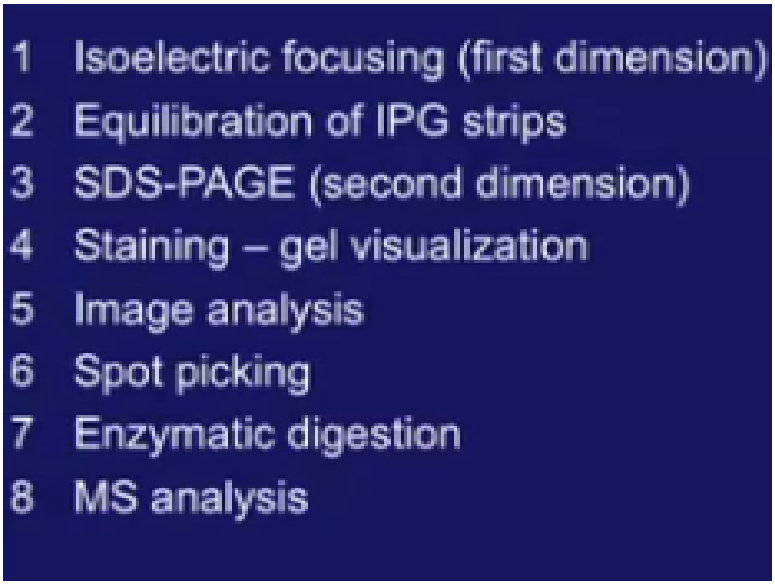
Introduction to Proteomics
Dr. Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology – Bombay

Lecture – 10
Introduction to 2-DE

So, now let us go to the detailed part of each of the processes involved in studying 2 dimensional electrophoresis; the experimental workflow. There are different steps, which are involved in making a good gel, 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.

(Refer Slide Time: 01:28)

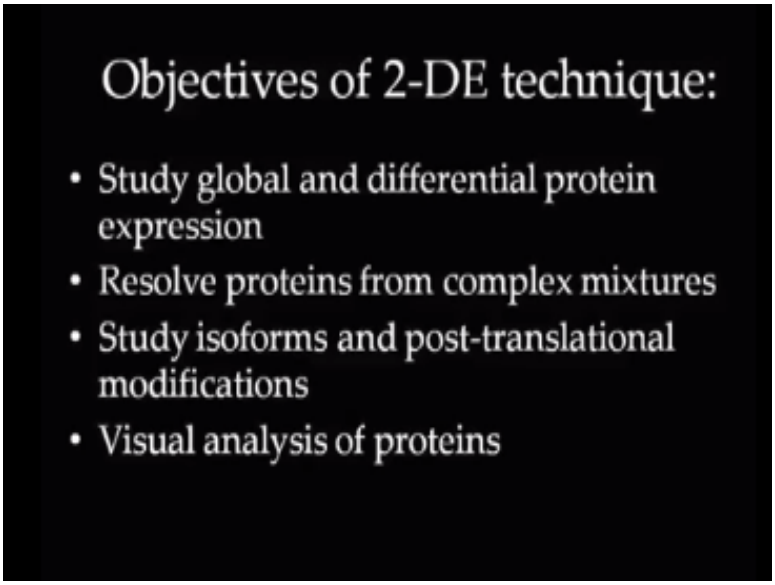


When you are running an SDS page gel, you make your protein denatured you want to separate that based on the molecular weight and you heat the samples, you apply different type of denaturant. Now, in this case when you are doing 2DE your first dimension separation has occurred on the IEF in the immobilized pH gradient strip. Now, you want to take this strip and want to apply that on an 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 this strip, then protein can be further separated due to SDS page, the third point; now fourth in the workflow is staining the gels and visualizing the gel images after staining with different type of a stains.

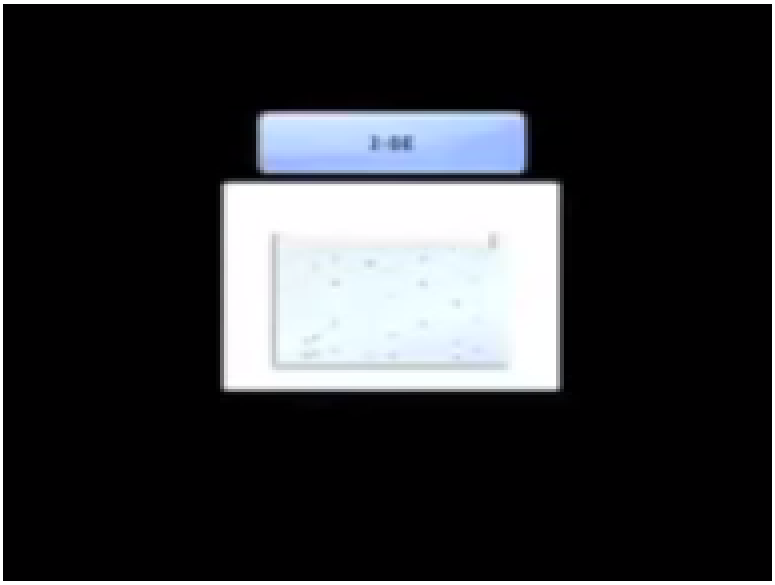
Image analysis, the fifth point is very crucial because you need to analyse all the protein spots so that the abundance of each of these proteins can be calculated and more detailed statistical analysis can be performed. Once you are confident that these are the proteins which are highly significant, then you like to pick those spots by a process, mention a 6 point, which is a spot picking.

(Refer Slide Time: 03:03)



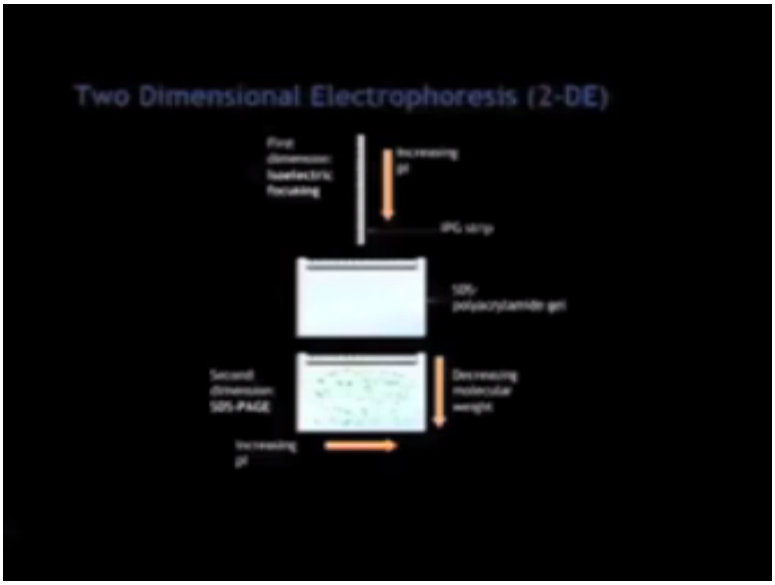
After that, if you want to identify and know that what this protein is, then you need to do the in gel digestion or enzymatic digestion of these proteins obtained from the gels. Then the last point will be the mass spectrometry analysis, which will be different series of lectures in the other module based on the mass spectrometry. So, I will now show you an animation for 2 dimensional forces which will give you an overview of different steps involved in 2DE experiment.

(Refer Slide Time: 03:31)

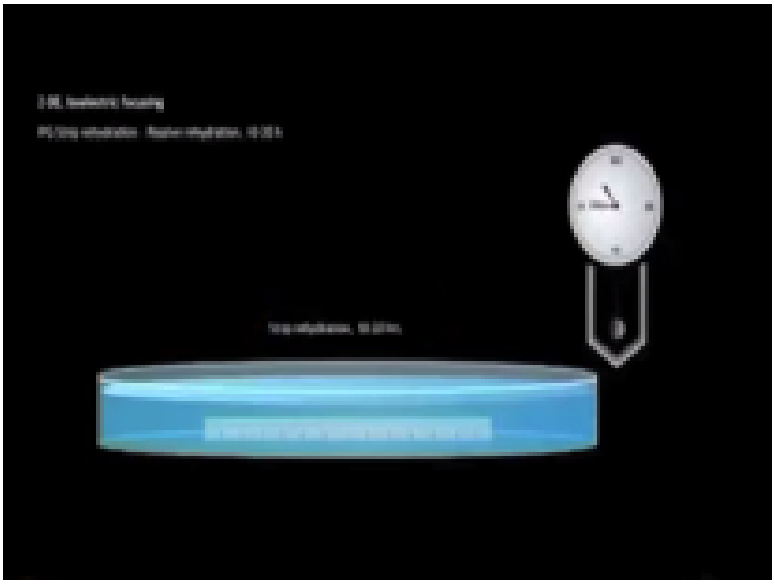


So, in this animation, I will describe you the 2 dimensional electrophoresis processes.

(Refer Slide Time: 03:39)

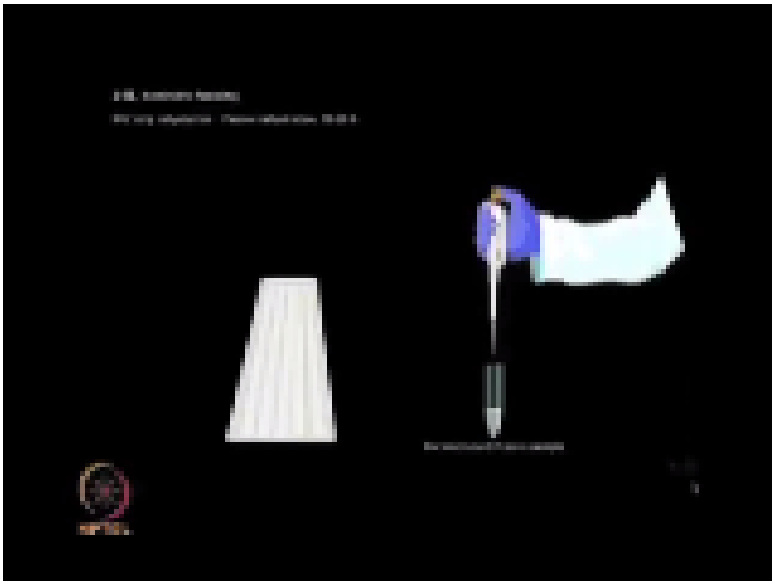


(Refer Slide Time: 03:44)



Prior to isoelectric focusing in 2-dimension electrophoresis, the commercially available IPG strips must be rehydrated. This process can be done either by the passive rehydration or active rehydration.

(Refer Slide Time: 04:07)

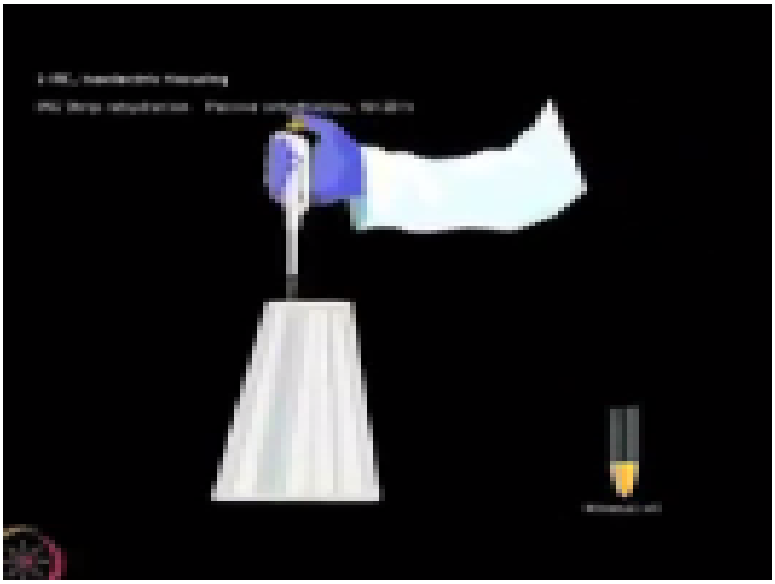


In passive rehydration, the IPG strip is placed with a gel site downwards in a well containing the protein sample reconstituted with a suitable buffer, as you can see the animation, you can apply the protein sample.

