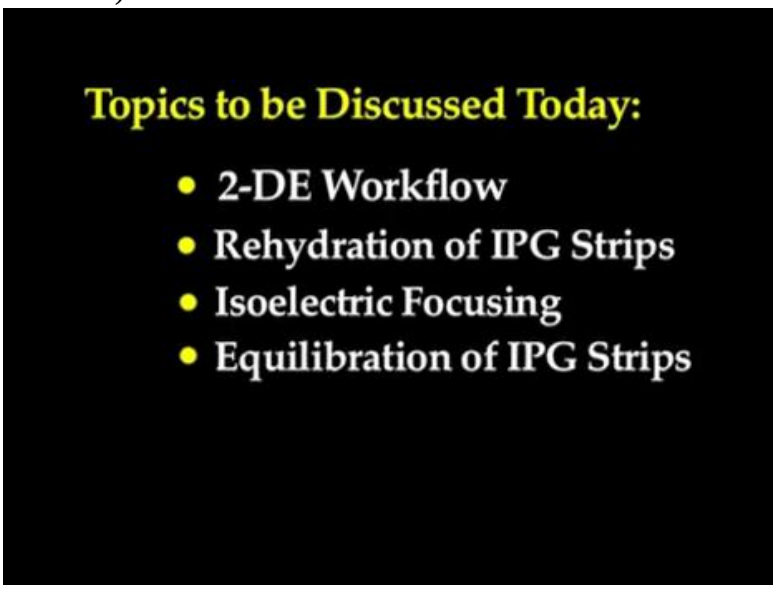

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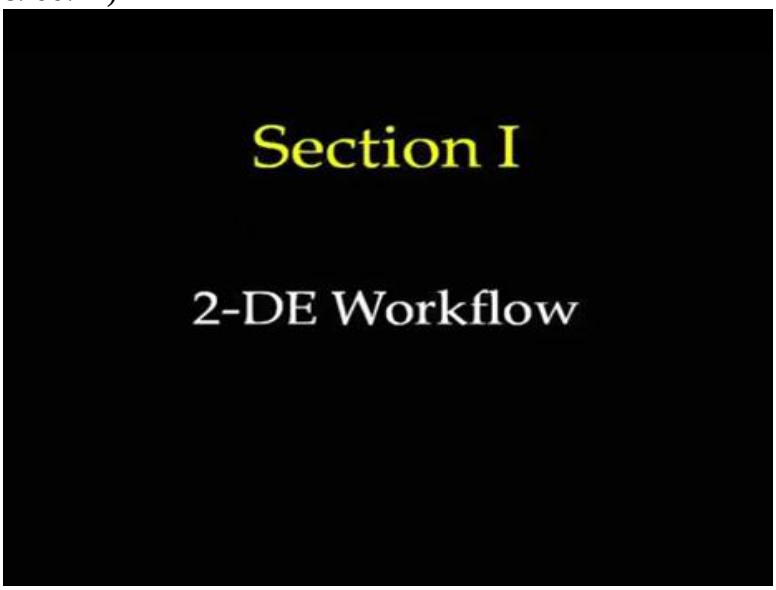

So, now let's go to the detail part of each of the processes involved in studying two-dimensional electrophoresis, the experimental workflow.

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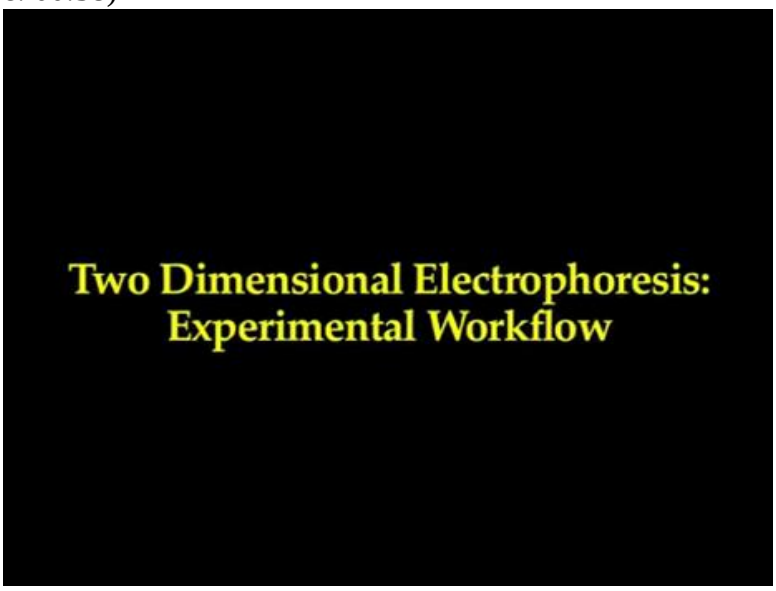

There are different steps which are involved in making a good gel.

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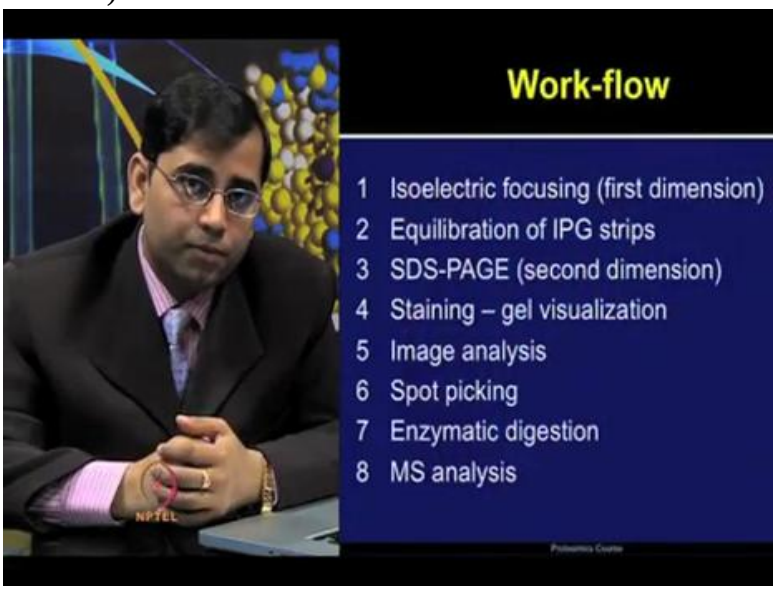

So the first point in that workflow is Isoelectric focusing which is the first dimension separation of the proteins based on Isoelectric Point. Second step is Equilibration of immobilized Ph Gradient Strip. When you are running an SDS PAGE gel, you make your protein denatured. You want to separate that based on the molecular weight.

And heat the sample; you apply different types of denaturants. Now in this case when you are doing 2DE, your first dimension separation has occurred on the IEF in the immobilized Ph Gradient Strip. Now you want to take these strips and want to apply that on SDS PAGE gel, you cannot do the heat treatment.

So you want to make these strips prepared to be separated based on the molecular weight in the second dimension, and that is where the equilibration process is useful. Once you have done the equilibration of the strip, then protein can be further separated with SDS PAGE, the third point.

Now fourth in the workflow is staining the gels and visualizing the gel images after staining with different types of staining. Image analysis, the fifth point, is very crucial because you need to analyze the all the protein spots, so that the abundance of each of these proteins can be calculated and more detailed statistical analysis can be performed.

Once you are confident that these are the proteins which are highly significant, then you would like to pick those spots by process's sixth point which is spot picking. After that if you want to identify and know that what this protein is, then you need to do the in-gel digestion or enzymatic digestion of these proteins obtained from the gels.

Then the last point will be the mass spectrometry analysis, which will be different series of lectures in the other module based on the mass spectrometry.

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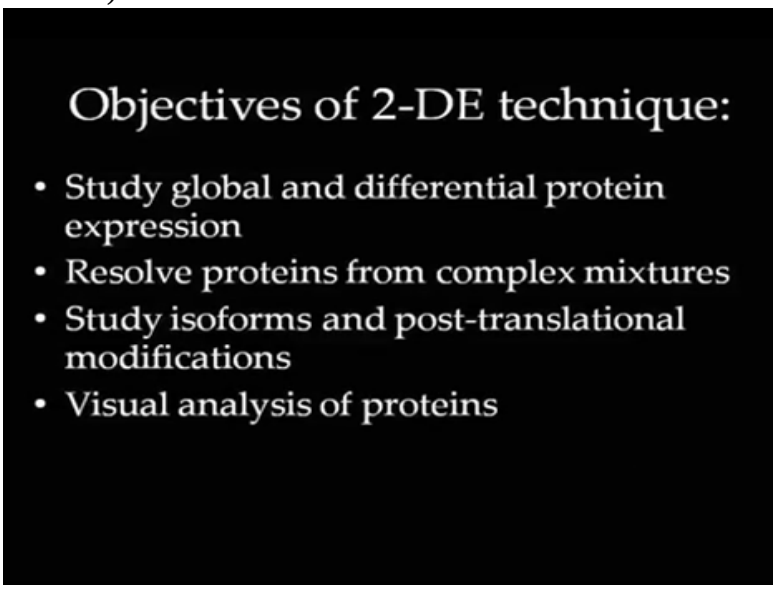

So I will now show you an animation for two-dimensional electrophoresis which will give you an overview of different steps involved in 2DE experiment.

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So in this animation, I will describe you the two-dimensional electrophoresis process.

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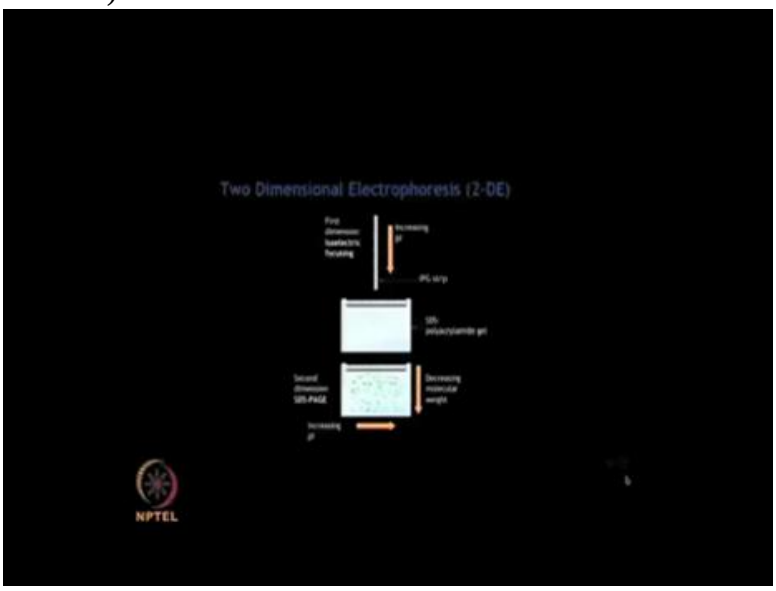

Prior to Isoelectric Focusing in two-dimensional electrophoresis…

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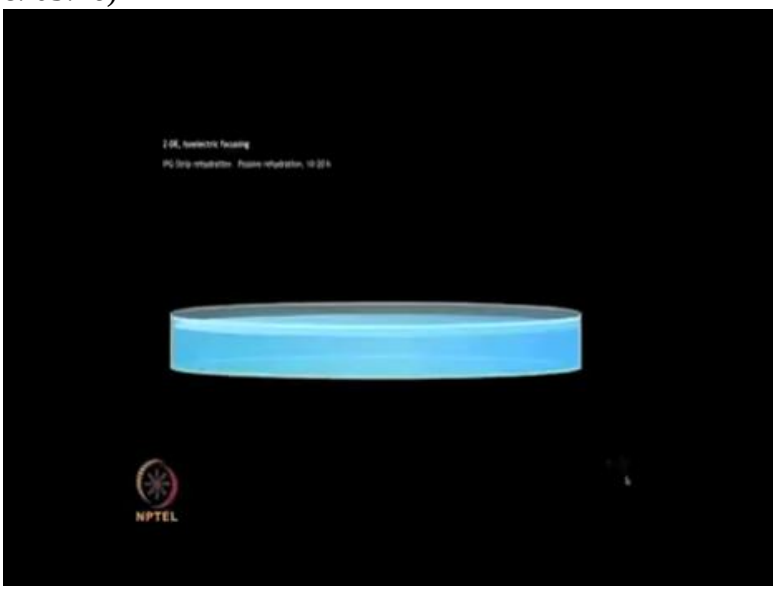

…the commercially available IPG Strip must be rehydrated. This process can be done either by the passive rehydration or active rehydration.

