**Proteomics: Principles And Techniques**
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**Module No.# 13**
**Lecture No. # 13**
**Gel-based Proteomics**
**Two-dimensional electrophoresis (continued)**

Welcome to the Proteomics course. In the previous lecture, we started discussing about the Gel Based Proteomics.

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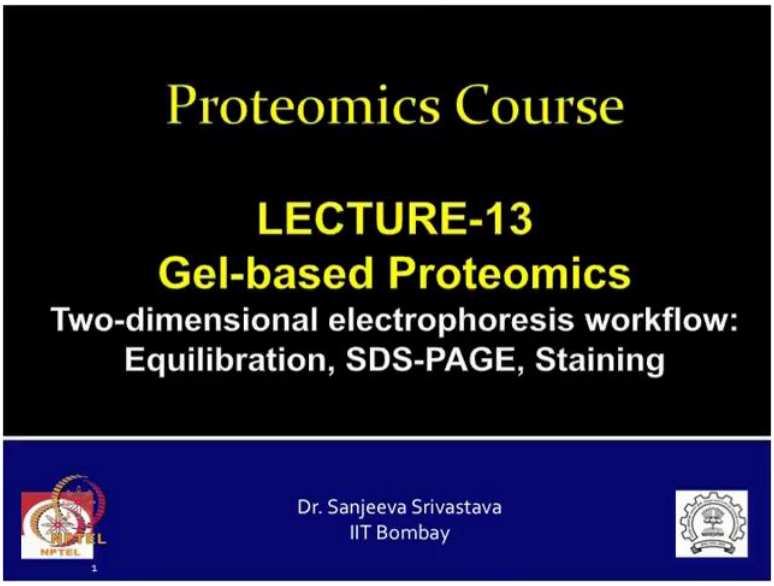


In that work flow, we talked about two dimensional gel electrophoresis and there are different steps which are involved in performing the two dimensional electrophoresis. As I mentioned, the gel based proteomics include various techniques such as SDS page, blue native page, two dimensional electrophoresis and advance formsof electrophoresis such as difference in gel electrophoresis or dyes.

In the last lecture, I described you to perform a two dimensional electrophoresis experiment, you need to follow a workflow and I gave you different steps which are involved to perform the 2 D. The first step was isoelectric focussing,which means you want to focus the proteins based on its isoelectric point. I have demonstrated you some video to give you the

laboratory feel of the experiment, how it can be performed and now we are continuing from the previous lecture.

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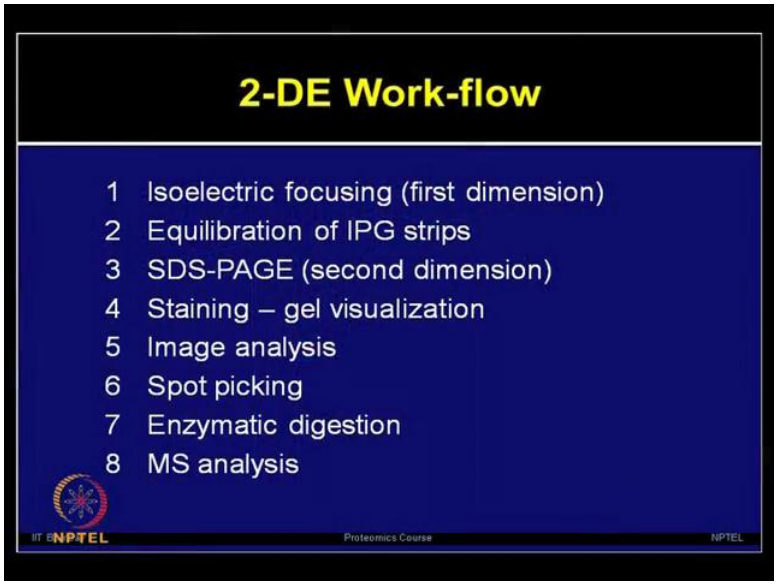


In today's lecture, we will talk about the work flow of two dimensional electrophoresis, the first step was already described in the last lecture; and then we will discuss few more steps today. So, today's lecture we will focus on equilibration step how to equilibrate the LPG strip how to perform SDS page, which is protein separation in second dimension based on the molecular weight. Now, for the step the staining procedure how to visualise your gel, because many time you have problems of low abundance protein; or sometime you are lucky and you have good protein amount. So, you can try different type of staining methods depending upon your requirement and your protein yield.