(Refer Slide Time: 04:21)

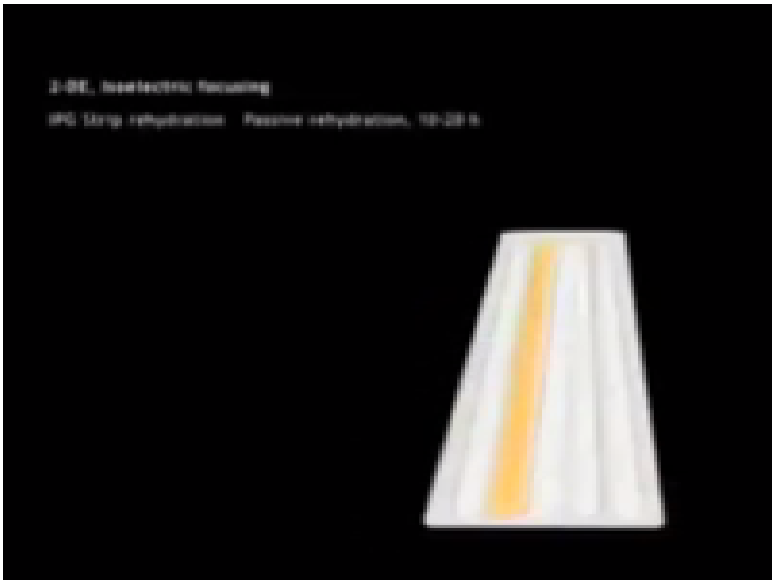
And then add the IPG strip, so that it can absorb the protein solution.

(Refer Slide Time: 04:35)

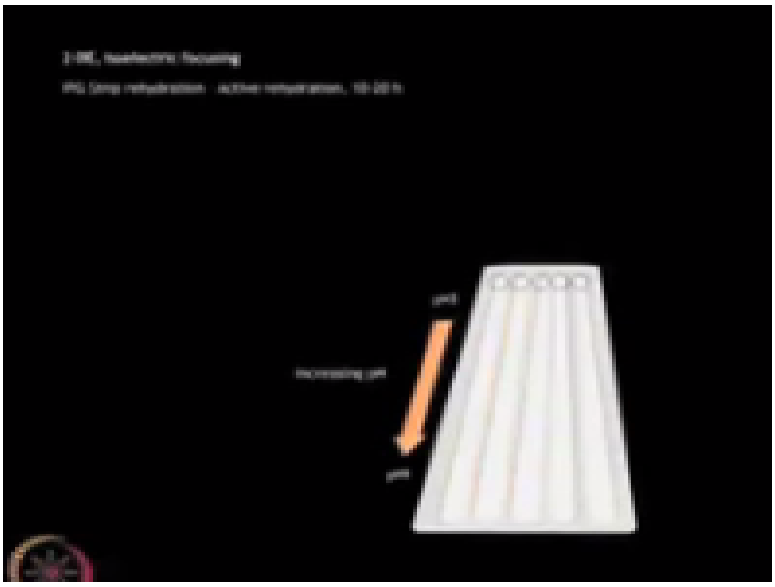


This process can be done for 10 to 20 hours depending upon your length of the IPG strip.

(Refer Slide Time: 04:43)



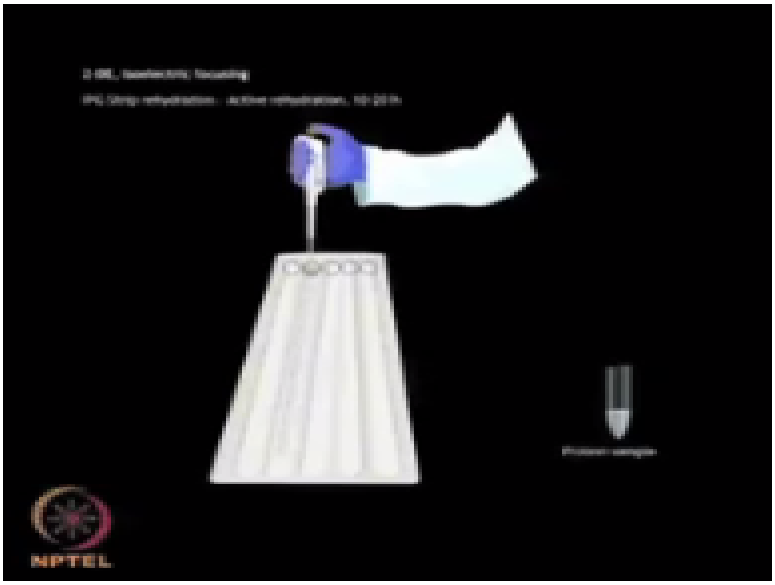
(Refer Slide Time: 04:47)



This is then covered with mineral oil to prevent the gel for drying up and left overnight.

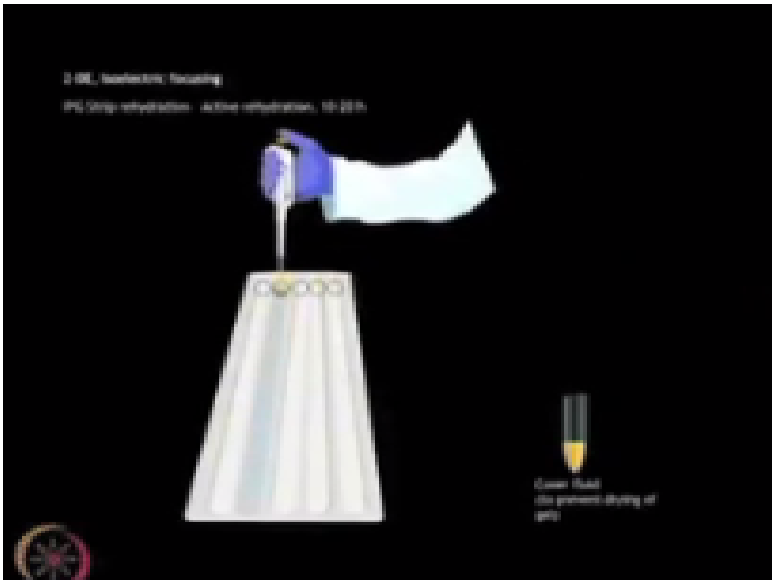
(Refer Slide Time: 04:52)

(Refer Slide Time: 05:02)



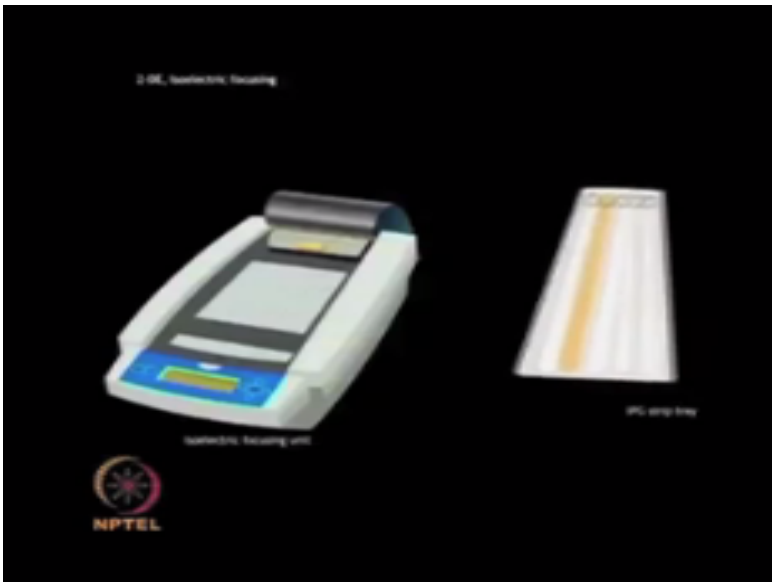
Other process, known as active rehydration where the protein sample is added to the strip via a sample cup followed by the cover fluid to prevent the gel from drying up.

(Refer Slide Time: 05:15)



The protein sample being applied, a very low voltage condition is provided and then cover fluids can be added.

(Refer Slide Time: 05:29)

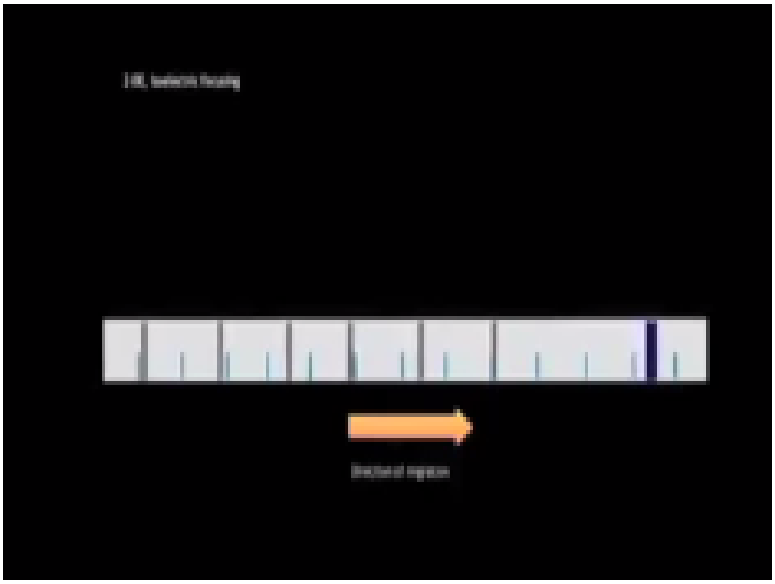


This is then placed in the isoelectric focusing instrument and low voltage is applied.

(Refer Slide Time: 05:32)

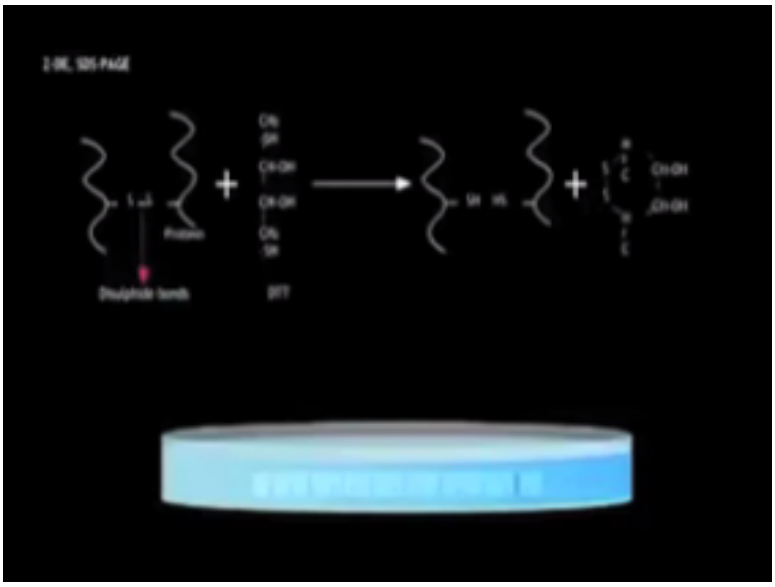
Process can be performed from 10 to 20 hours.