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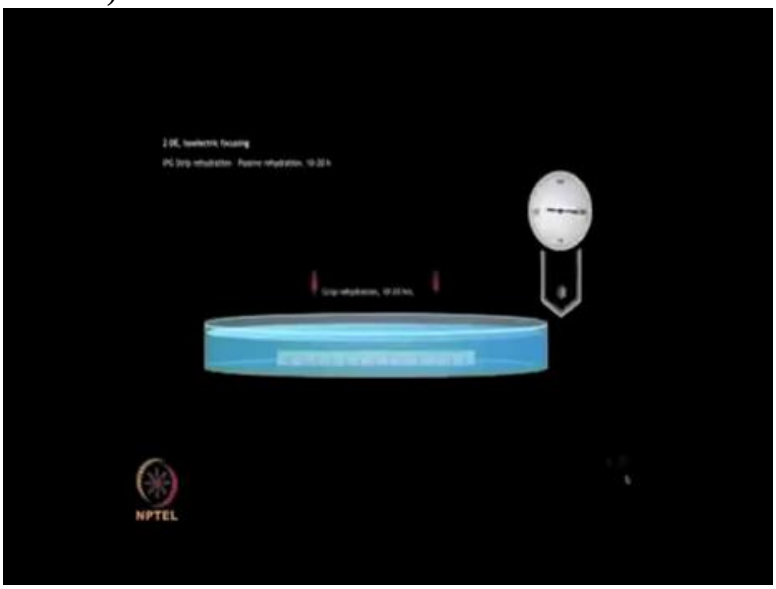

In passive rehydration, the IPG strip is placed with its gel side downwards in a well containing the protein sample reconstituted with suitable buffer.

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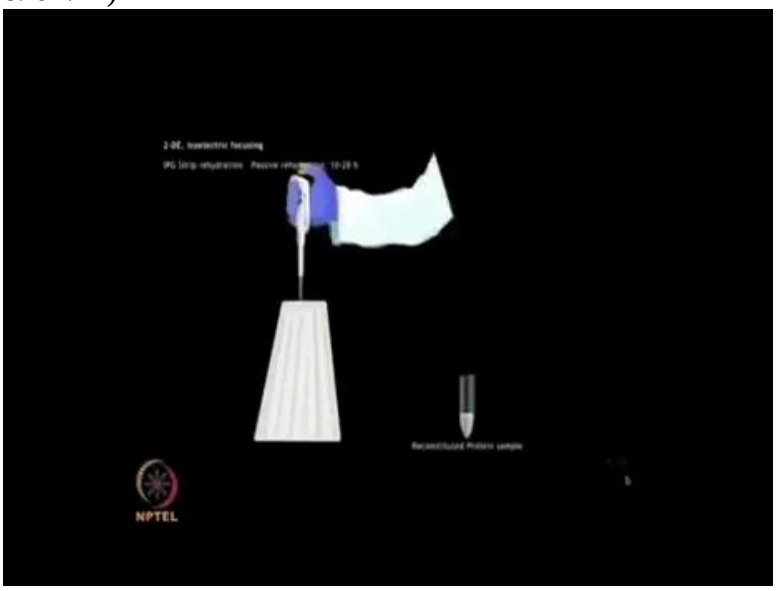

As you can see in the animation, you can apply the protein sample…

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… and then add the IPG Strip, so that it can absorb the protein solution. This process can be done for 10 to 20 hours depending upon your length of the IPG Strip.

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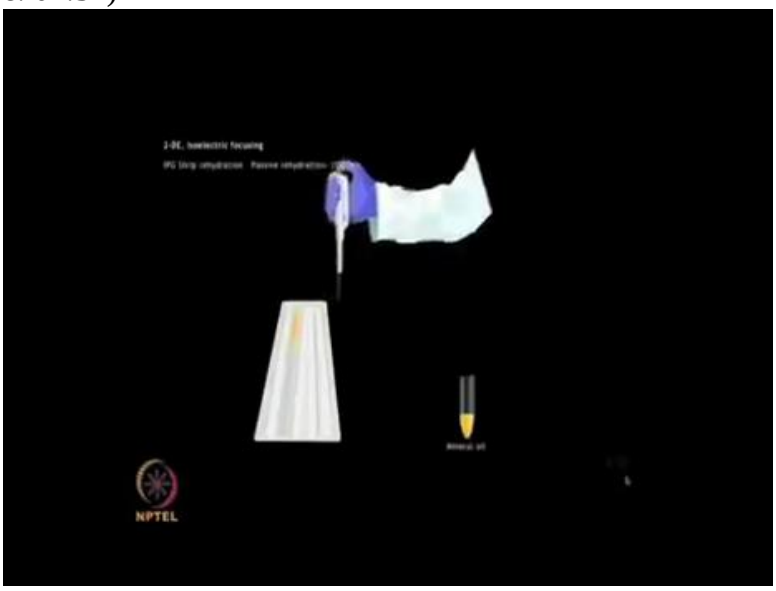

This is then covered with mineral oil to

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…. prevent the gel from drying up…

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... and left overnight.

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Other process known as active rehydration….

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… where the protein sample is added to the strip via a sample cup…

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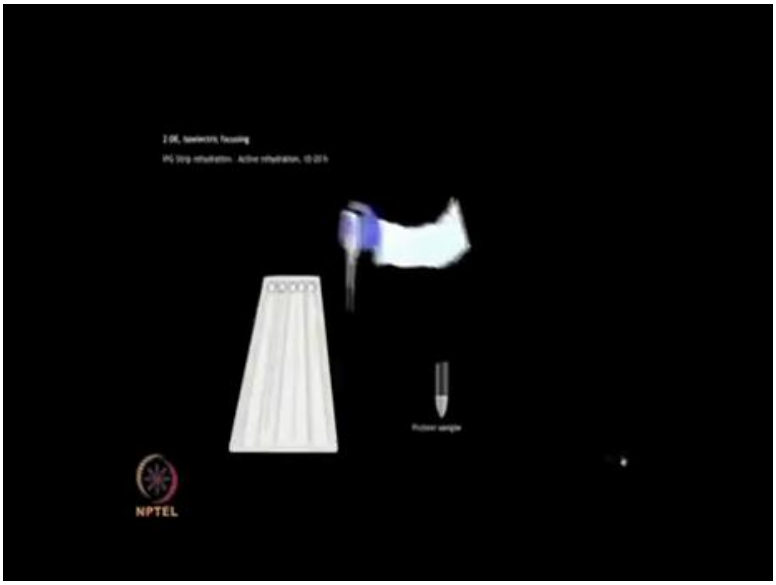

…followed by the cover fluid to prevent the gel from drying up The protein sample being applied, a very low voltage condition is provided…

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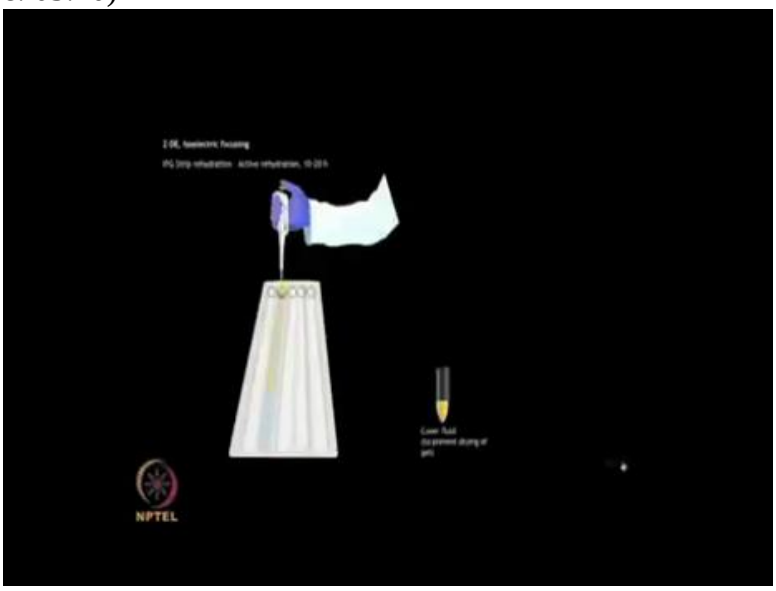

…and then cover fluid can be added.

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This is then placed….

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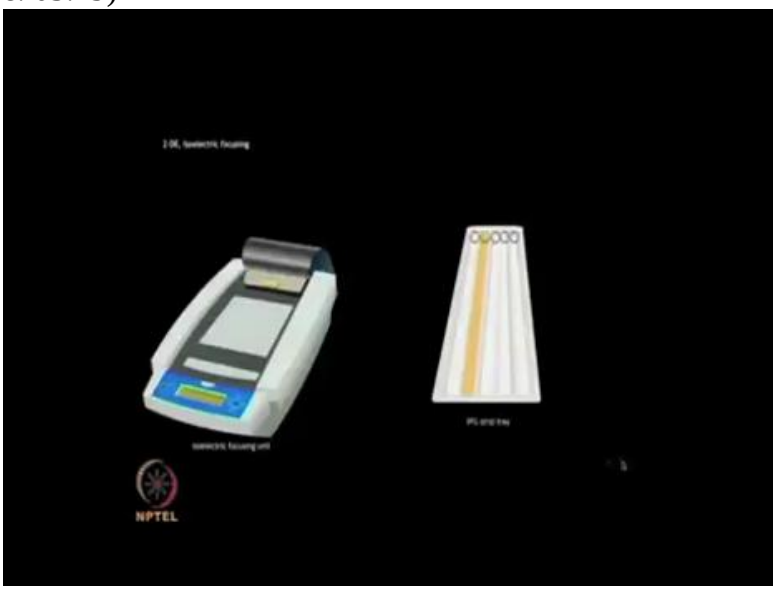

…in the Isoelectric Focusing Instrument and low voltage is applied.

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Process can be performed from 10 to 20 hours.

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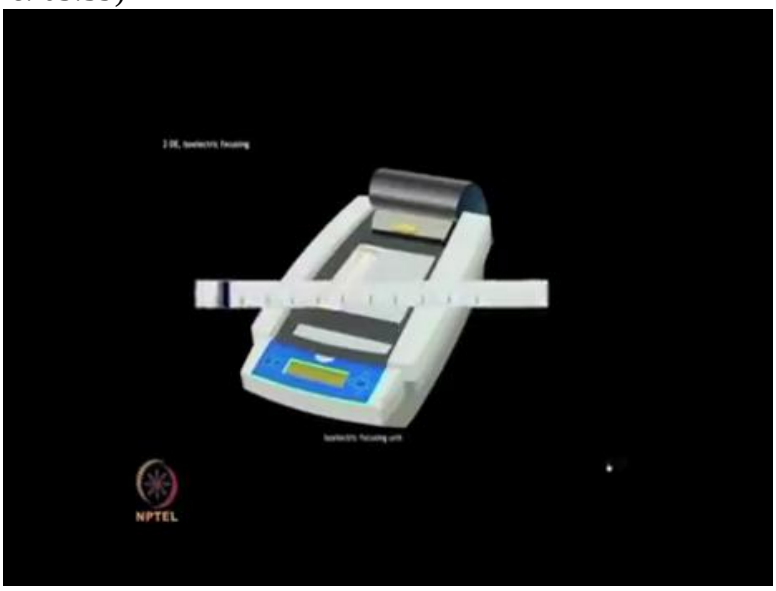

These loaded strips are then focused on an Isoelectric Focusing Unit by passing the current.

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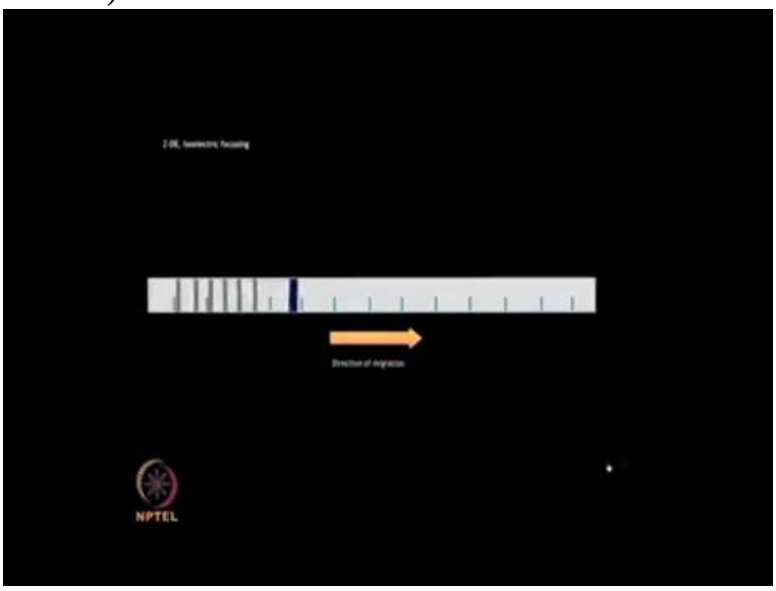

The various proteins of the sample mixture migrate in the electric field…

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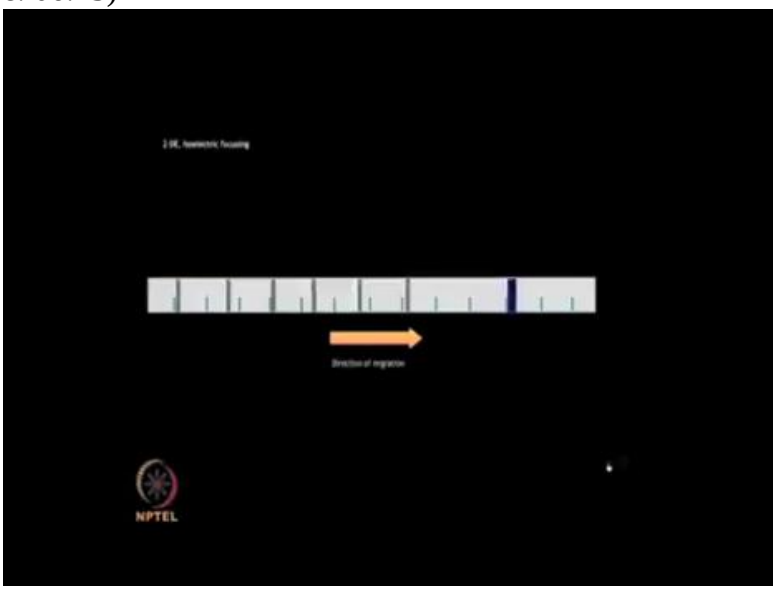

…and come to rest when the pH is equal to their isoelectric point or pI. So they become neutral and are no longer being affected by the electric field.

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The progress of electrophoresis is monitored by adding a tracking dye which you can see in the animation moving ahead of the proteins.

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The IPG strip is then equilibrated in a reducing agent such as DTT…

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…followed by an alkylating agent iodoacetamide…

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… which prevents re-formation of reduced bonds.

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This strip containing the separated proteins is then placed on SDS PAGE gel slab for further protein separation in the second dimension based …

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… on the molecular weight

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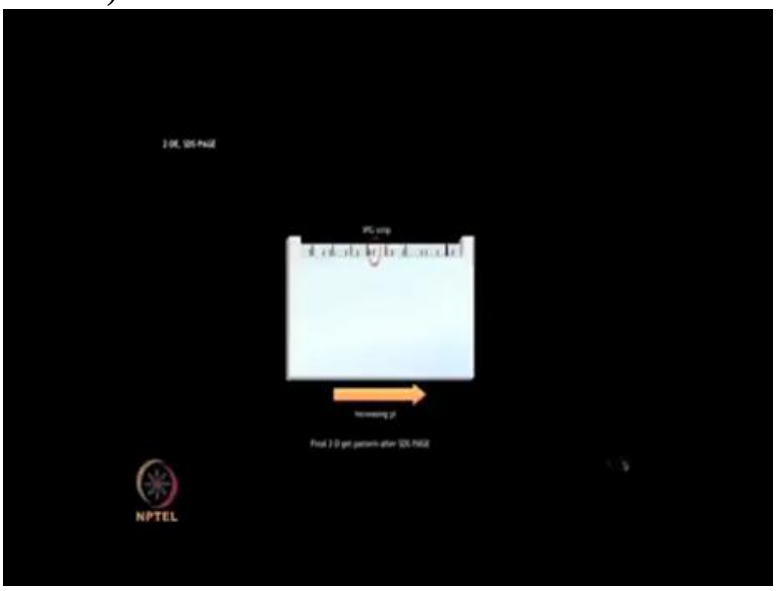

The proteins on the IPG strip are subjected to SDS PAGE by applying the direct current between 100 to 350 Volts depending upon the size of the gel. Any protein that may have been present as a single band on the IPG Strip, as you can see in the red circle, due to similar Isoelectric Point…

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…can then be separated based on the molecular weight…

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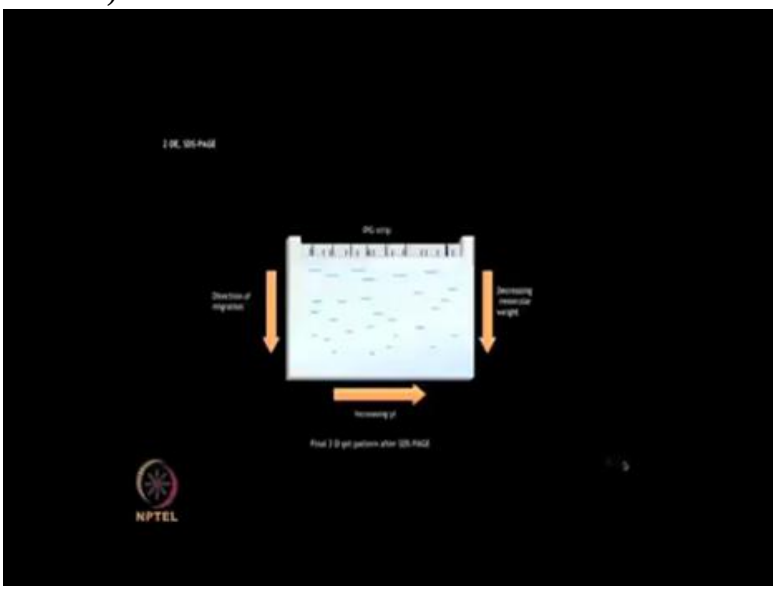

… where smaller proteins will migrate the farthest.

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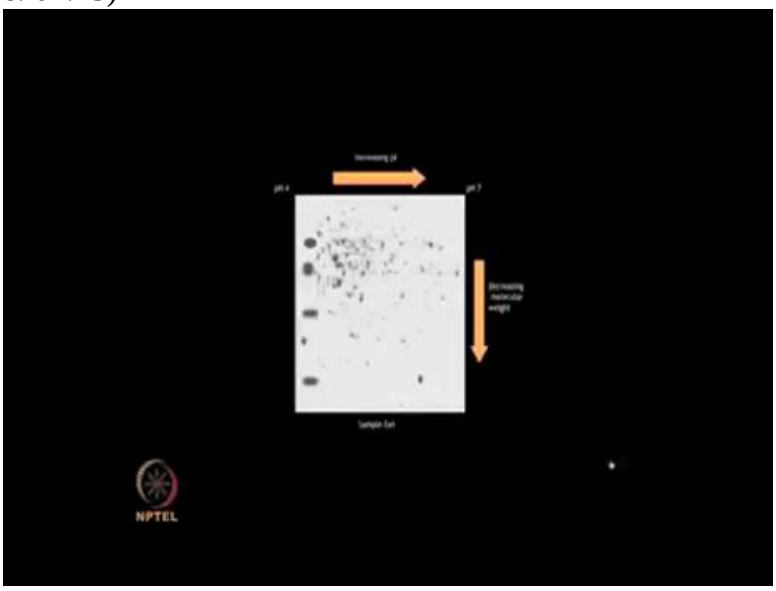

This is view of a sample gel which has been run by two-dimensional electrophoresis and stained with Coomassie blue; each spot provides the information about the molecular weight and Isoelectric point of the protein.

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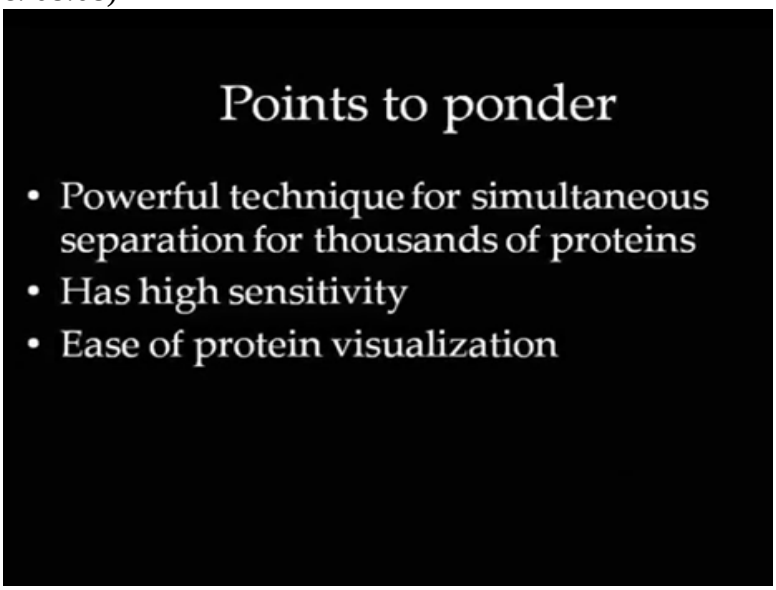

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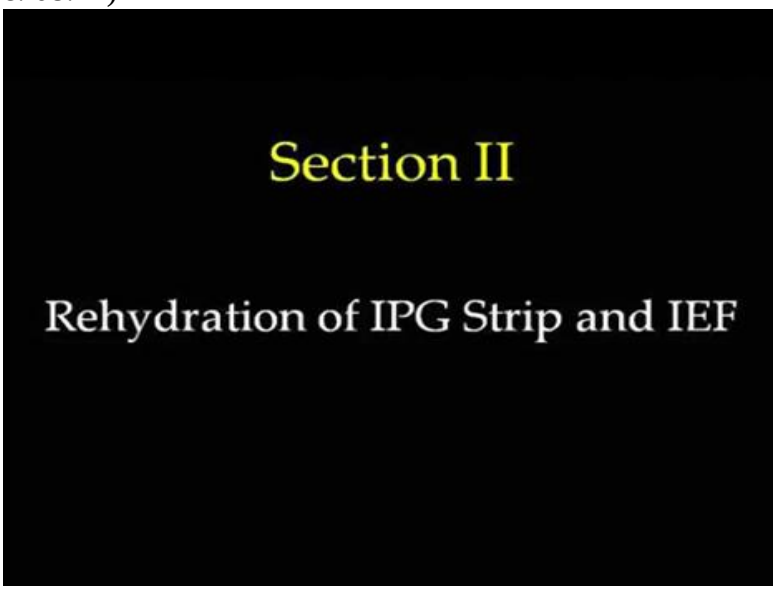

So let's talk about IsoElectric Focusing or IEF. In the IEF process, proteins are separated according to the Isoelectric Point.