(Refer Slide Time: 05:37)

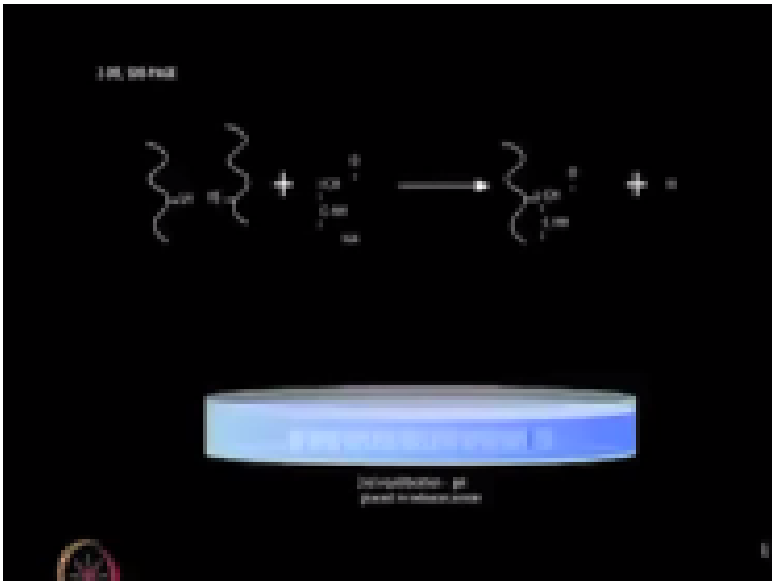


These loaded strips are then focused on an isoelectric focusing unit by passing the current. The various proteins of the sample mixture migrate in the electric field and come to rest, when the pH is equal to their isoelectric point or PI, so they become neutral and are no longer being affected by the electric field. The progress of electrophoresis is monitored by adding a tracking dye which you can see in the animation moving ahead of the proteins.

(Refer Slide Time: 06:20)

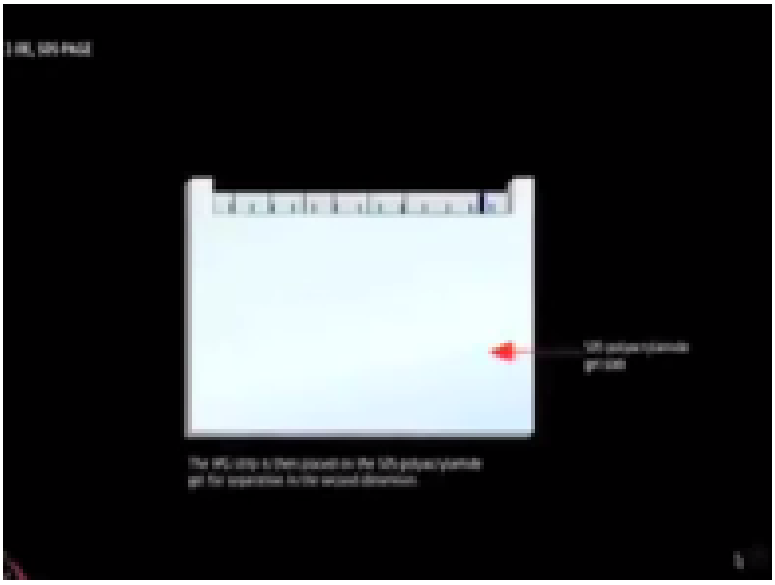


(Refer Slide Time: 06:33)

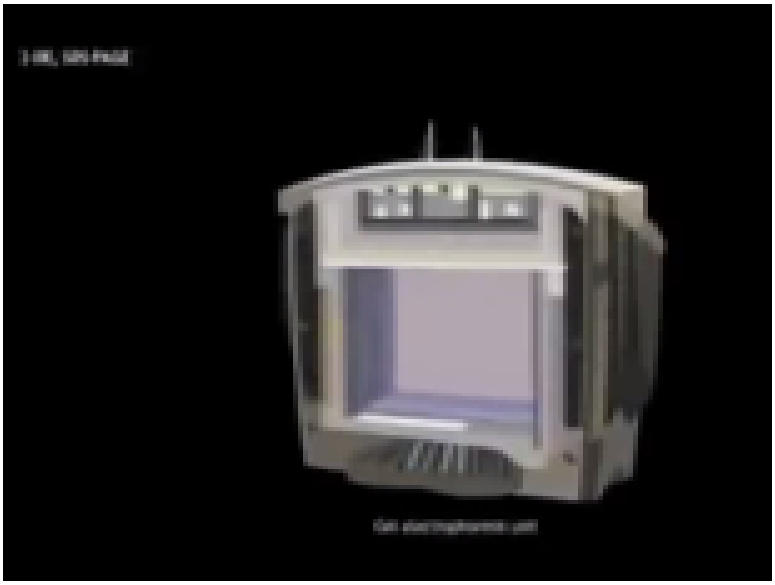


Type EG strip is then acclimated in a reducing agent such as DTT followed by an alkylating agent; Iodoacetamide, which prevents reformation of reduced bonds.

(Refer Slide Time: 06:42)

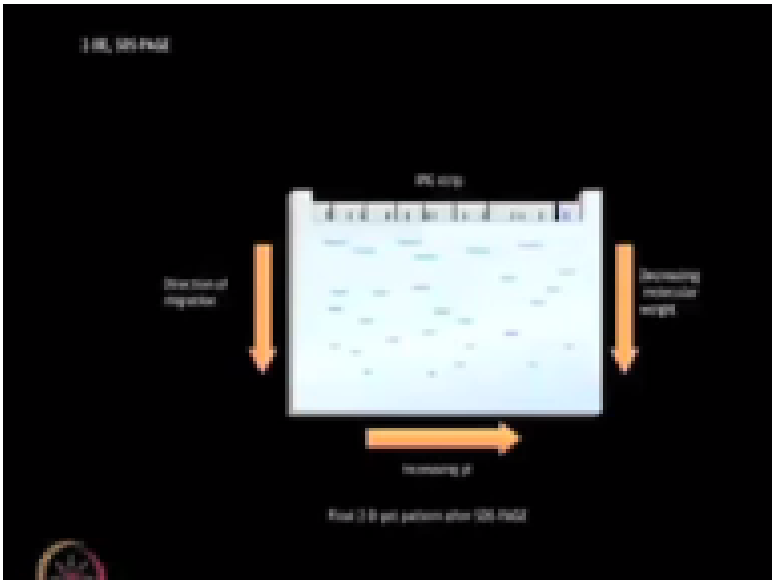


(Refer Slide Time: 06:57)



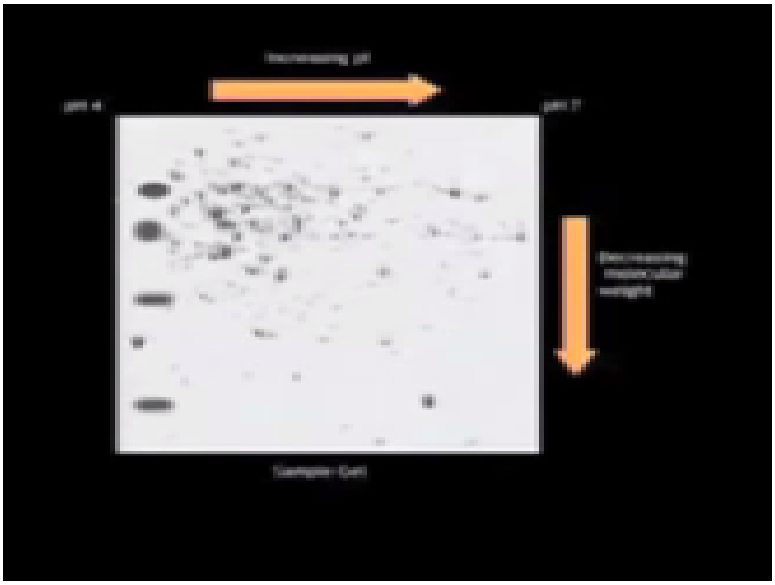
This is strip containing the separated proteins is then placed on SDS page gel slab for that protein separation in the second dimension based on the molecular weight.

(Refer Slide Time: 06:59)



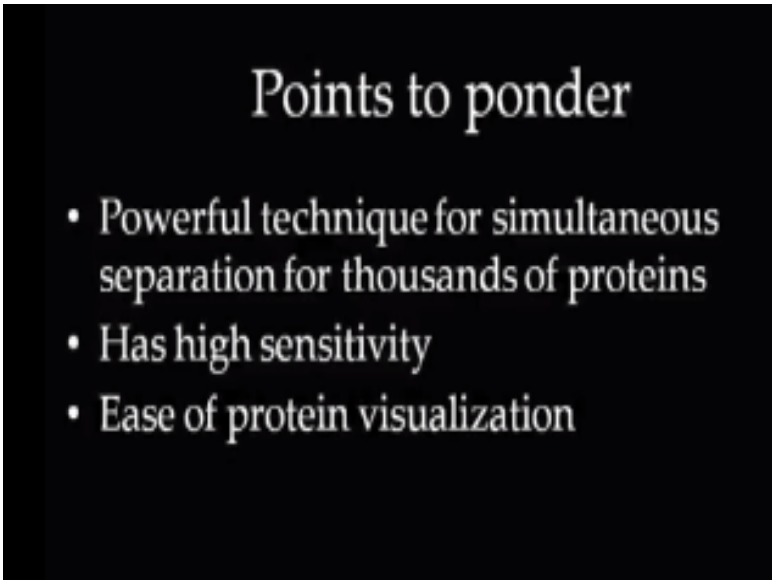
The proteins on the IPG strip are subjected to SDS page by applying a direct current between 100 to 350 volts depending upon the size of the gel. Any protein that may have been present as a single band on the IPG strip as you can see in the red circle due to similar isoelectric point can then be separated based on the molecular weight, where the smaller proteins will migrate the farthest.

(Refer Slide Time: 07:41)

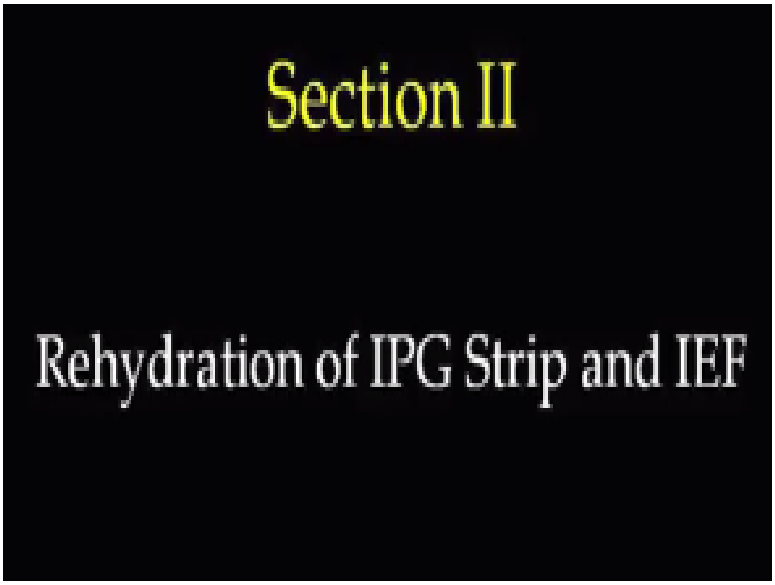


This is view of a sample gel which has been run by 2-dimension electrophoresis and stained with Coomassie blue, each spots provide the information about the molecular weight and isoelectric point of the protein.