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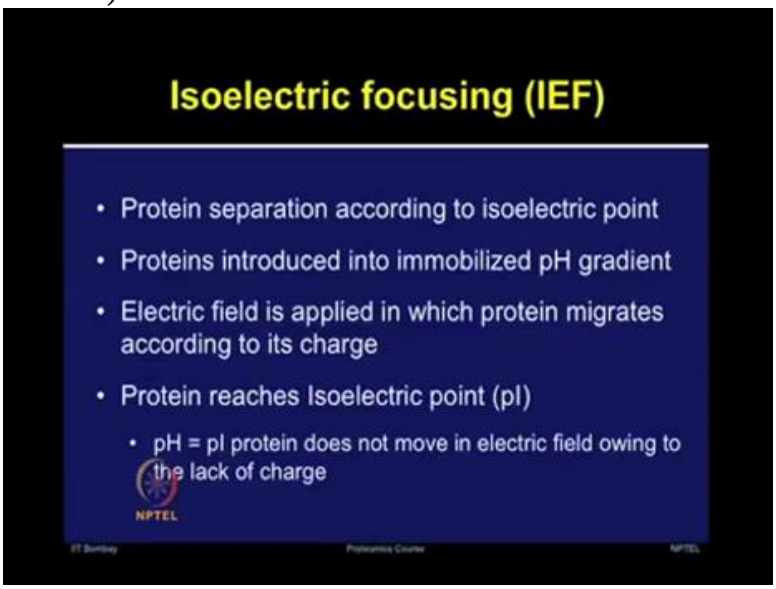

You can take the Immobilized pH Gradient Strip and apply the protein solution on the IPG strip so that protein is absorbed on this strip. And then after applying the electric field, the proteins will migrate according to its charge.

When protein is reaching to its Isoelectric Point where pH becomes equal to Isoelectric Point or pI, then proteins do not move in the electric field because of lack of charge. So when pH equals to pI, then protein will not move any further.

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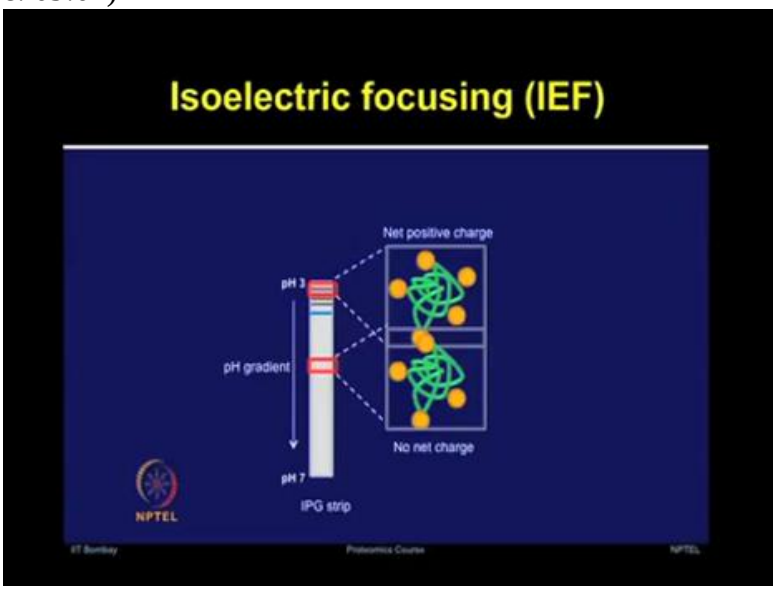

Let me show this process in this animation here. You have an IPG Strip with pH 3 to 7 Gradient. When you are moving in the electric field from the positive charge towards the negative charge, when pH becomes equal to its pI then there is no net charge. And protein will remain over there. So this will be the Isoelectric Point.

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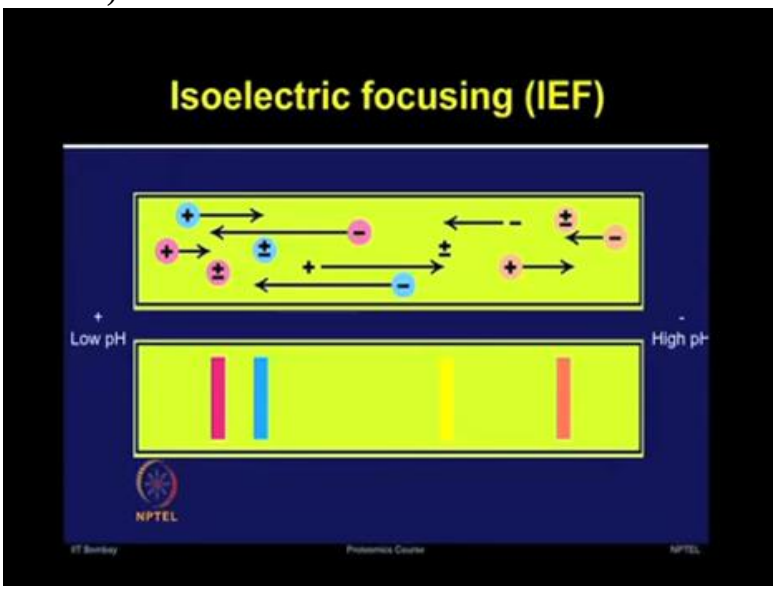

And this view you can see from the low pH to the high pH, proteins are moving in the electric field and once they reach to a stage where there is no net charge or pH becomes equal to their Isoelectric point pI, then these discrete bands can be seen.

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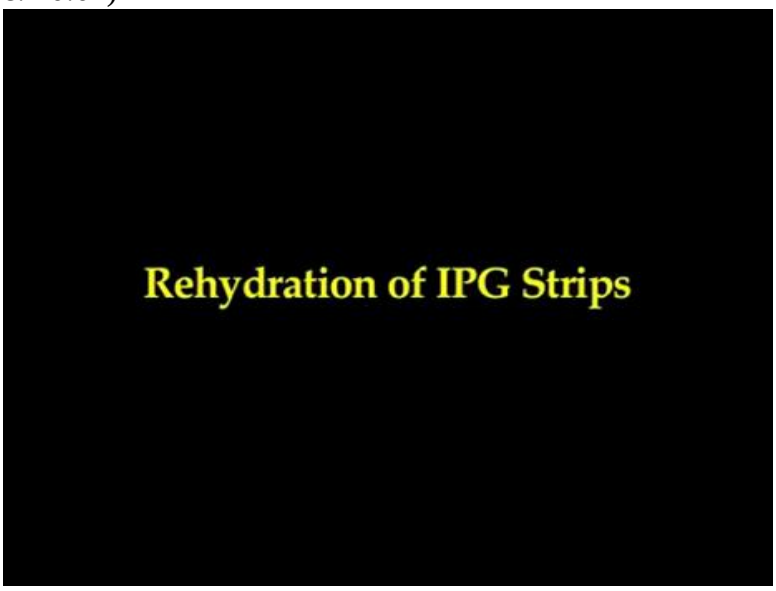

So the rehydration is an important step before applying the electric field in the IEF process.

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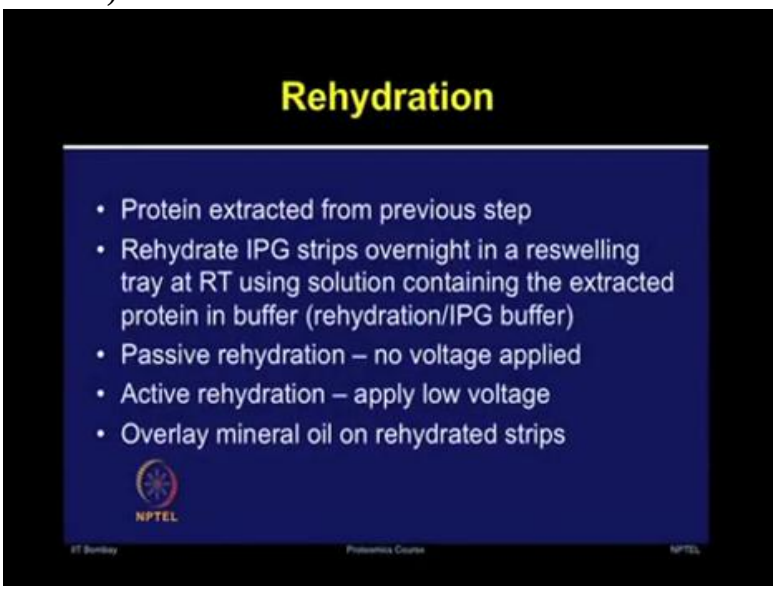

As you have seen in the previous animation that, to rehydrate the IPG strip, you have to apply the protein solution on the IPG strip and different methods can be used for this, whether passive rehydration where no voltage is applied or active rehydration where you apply the protein sample and very low voltage is applied to resolve the proteins.

Now we have already discussed that none of these methods can be compared and said as a superior method, one has to really use that in their own biological sample and see where the protein separation can be better, based on whether passive rehydration or active rehydration.

Once you have done the rehydration process, then one can apply the mineral oil to avoid any protein evaporation from these IPG strips.

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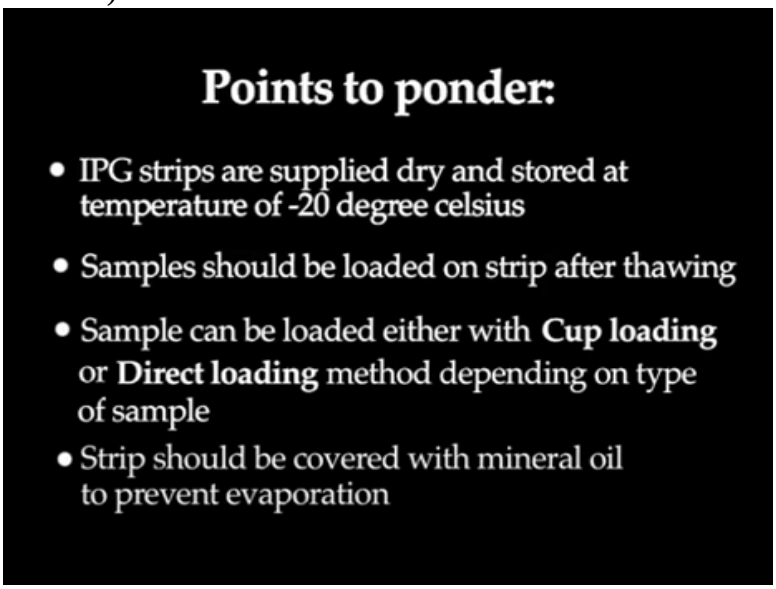

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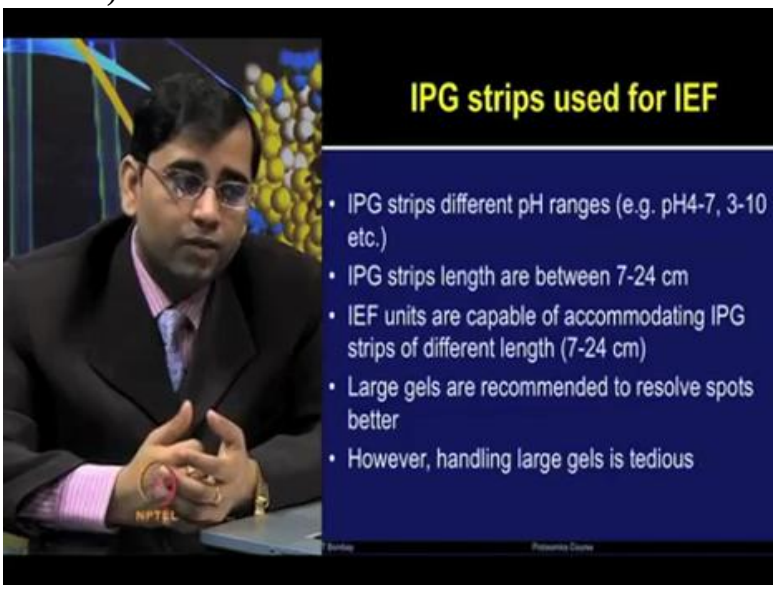

Now different types of strips can be used during the IEF process, whether it is pH gradient of 4 to 7, or 3 to 10 or some other pH range.