(Refer Slide Time: 08:00)

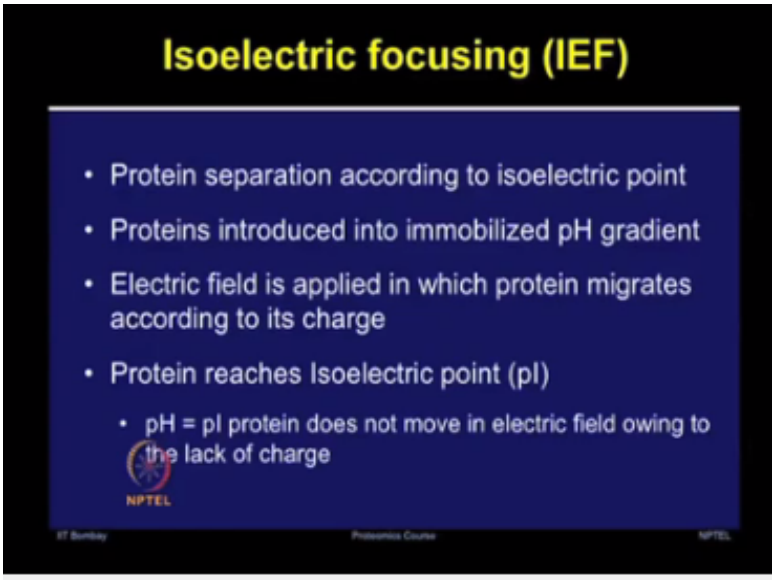


(Refer Slide Time: 08:11)



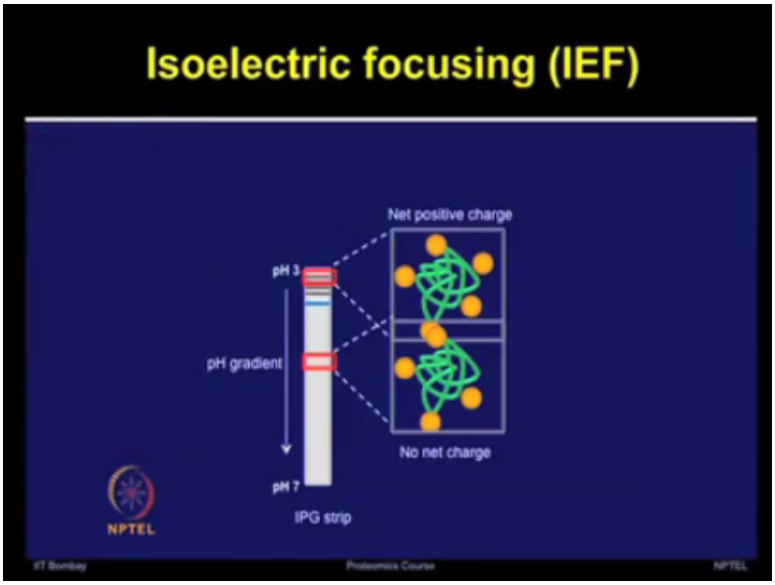
So, let us talk about isoelectric focusing or IEF. In the IEF process, proteins are separated according to the isoelectric point.

(Refer Slide Time: 08:27)



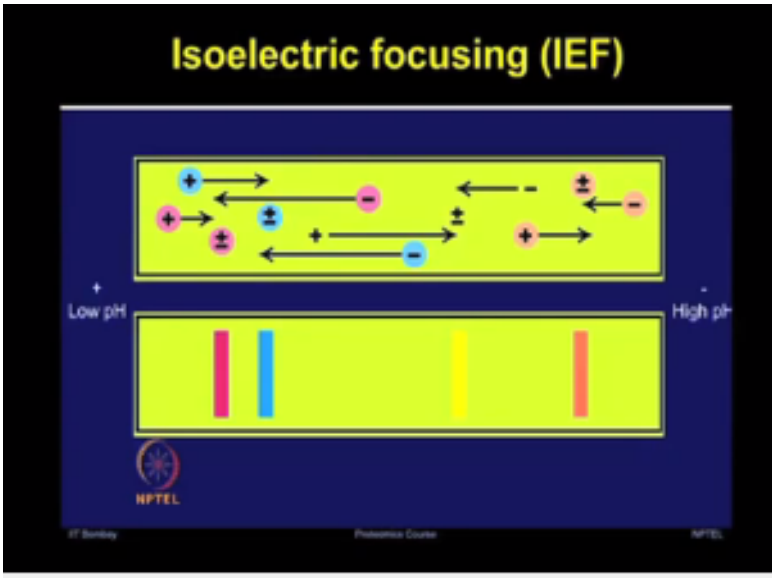
You can take the immobilise pH gradient strip and apply the protein solution on the IPG strip, so that protein is absorbed on the strip and then after applying the electric field, the proteins will migrate according to its charge. When protein is reaching to its isoelectric point, where pH becomes equal to isoelectric point or PI, then proteins do not move in the electric field because of the lack of charge.

(Refer Slide Time: 09:03)



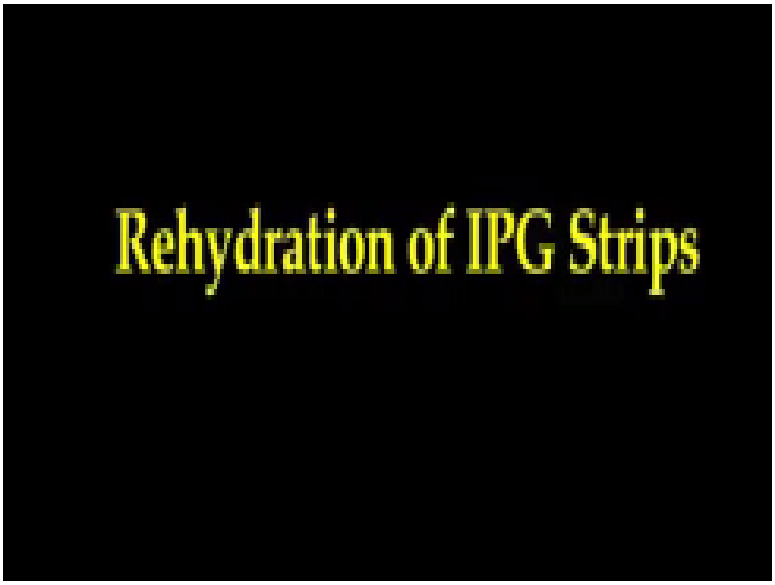
So, when pH equals to PI, then protein will not move any further. Let me show you this process in this animation here, you have an IPG strip with pH 3 to 7 gradient, when you are moving in the electric field from the positive charge towards the negative charge, when pH becomes equals to its PI, then there is no net charge and protein will remain over there. So, now this will be the isoelectric point.

(Refer Slide Time: 09:37)

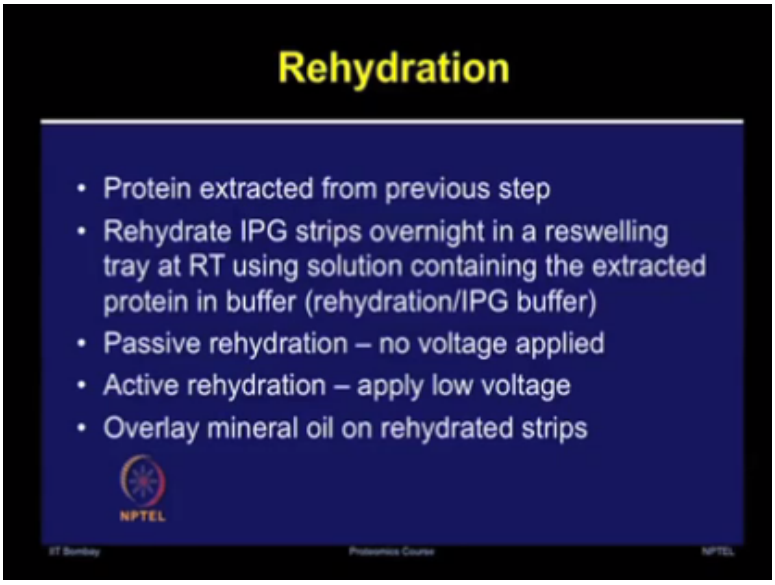


And this view, you can see from the low pH to the high pH proteins are moving in an electric field and once they reach to a stage, where there is no net charge or pH becomes, it goes to their isoelectric point PI, then these discrete band can be seen.

(Refer Slide Time: 09:58)



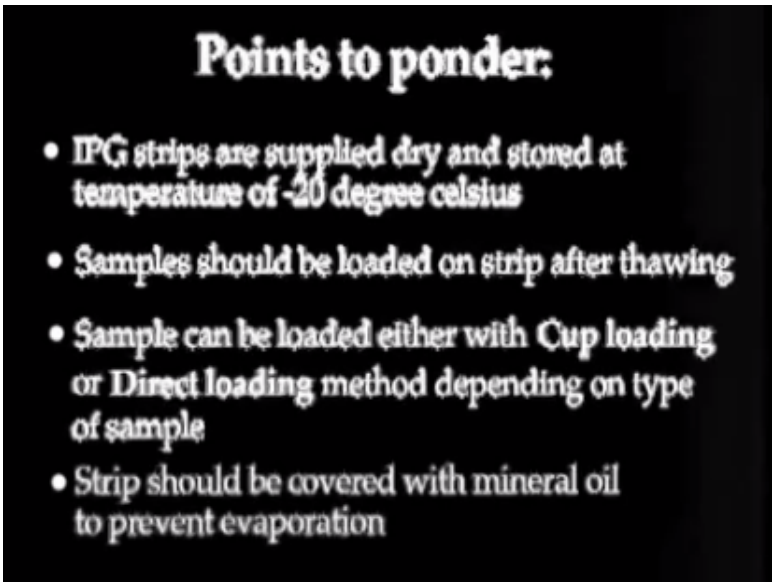
(Refer Slide Time: 10:19)



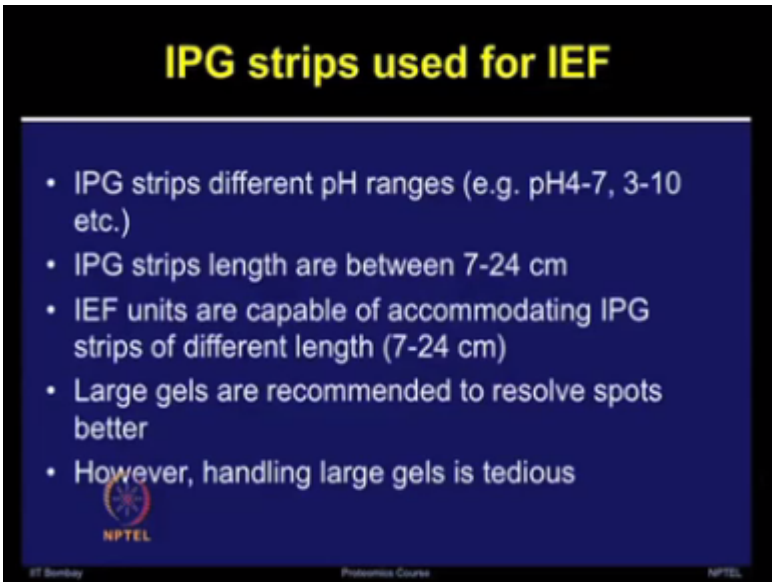
So, the rehydration is one of the important step before applying the electric field in the IEF process as you have seen in the previous animation that to rehydrate the IPG strip, you can apply the protein solution on the IPG strips and different methods can be used for this, whether passive rehydration, where no voltage is applied or active rehydration, where you apply the protein sample and a very low voltage is applied to resolve the protein.

Now, we have already discussed that none of these methods can be compared and said in the superior method, one has to really use that in their own balance or 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.

(Refer Slide Time: 11:15)



(Refer Slide Time: 11:33)



Now, different type of strips can be used during the IEF process, whether it is pH gradient of 4 to 7 or 3 to10 or some other pH range, as I described in my previous slide depending on 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 metal suppression of the protein but you may lose your 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 of strip like 3 to 10, now what type of length of IPG strip should be used? One can use starting from 7 centimetres till 24 centimetres, there are different types of a 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.

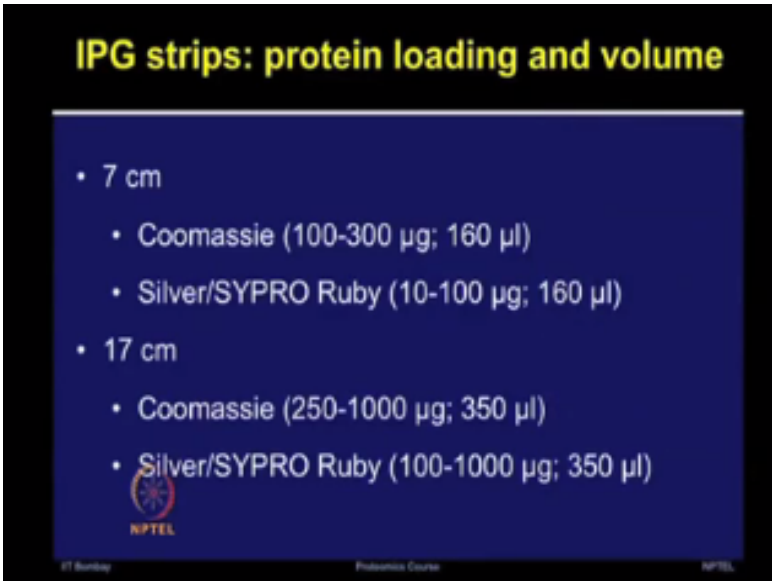
So, that you know that your IEF process is going fine and your protocol is looking good. you can separate the proteins. Now, once you know that your extract is good, then you can apply that to separate the proteins in the long strip length, for example 17 or 24 centimetre. Now, these IEF units or isoelectric focusing units obtained from different vendors commercial manufactured.

They are capable of taking the IPG strips of different varying pH lengths, few instruments take a flexible length from 7 to 24 centimetre, other commercial instruments they are able to take different type of trace, design for each type of a strip of length. Now, depending on your

proteins; how much protein sample you have in your protein with in the sample mixture, it is always better or good idea to resolve that on the very large gels.

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

(Refer Slide Time: 14:50)



But overall the largest are recommended for doing any type of differential proteomic analysis when you want to compare your controls and treatment response but handling the larger is very tedious, so I will show you some lab demonstration; the video for that, so that you can be well prepared to perform the experiments in your lab. These are some recommendations for how much protein one should note on the IPG strips.

For example, if you have a small strip like 7 centimetre length, you can apply between 100 to 300 micro gram of protein and maximum volume could be 150 to 160 micro litres. 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 sensitivity stain such as silver or SYPRO Ruby.

One can also apply the largest strip like 17 centimetre or 24 centimetre strip but then you have to increase the protein amount to be loaded and separated on the IPG strip. For example, between 250 to 1000 micro gram of protein can be loaded on the largest strip and one can visualize that by using Coomassie stain, total reaction volume should not be more than 350 microliters.

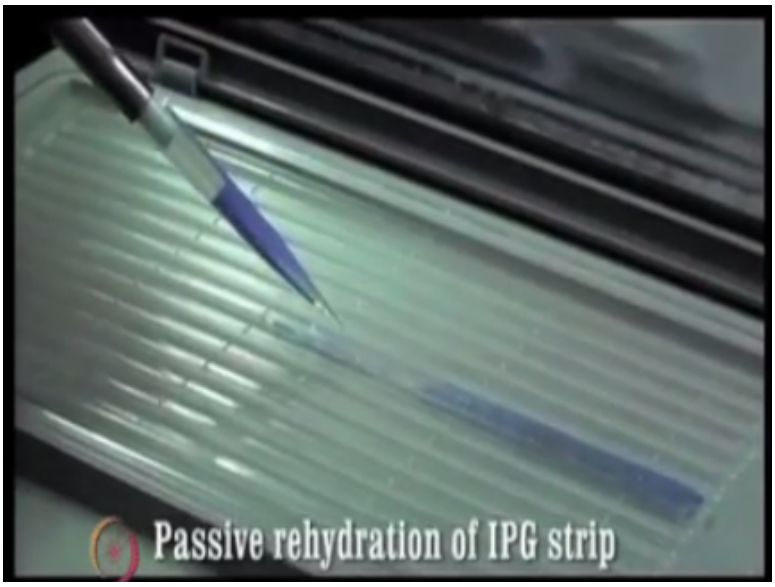
(Refer Slide Time: 16:24)

But if you do not have too much protein fill it in your sample, then still you can apply these methods, you can apply the larger strip but then you have to stain with silver or SYPRO Ruby in that case, you can load between 100 to 1000 microgram of protein. So, I will give you the laboratory demonstration of isoelectric focusing, so that you can be very clear about different intricate strips involved in performing IEF.

(Refer Slide Time: 16:45)

Isoelectric focusing; this process involves 2 major steps; rehydration of the IPG strips and focusing of the rehydrated strips.

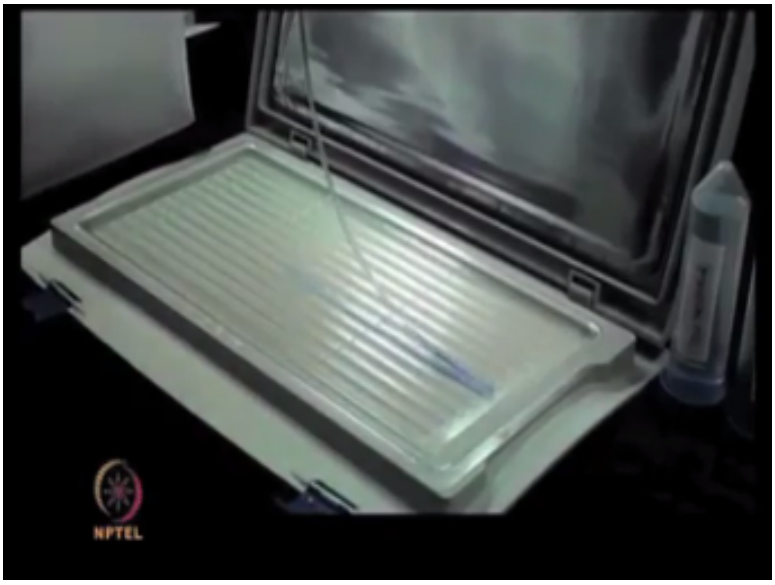
(Refer Slide Time: 16:58)



IPG strip rehydration; clean all the apparatus thoroughly before use to avoid any contamination, add the reconstituted protein sample in a well of the rehydration tray.

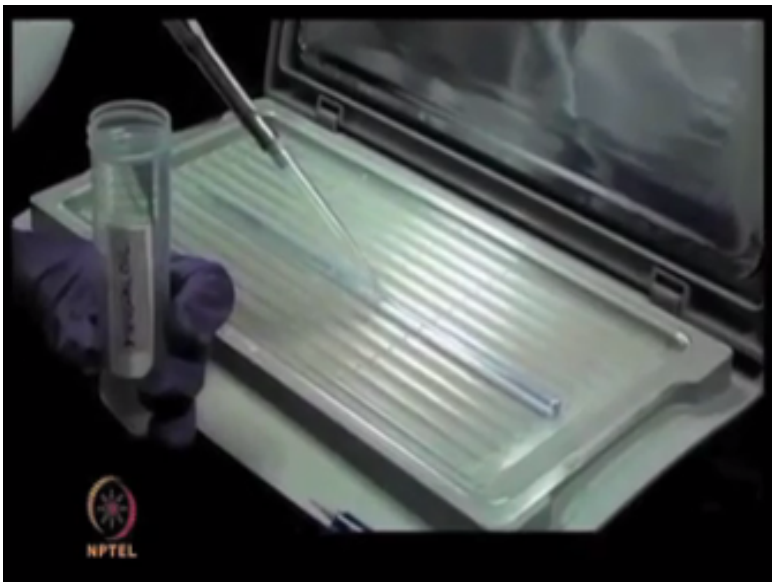
(Refer Slide Time: 17:16)

(Refer Slide Time: 17:21)



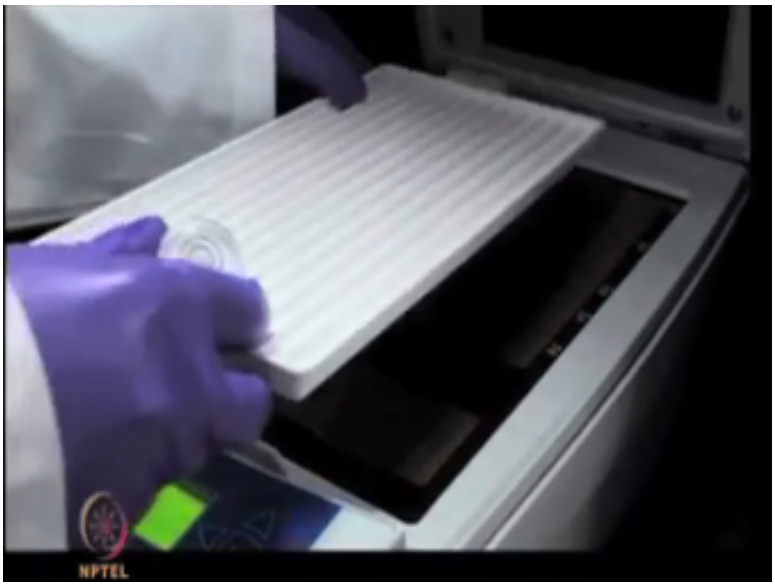
Then, remove the IPG strip from its cover and place it carefully in the well. IPG strips are used for separation of proteins based on their isoelectric point. These are acrylamide coated plastic strips containing immobilised of various pH spread across it. They have successfully replaced conventional tube gels due to their reproducibility they can achieve.

(Refer Slide Time: 17:45)



After around 30 minutes, pour some mineral oil over the strip to prevent it from drying up, cover the tray and leave it overnight to allow rehydration to occur.