As I described in my previous slide, depending upon your biological question, if you are mainly interested in separating the proteins in their biological pH range, then 4 to 7 could be a good choice where you can have better separation of the protein.

But you may lose few proteins which could be in the extreme region of acidic and basic. Now if you want to know all the proteins which could be present in your sample, you can apply a very broad range strip like 3 to 10.

Now what type of length of IPG Strip should be used? One can use, starting from 7 centimeters till 24 centimeters. There are different types of strips being manufactured from different commercial companies.

Now when you are optimizing a protein extraction protocol, it is better if you use a smaller strip so that you know that your IEF process is going fine and your protocol is looking good. You can separate the protein. Now once you know that your extract is good then you can apply that to separate the proteins in the long strip length, for example 17 or 24 centimeters.

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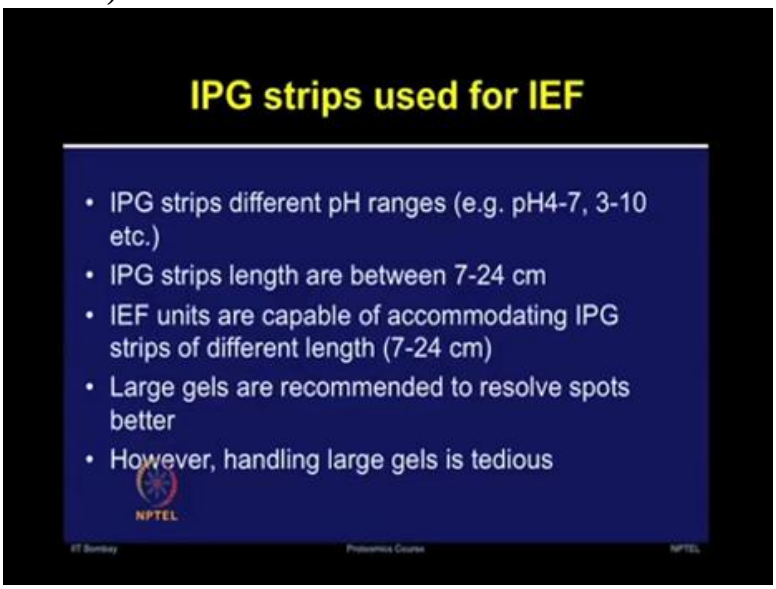

Now these IEF units or Isoelectric Focusing Units obtained from different vendors, commercial manufacturers, they are capable of taking the IPG strips of different varying pH length.

Few instruments take a flexible length from 7 to 24 centimeters, other commercial instruments; they are able to take different types of trays designed for each type of strip length.

Now depending on your proteins, how much proteins sample you have in your protein sample mixture, it is always better or good idea to resolve that on the very large gel, for example 18 or 24 should be a better choice. But if you do not have enough protein to separate, then you have to restrict yourself to smaller strips.

Or one can still go with the large strip but apply different, more sensitive stain. So when we talk about different types of staining methods then we will talk of...even if you have low protein, you can separate on the large gels but then apply more sensitive stain such as silver or SYPRO Ruby.

But overall, the large gels are recommended for doing any type of differential proteomic analysis when you want to compare your controls and treatment spots. But handling the large gel is very tedious. So I will show you some lab demonstration in the video for that so that you can be well-prepared to perform these experiments in your labs.

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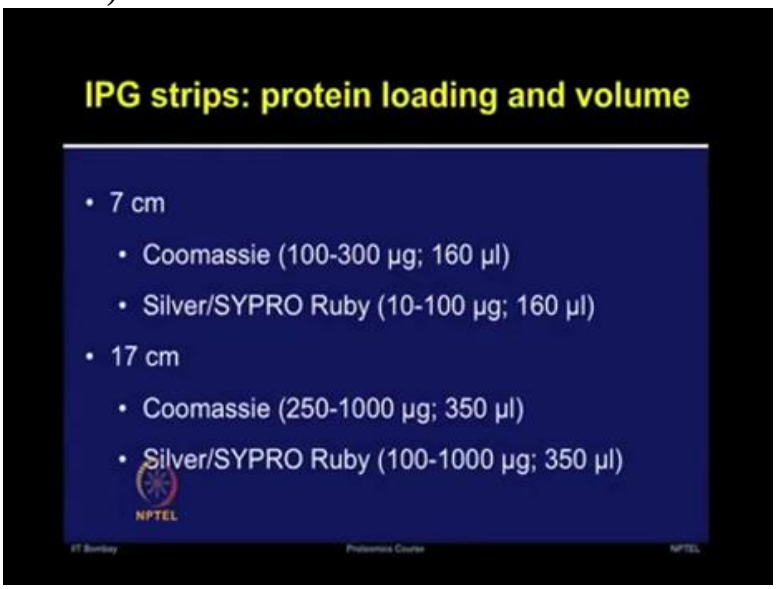

These are some recommendations for how much proteins one should load on the IPG strips. For example, if you have small strip like 7 centimeter length, you can apply between 100 to 300 microgram of protein and maximum volume could be 150 to 160 micro liters. Usually Coomassie stain is a good choice and if you have Coomassie stain, you can stain that with this much protein.

If your protein sample is having very less amount of protein, for example 10 to 100 microgram, at that time, you have to apply the sensitive stain such as silver or SYPRO Ruby. One can also apply the large strip such as 17 centimeter or the 24 centimeter strip but then you have to increase the protein amount to be loaded and separated on the IPG strip.

For example between 250 and 1000 micro gram of protein can be loaded on the large strip and one can visualize that by using the Coomassie stain. Total reaction volume should not be more than 350 micro liters.

But if you do not have too much proteins present in the sample, then still you can apply these methods. You can apply the large strip but then you have to stain with silver or SYPRO Ruby. In that case, you can load between 100 and 1000 micro grams of protein.

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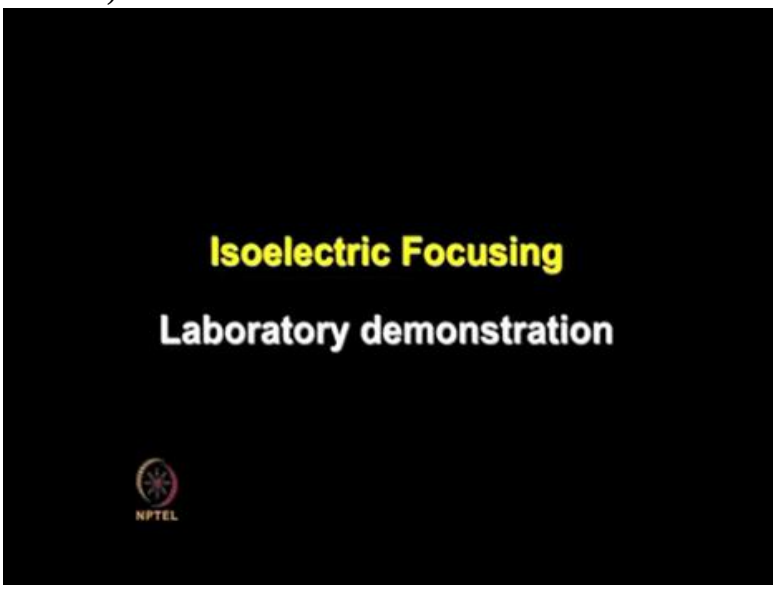

So I will give you the laboratory demonstration of IsoElectric Focusing so that you can be very clear about different intricate steps involved in performing IEF.

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Isoelectric Focusing

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This process involves two major steps; Rehydration of the IPG strips and focusing of the rehydrated strips

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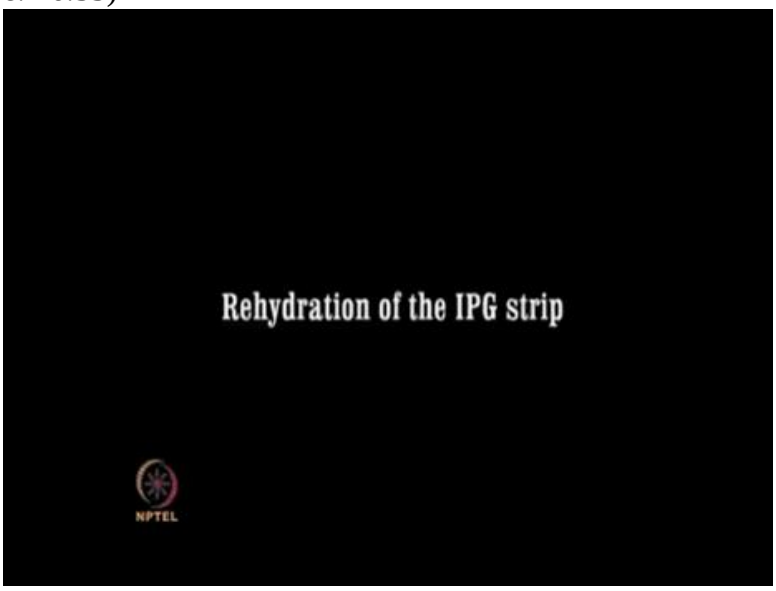

IPG strip rehydration

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Clean all the apparatus thoroughly before use to avoid any contamination. Add the reconstituted protein sample in a well of the rehydration tray.

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Then remove the IPG Strip from its cover and place it carefully in the well.

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IPG strips are used for separation of proteins based on their Isoelectric Point.

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These are Acrylamide coated plastic strips containing immobilization of various pH spread across it. They have successfully replaced conventional tube gels due to their reproducibility they can achieve.

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After around 30 minutes…

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… pour some mineral oil over the strip to prevent it from drying up.

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Cover the tray and leave it overnight to allow rehydration to occur.

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Isoelectric focusing

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Initialize the instrument and clean the surface well with a dust free cloth.

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Place the focusing tray on the instrument and…

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…ensure it is properly balanced.

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Cut the paper wicks required for focusing to a suitable length…

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…and wet them with a small amount of water before use.

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Carefully remove the IPG strip from the rehydration tray and drain out any excess oil by blotting it on a tissue paper.

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Place the strip in the focusing tray and immerse it with mineral oil.

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Then place the two wicks at either end of the IPG strip…

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…followed by an electrode at each end

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Fill all the adjacent wells with mineral oil to ensure uniform current flow.