(Refer Slide Time: 18:33)

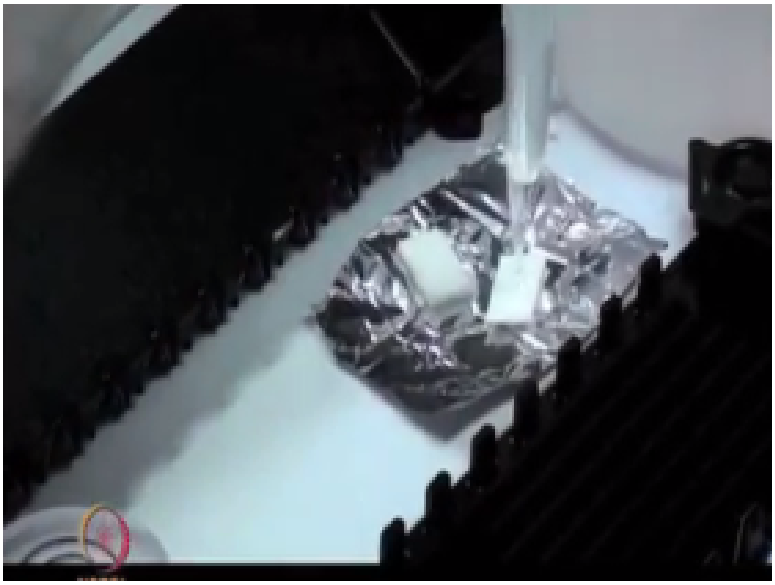


Isoelectric focusing; initialize the instrument and clean the surface well with a dust free cloth, place the focusing tray on the instrument and ensure that it is properly balanced.

(Refer Slide Time: 19:30)

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

(Refer Slide Time: 19:39)

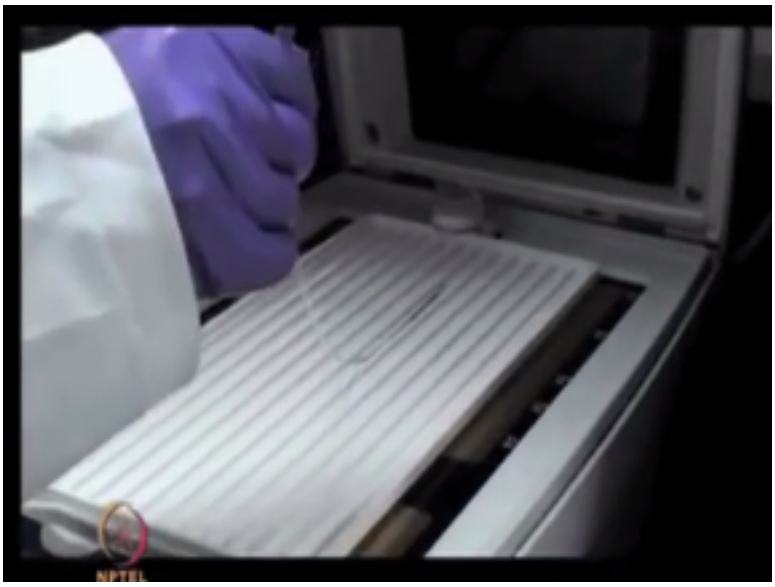


And wet them with a small amount of water before use.

(Refer Slide Time: 19:59)

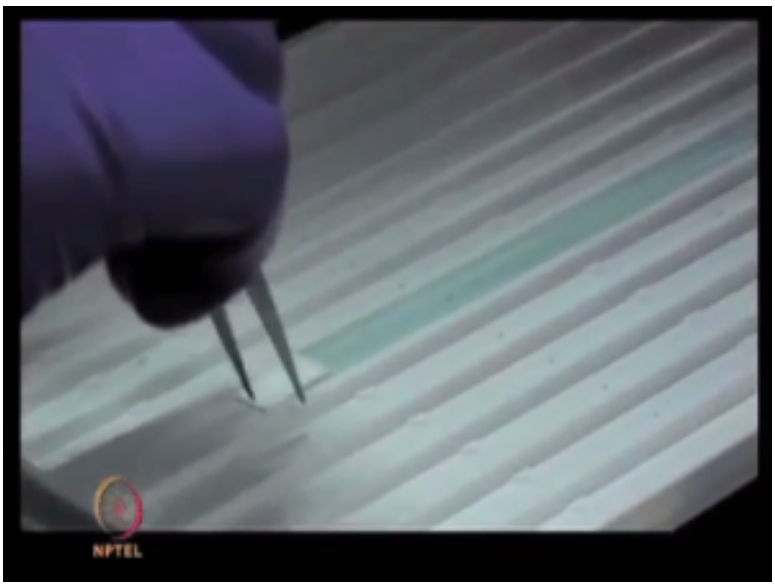
Carefully remove the IPG strip from the rehydration tray and drain out any excess oil by blotting it on a tissue paper.

(Refer Slide Time: 20:45)



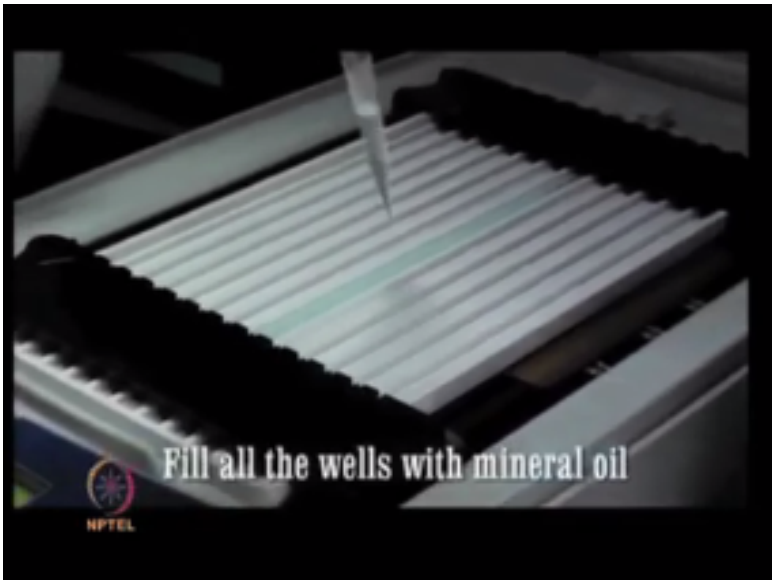
Place the strip in the focusing tray and immerse it with mineral oil.

(Refer Slide Time: 20:57)

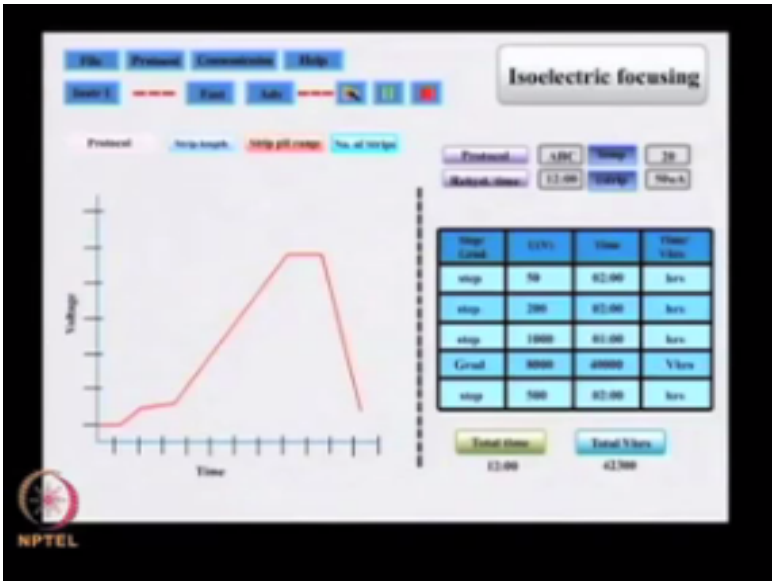


Then place the two wicks at either end of the IPG strip followed by an electrode at each end.

(Refer Slide Time: 21:38)

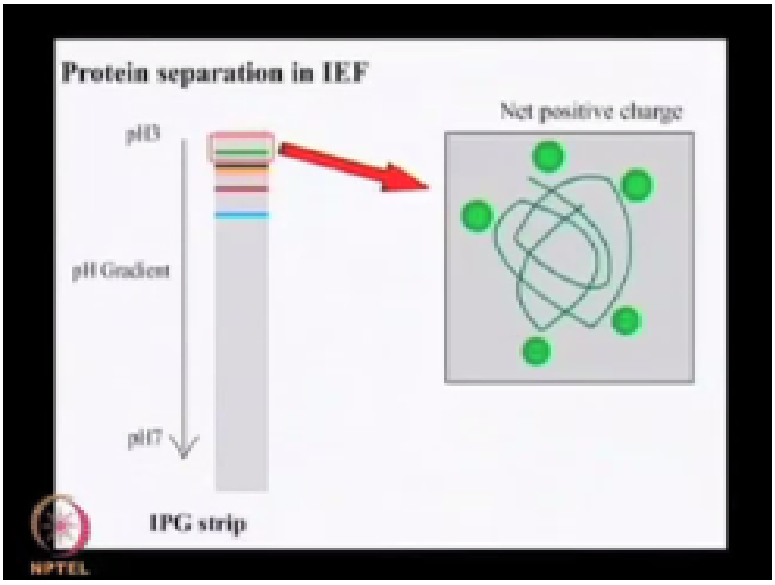


(Refer Slide Time: 21:41)



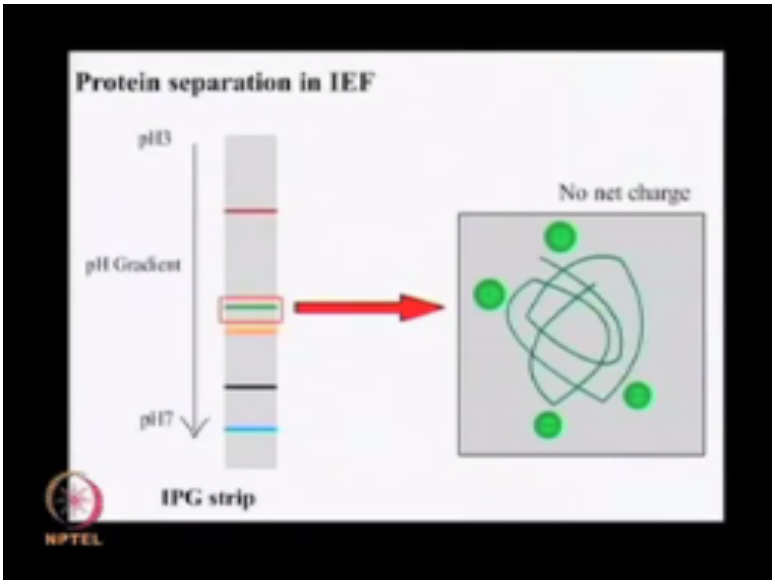
Fill all the adjacent wells with mineral oil to ensure uniform current flow. Then input the desired protocol on the instrument software along with details of strip length, pH range and number of strips and start the focusing process. The voltage time curve will appear based on the protocol that has been set.

(Refer Slide Time: 22:05)



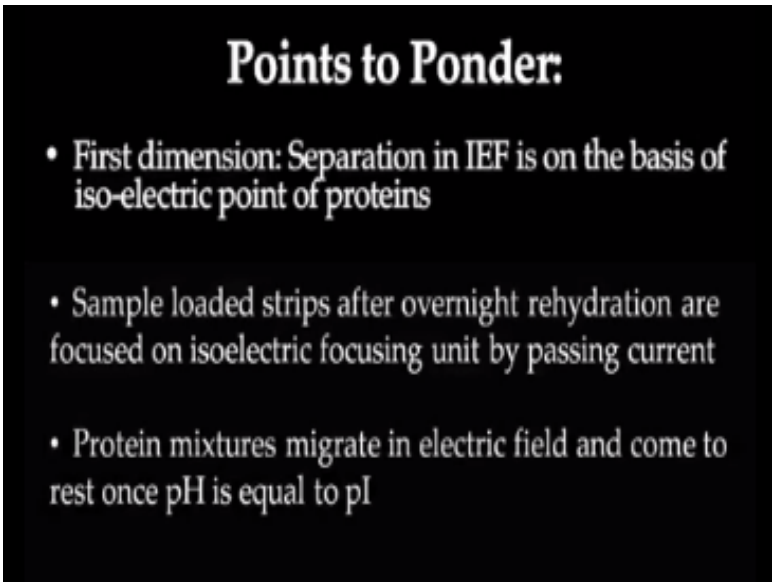
Protein separation occurs on the basis of net charge of the protein.

(Refer Slide Time: 22:09)

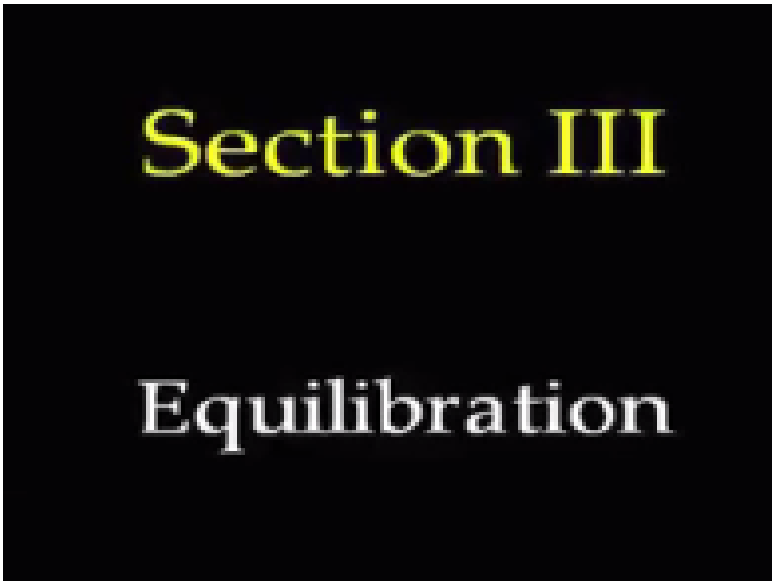


Proteins will migrate along the strip and come to rest at a point when their net charge becomes 0 known as their isoelectric point.

(Refer Slide Time: 22:21)



(Refer Slide Time: 22:33)

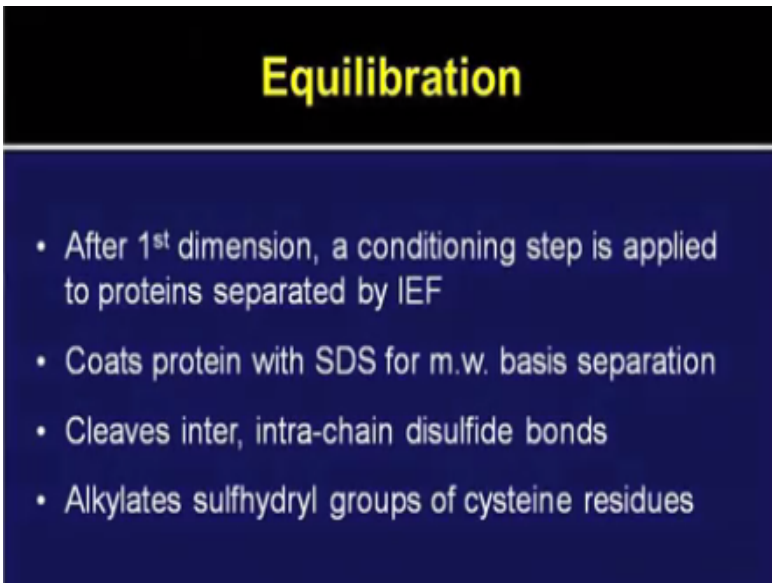


Once you have performed the isoelectric focusing or IEF, you need to prepare your IPG strip for the next step, which is SDS page. When you are doing the conventional SDS page, you pour the gel both resolving and stacking and then you want to make some wells by using a comb and then you load your protein samples in different wells. Now, when we talk about 2 dimensional electrophoresis it is different than only doing 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 staking 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.

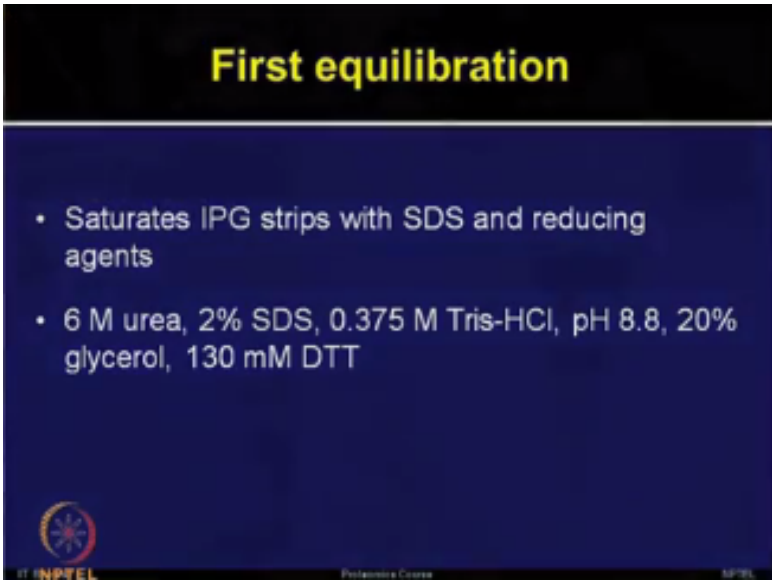
Now, you need to add SDS to provide the negative charge and you need to do some treatment for reduction and alkylation. When you are taking the protein sample alone, you have flexibility of boiling the protein sample in the tubes and do all those treatments to denature the protein separately but now, your protein is already focused inside the IPG strip, so you do not have that flexibility here.

(Refer Slide Time: 24:34)



So, all you need; you need to prepare your IPG strip in such a way that you can apply that on top of a SDS page gel. Shall I show you what strips are required in the equilibration step? So, after the first dimension separation is done, you need to coat the protein with SDS page for making it compatible for SDS page, so you need to coat with SDS sodium dodecyl sulfate, which is an anionic detergent and it provides negative charge later on for separation in the electrophoretic field.

(Refer Slide Time: 25:27)

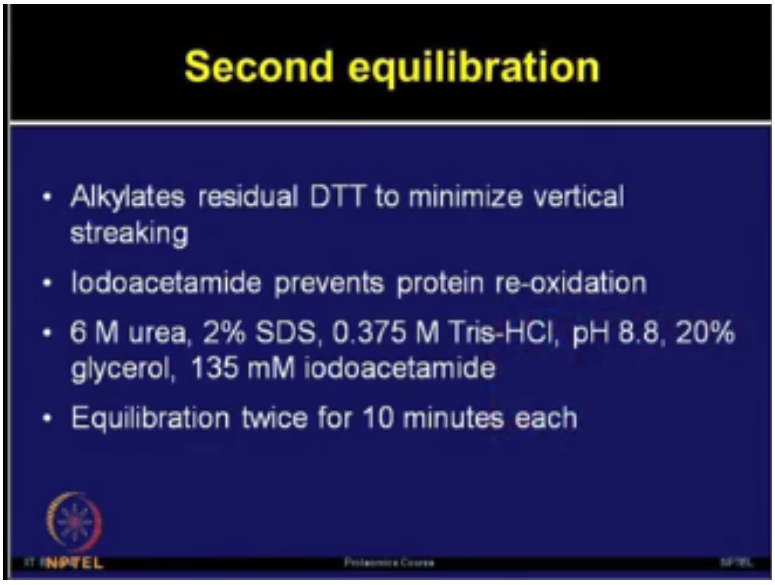


The equilibration step performs cleavage of both inter and intra chains of disulphide bonds and alkylates sulfhydryl groups of the cysteine residues. So, there are 2 equilibration steps, which are required for this preparatory step; the first equilibration step and the second equilibration step, your 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 step can 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.

(Refer Slide Time: 26:49)

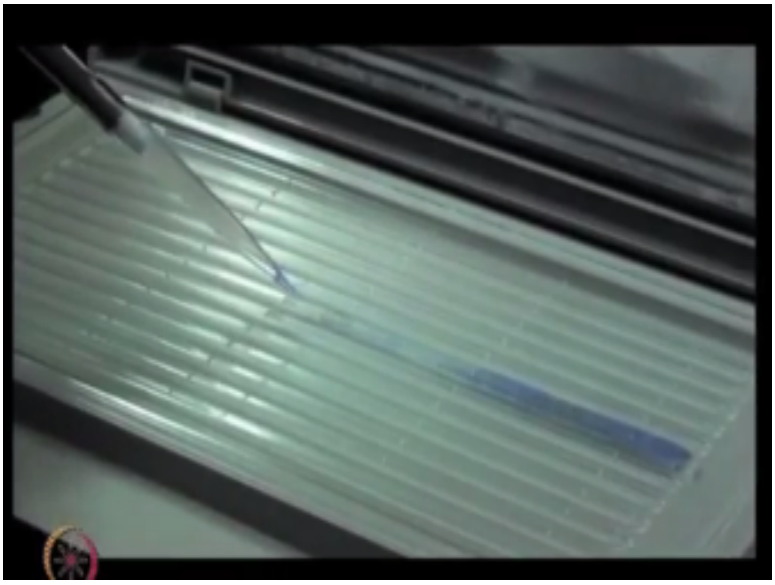


Now, this buffer recipe is also exactly similar to the last one except one chain DTT is replaced with Iodoacetamide. So, you can add 135 millimolar of Iodoacetamide during the second equilibration step. Now, this is very important because IAA can alkylate the residual DTT and it minimizes the vertical streaking, it also prevents the reoxidation, 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. I will show you in this slide; the brief strip wise 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.

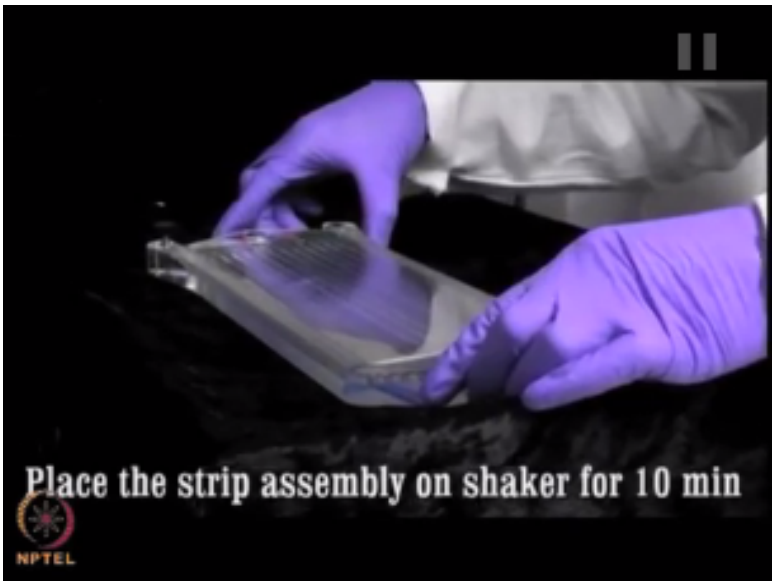
And then you change that with the second equilibration solution, the DTT reductant will be used for the first strip followed by IAA; Iodoacetamide, which functions as a scavenger of the excess reductant and alkalytes and prevent the reoxidation, so here you can see how different bonds are broken from the disulfide and IAA is preventing the reoxidation; alkylating these residues.