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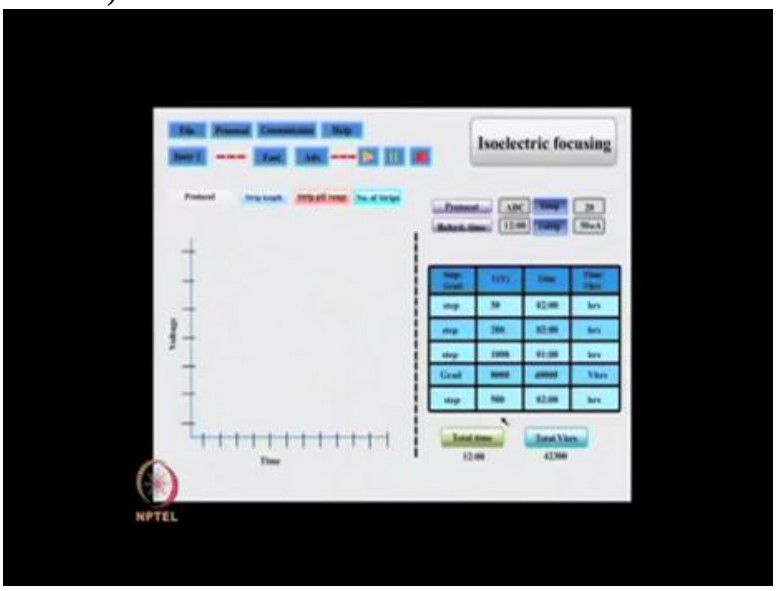

Then input the desired protocol on the instrument software along with details of strip length, pH range and number of strips and start the focusing process.

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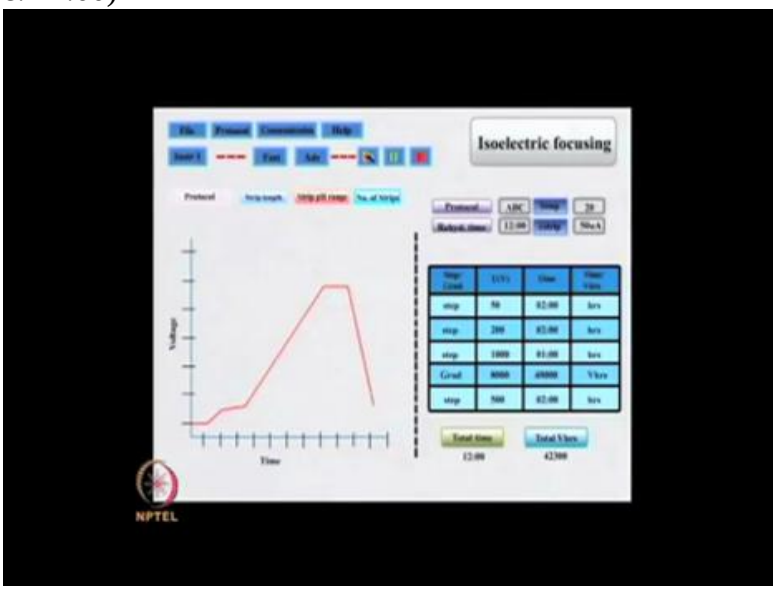

The voltage time curve will appear based on the protocol that has been set.

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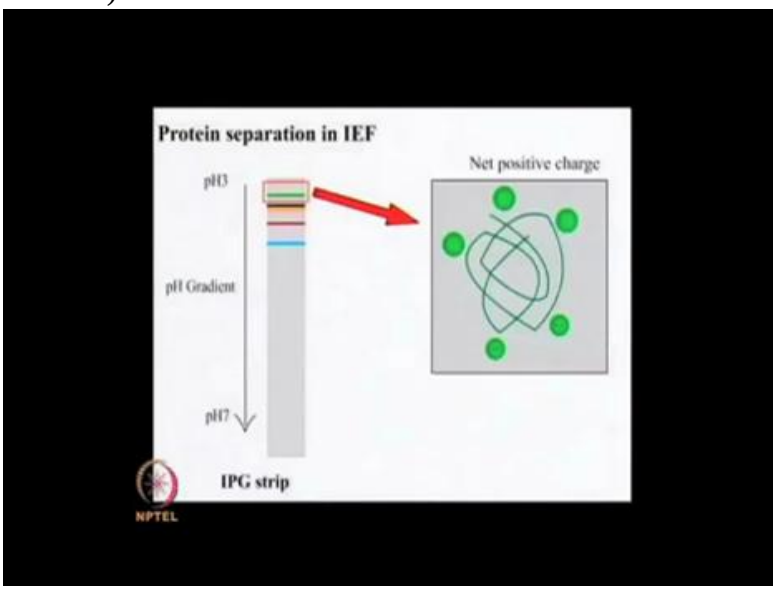

Protein separation occurs on the basis of the net charge of the protein.

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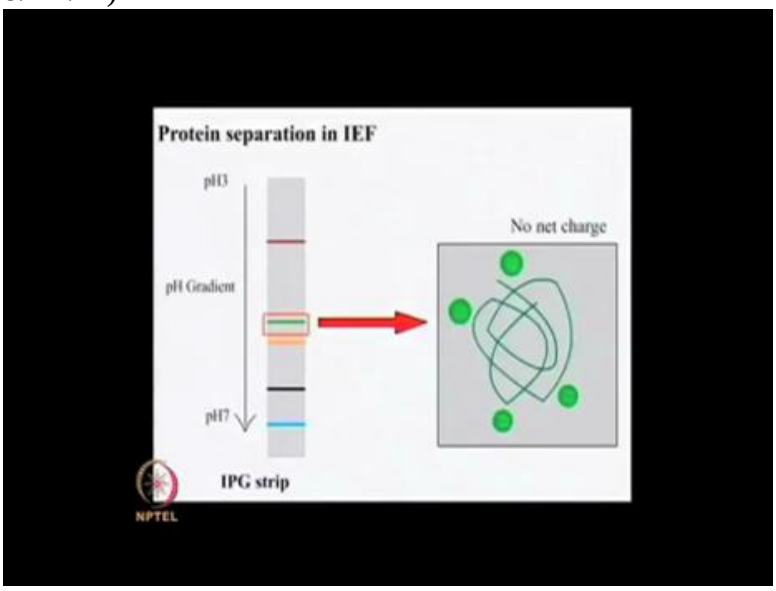

Proteins will migrate along the strip and come to rest at a point when their net charge becomes zero known as their Isoelectric point.

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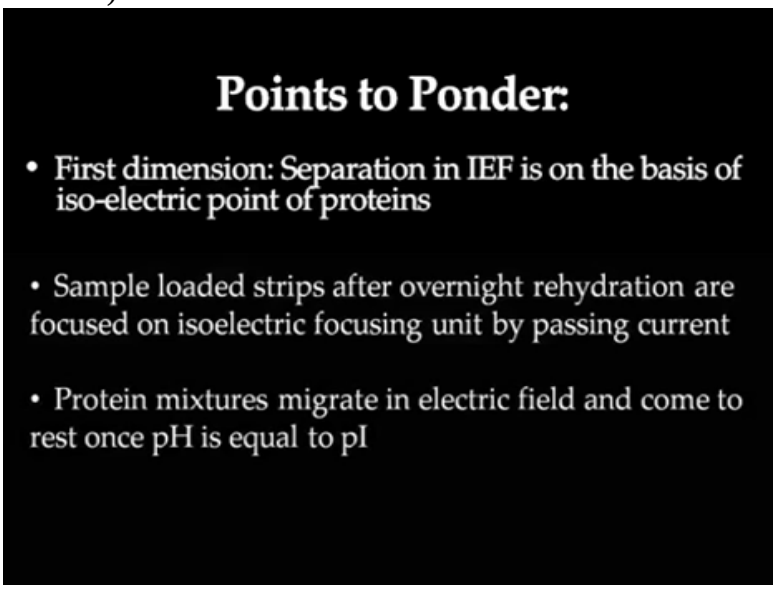

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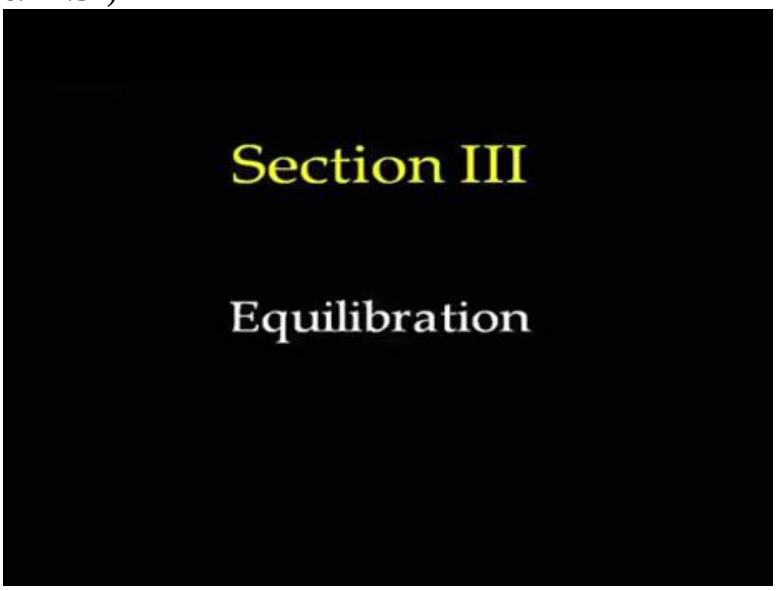

Once you have performed the isoelectric focusing or IEF, you need to prepare the IPG Strip for the next step which is the SDS PAGE.

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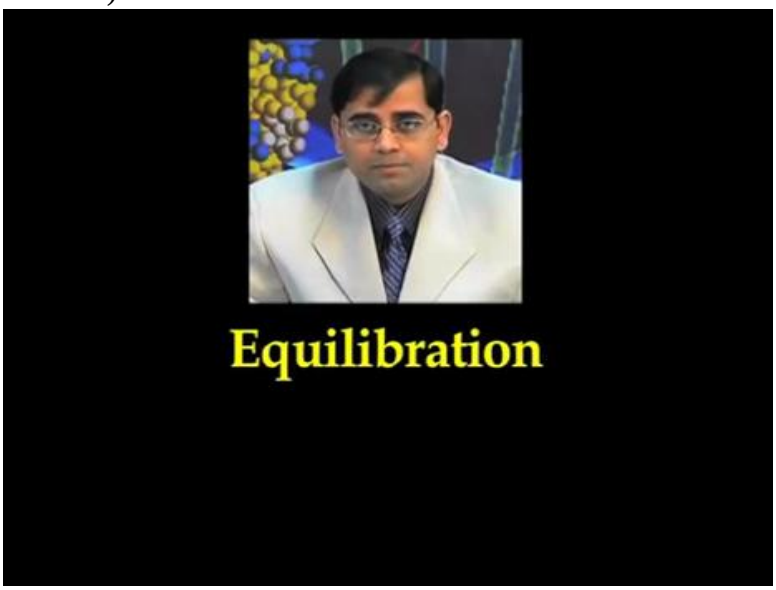

When you are doing the conventional SDS PAGE, you pour the gel, both resolving and stacking and then you want to make some wells by using a comb. And then you load your protein samples in different wells. Now when we talk about two-dimensional electrophoresis, it is different from doing only the SDS PAGE alone.

The concept of SDS PAGE will be involved which will be same but here there will be variation. You do not need to add the stacking gel. You need to prepare your protein sample which is already focused inside the strip in the first dimension.

Now this IPG Strip which was already put in the electrophoretic unit, in the IEF instrument, after that you want to prepare that so that protein can be separated further based on the molecular weight.

Now you need to add SDS to prevent the negative charge And you need to do some treatment for reduction and alkylation. When you are taking the protein sample alone, you have flexibility of boiling the protein sample in the tubes and do all those treatments to denature the protein separately.

But now your protein is already focused inside the IPG strip, so you do not have that flexibility here. So all you need, you need to prepare your IPG Strip in such a way that you can apply that on top of SDS PAGE gel. So I will show you what steps are required in the equilibration step.

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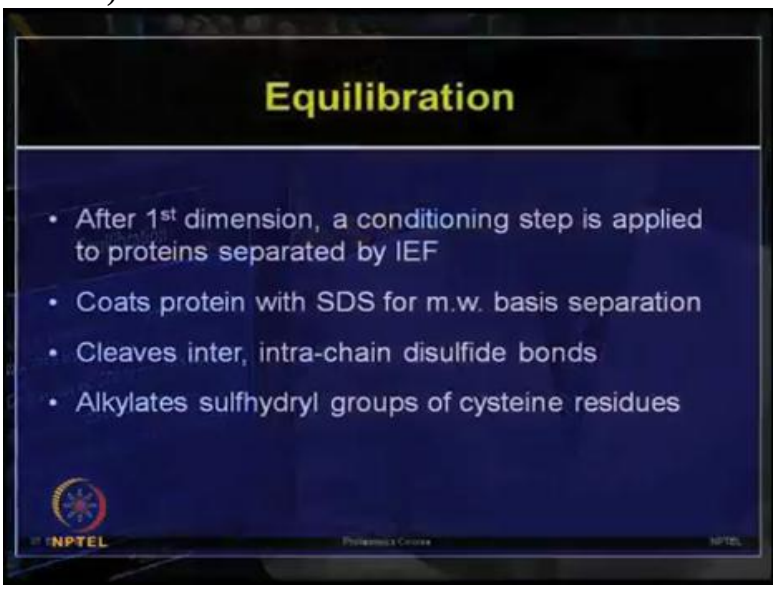

So after the first dimension separation is done, you need to coat the protein with SDS PAGE. For making it compatible with SDS PAGE, so you need to coat with SDS, Sodium Dodecyl Sulphide which is an anionic detergent and it provides negative charge later on for separation in electrophoretic field.

The equilibration step performs cleavage of both inter and intra chains of disulphide bonds. And it alkylates the sulf-hudryl group of the cysteine residues. So there are two equilibration

steps which are required for this preparatory step. The first equilibration step and the second equilibration step.

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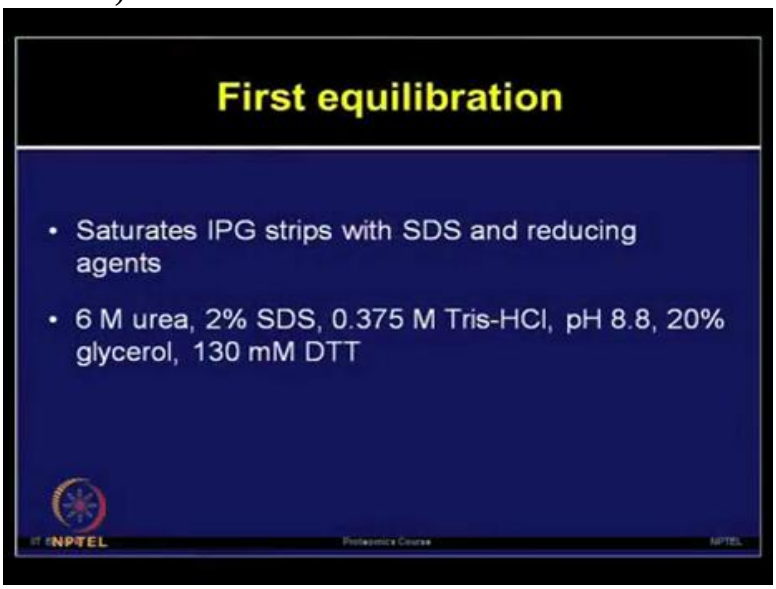

Your recipe for equilibration buffer is ideally similar in both, first and second equilibration which includes 6 Molar of urea, 2% of SDS, 0.375 molar of Tris-HCl pH 8.8, 20% of glycerol and then you need to make one variation in this buffer composition; either you add DTT dithiothreitol or you add IAA iodoacetamide.

Please note this recipe is given only for your reference. People try different slight modification of this concentration and recipe for doing the equilibration step.

Now, as I mentioned, in this buffer you can add DTT and that will be used in first equilibration step. The first equilibration will be performed for 10 or 15 minutes depending upon your strip length. Now once you have done the first equilibration, you need to remove that solution and add a new buffer for second equilibration.

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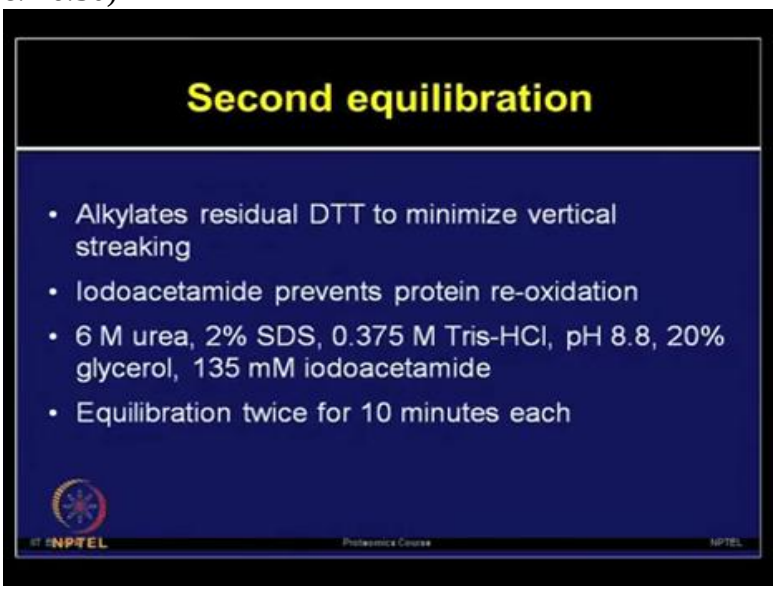

Now this buffer recipe is also exactly similar to the last one except one change, DTT is replaced with iodoacetamide. So you can add 135milli molar of iodoacetamide during the second equilibration step. Now this is very important because IAA can alkyl ate the residual DTT and it minimizes the vertical streaking. It also prevents the re-oxidation.

So you want to really ensure that your protein is denatured again after doing the IEF process, it is coated with SDS molecule so that now you can separate the proteins in the second dimension based on molecular weight. This step is also performed for 10 to 15 minutes depending upon the strip length and once both the strips are done, then you are ready to perform the SDS PAGE.

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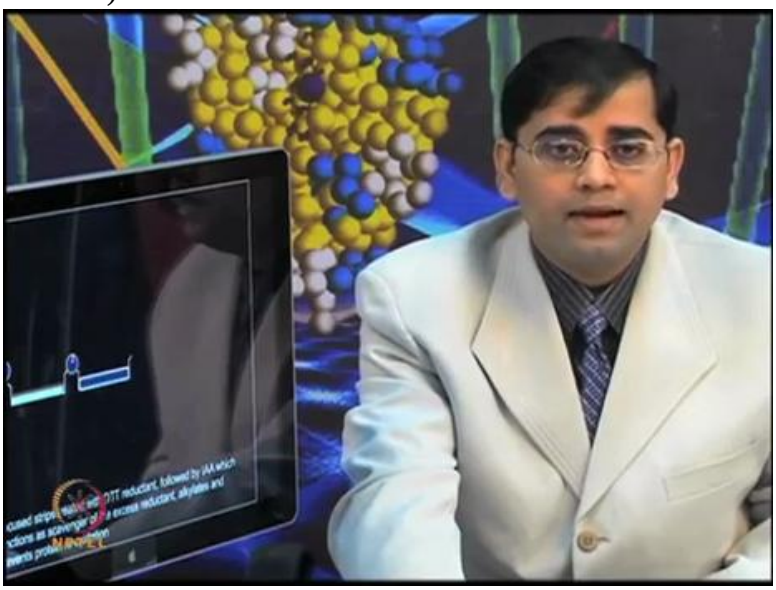

I will show in this slide, the brief stepwise procedure how you can do the first and second equilibration. So first strip can be equilibrated in the first equilibration buffer by adding inside a tray in a well…

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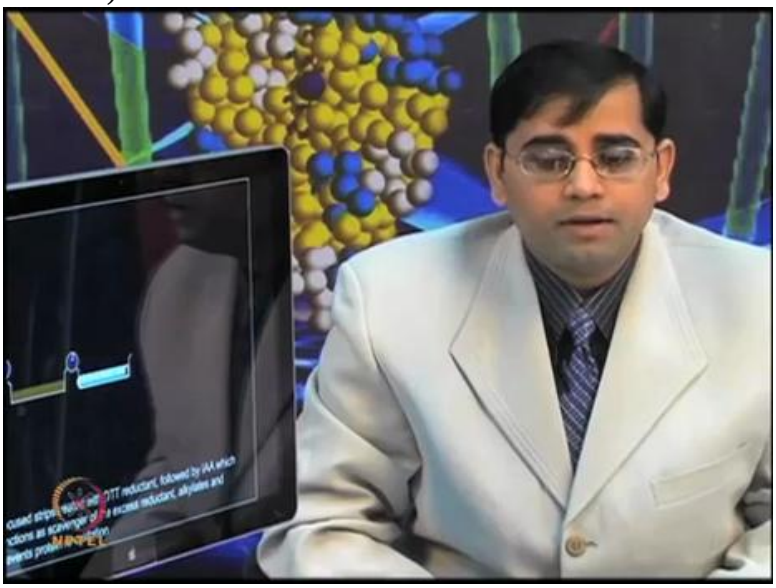

…and then you change that with the second equilibration solution. The DTT reductant will be used for the first strip followed by IAA iodoacetamide which functions as the scavenger of the excess reductant and alkylates and prevents the re-oxidation.

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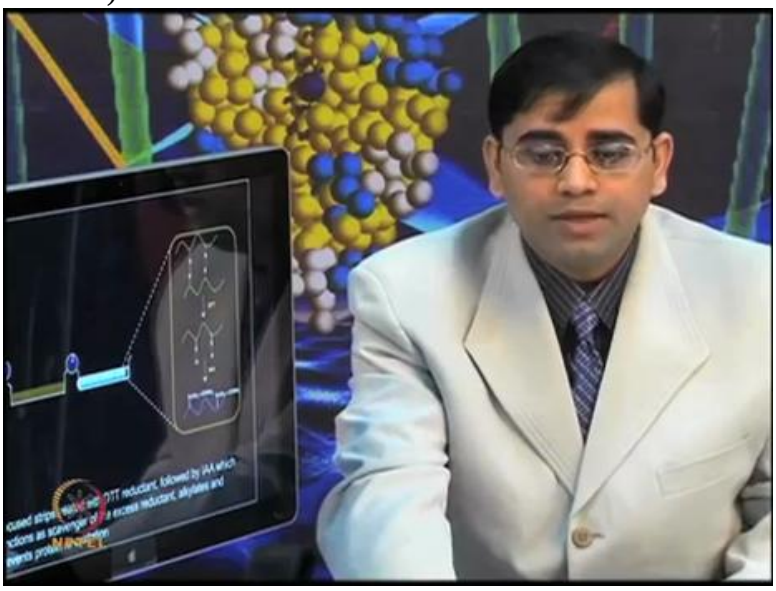

Here you can see how different bonds are broken from the disulfide and IAA is preventing the re-oxidation alkylating these residues.