(Refer Slide Time: 28:40)



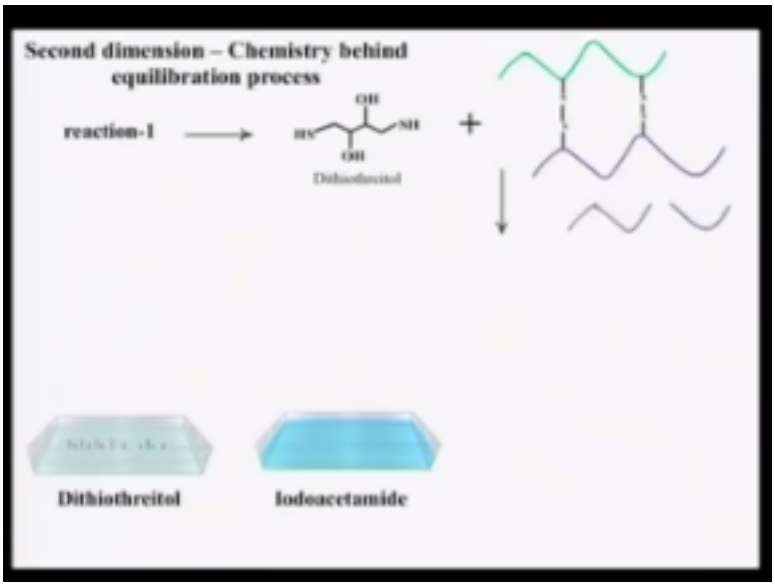
Place the third IPG strip in the tray and over it, pour the first equilibration solution consisting of urea, Tris-HCL of ph 8.8 glycerol SDS bromophenol blue along with dithiothreitol or DDT.

(Refer Slide Time: 29:17)



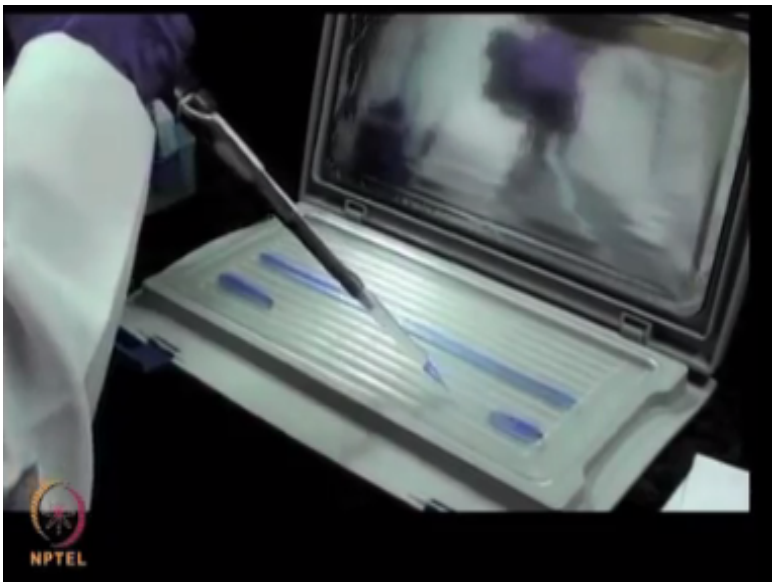
Place the assembly on a mechanical shaker for 10 minutes.

(Refer Slide Time: 29:21)



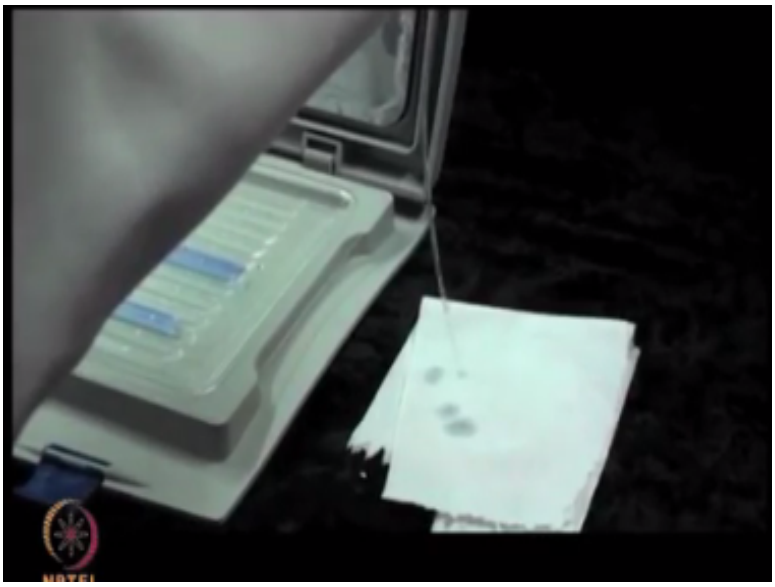
The dithiothreitol enables cleavage of all disulphide bonds present in the proteins on the IPG strip.

(Refer Slide Time: 29:31)



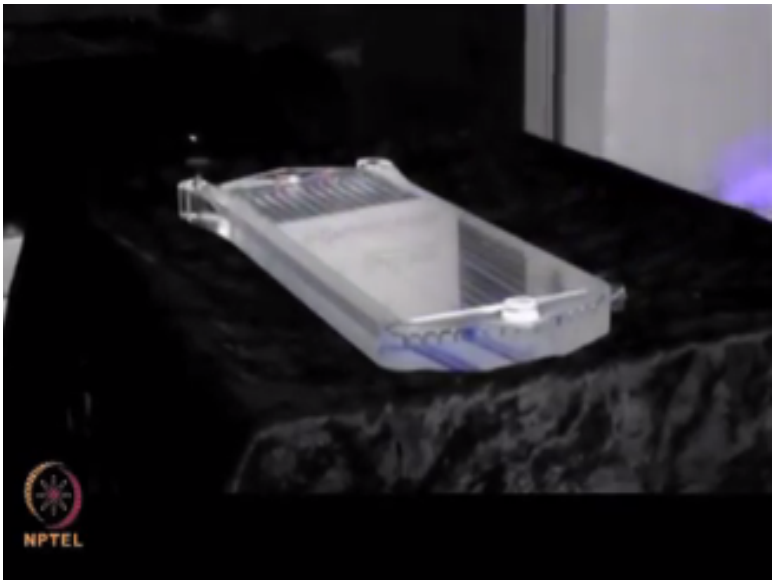
Next, add the second equilibration solution to another well of the tray, this solution has the same composition as the first except that the DTT is replaced by hydroacetamide.

(Refer Slide Time: 29:47)



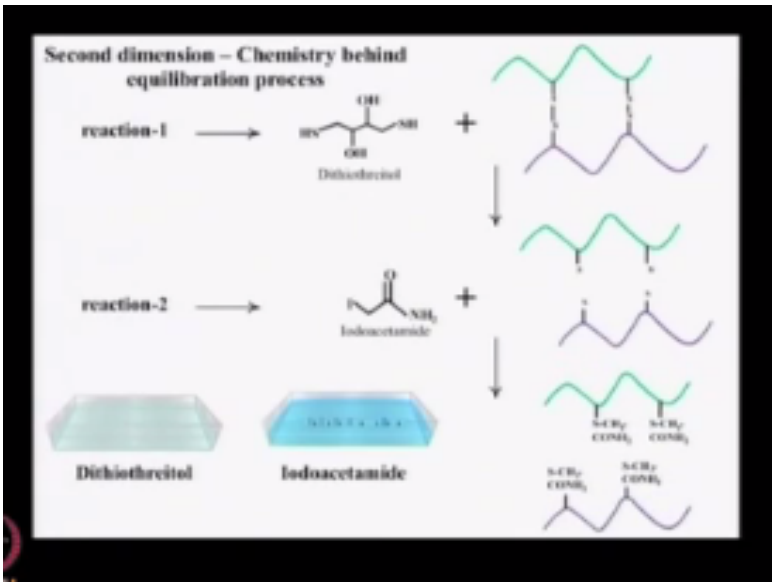
Transfer the IPG strip into this solution after draining off any excess liquid from the first.

(Refer Slide Time: 30:02)



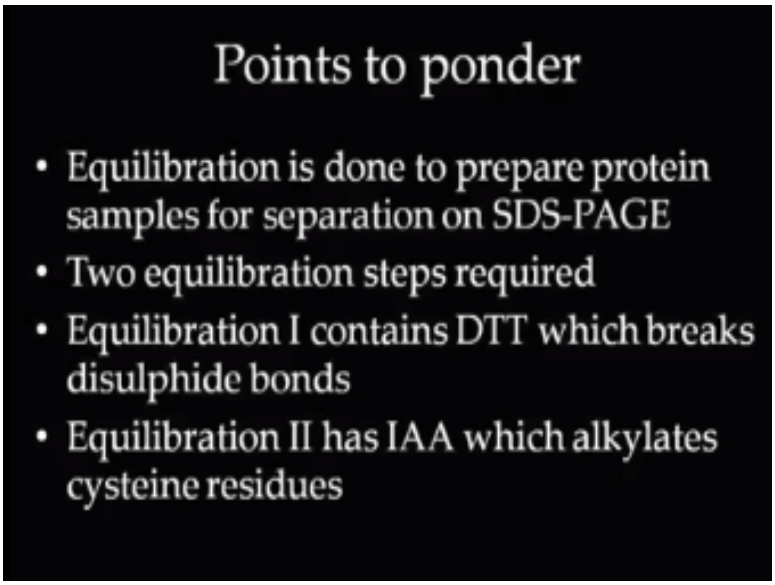
Again place the assembly on the shaker for 10 minutes.

(Refer Slide Time: 30:21)

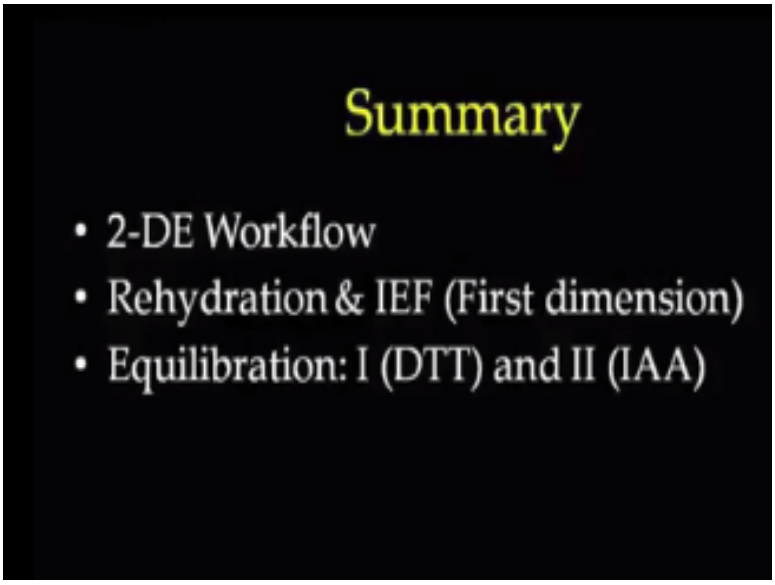


Iodoacetamide prevents reformation of the disulphide bonds by binding to the free sulphidal groups that were obtained after DDT treatment.

(Refer Slide Time: 30:33)



(Refer Slide Time: 30:49)



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