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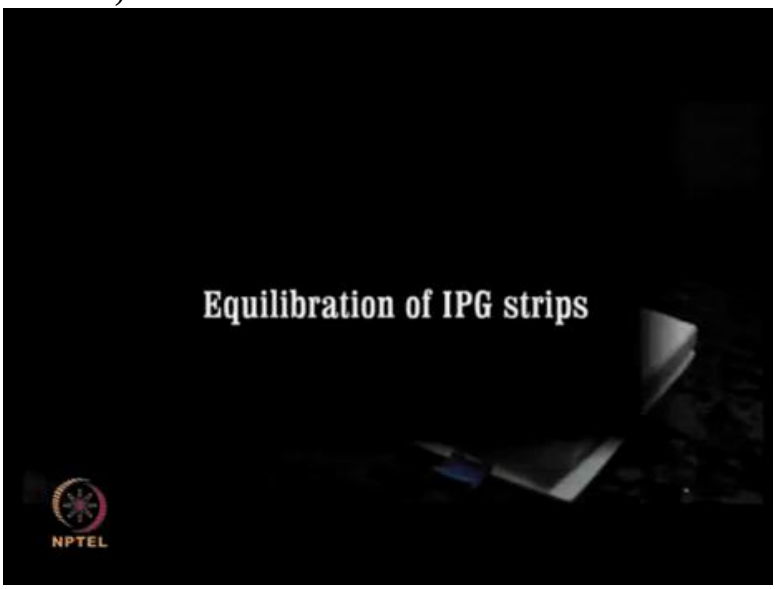

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Place the third IPG strip in the tray and over it; pour the first equilibration solution consisting of…

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…urea, Tris-HCl of pH 8.8, glycerol, SDS, bromophenol blue along with dithiothreitol or DTT.

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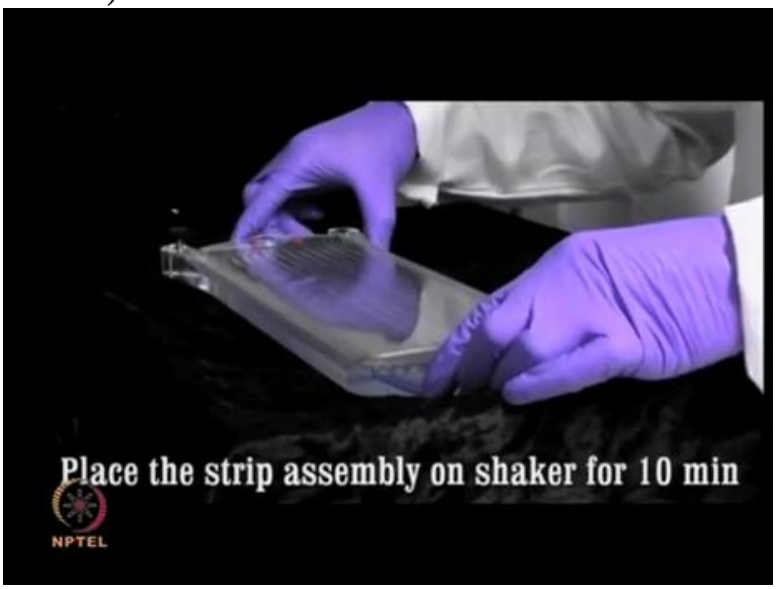

Place the assembly on the mechanical shaker for 10 minutes.

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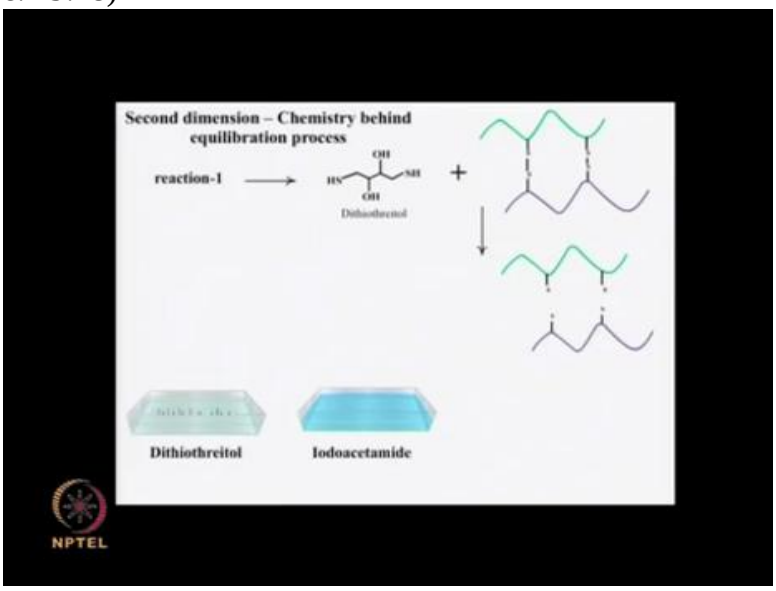

The dithiothreitol enables cleavage of all disulfide bonds present in the proteins in the IPG strip.

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Next, add the second equilibration solution to another well of the tray. This solution has the same composition as the first except that the DTT is replaced by iodoacetamide.

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. Transfer the IPG strip into this solution after draining off any excess liquid from the first.

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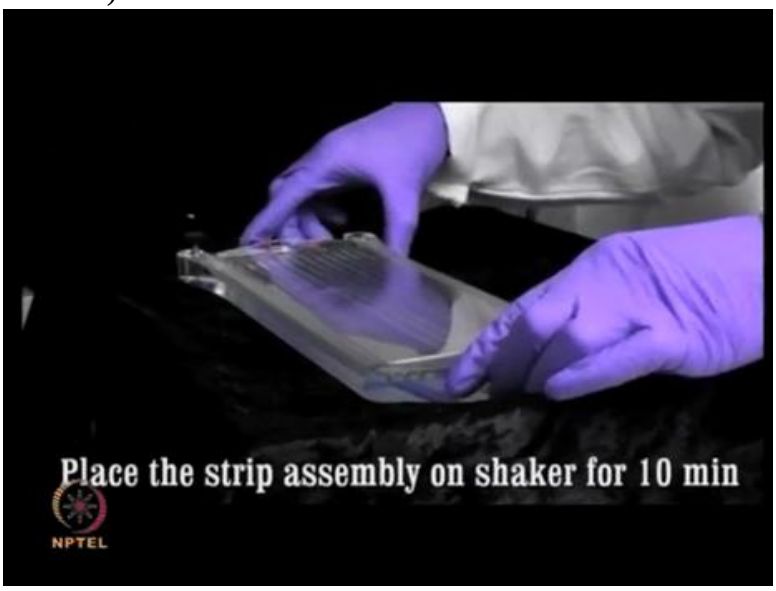

Again place the assembly on the shaker for 10 minutes

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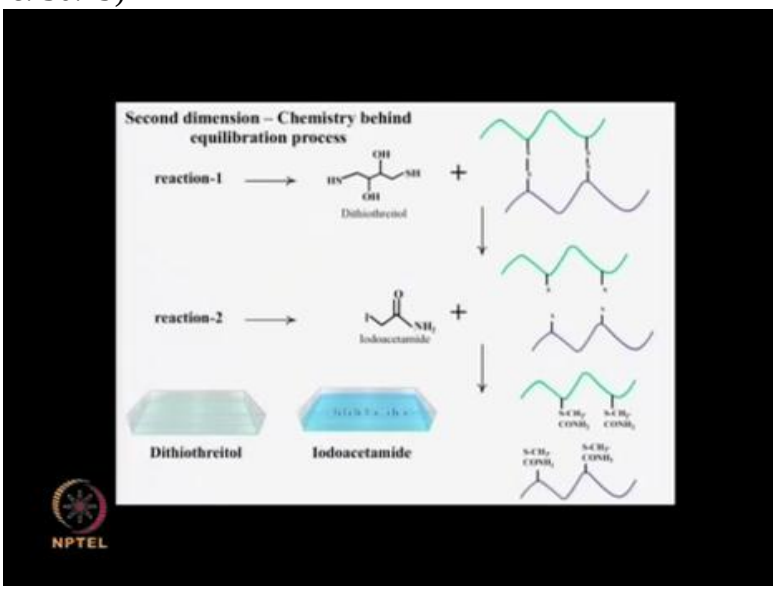

.Iodoacteamide prevents re-formation of the disulfide bonds by binding to the free sulfidryl groups that were obtained after DTT treatment.

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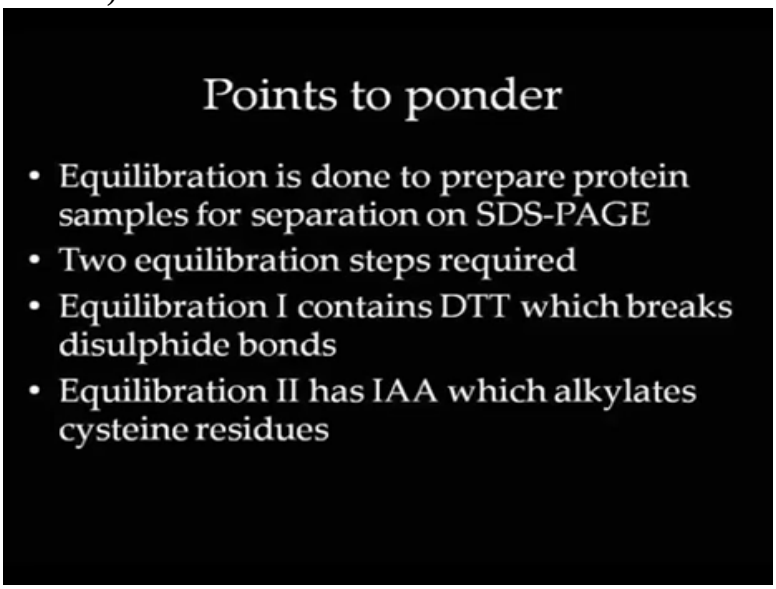

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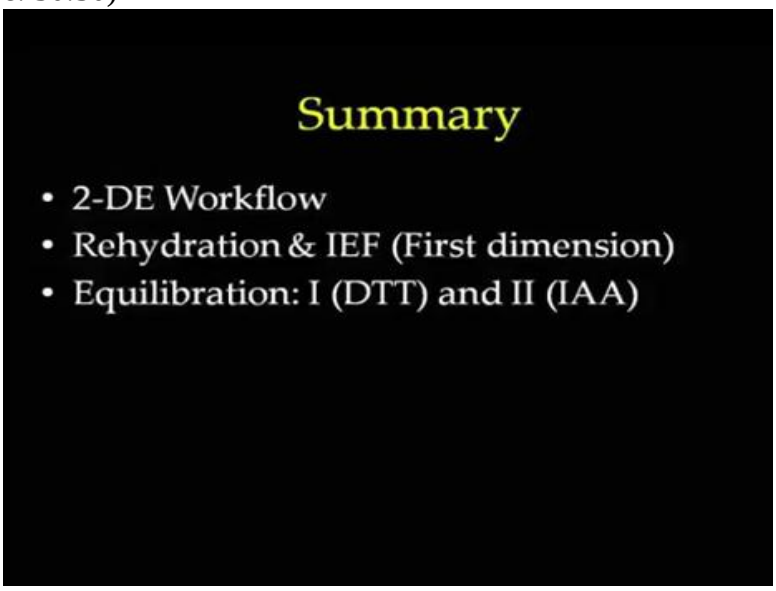

I hope now you are able to follow the workflow of 2DE and we will continue in the next class. Thank you