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We will discuss different type of staining methods which can be used to perform this step. So, let us start with the first point equilibration, as briefly I described in the previous lecture. Once you have performed the Iso Electric Focussing 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 stackingand 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 dimension 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 stacking gel; you need to prepare your protein sample, which is already focussed inside the strip in the first dimension. Now, this IPG strip which was already put in the elctrophoretic 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 focussed 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 a SDS page gel,so I will show you what steps are required in the equilibration step.

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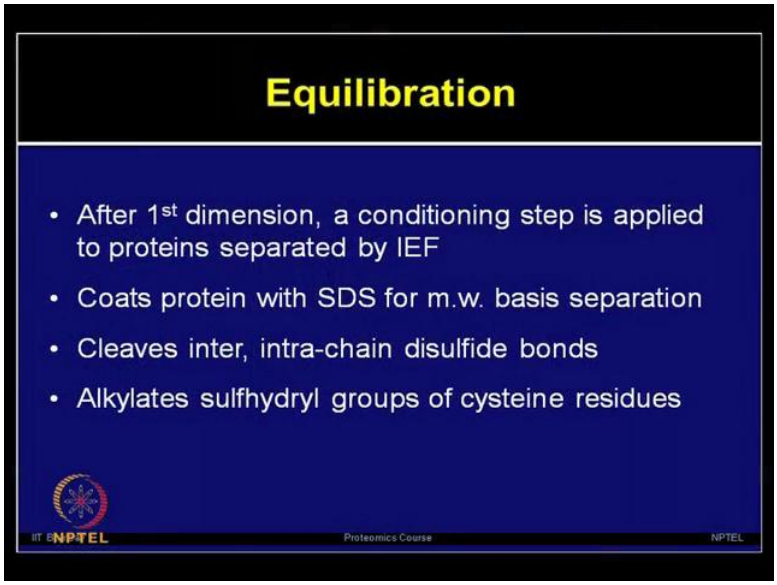


So, after the first dimension separation is done, you need to coat the protein with SDS page for making it compatible for SDS page, soyou need to coat with SDS sodium dodecyl sulfate which is an anionic detergent and it provides negative charge that are on for separation in the electrophoretic field. The equilibration step performs cleavage of both inter and intra chains of disulfide bonds and it alkylates the sulfhydryl groups of the cysteine residues.

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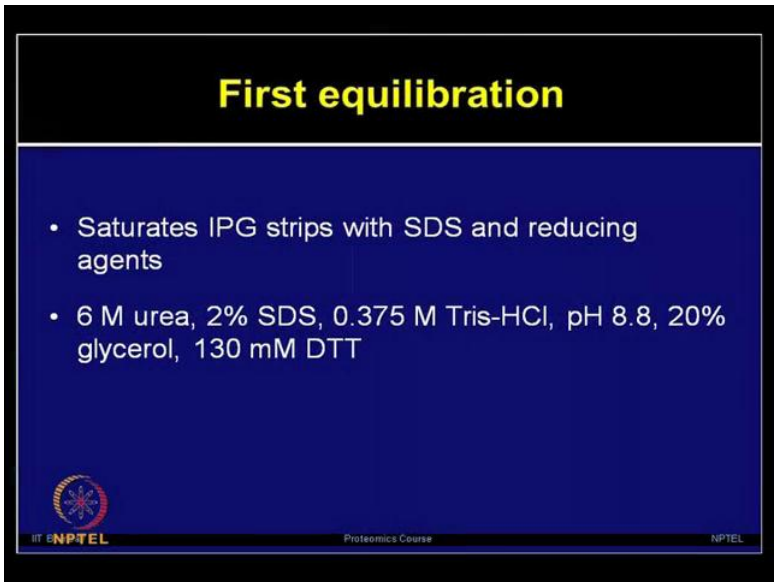


So, there are two equilibration steps which are required for this preparative step, the first equilibration step and the second equilibration step. Your recipe for equilibration buffer is ideally similar in bothfirst and second equilibration, which includes 6 molar of urea 2 percent of SDS 0.375 molar of tris HCLpH 8.8, 20 percent of glycerol and then you need to make one variation in this buffer composition, either you add DTT dithiothreitol or you add i a aiodoacetamide. 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. 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 milli molar of iodoacetamide during the second equilibration step.

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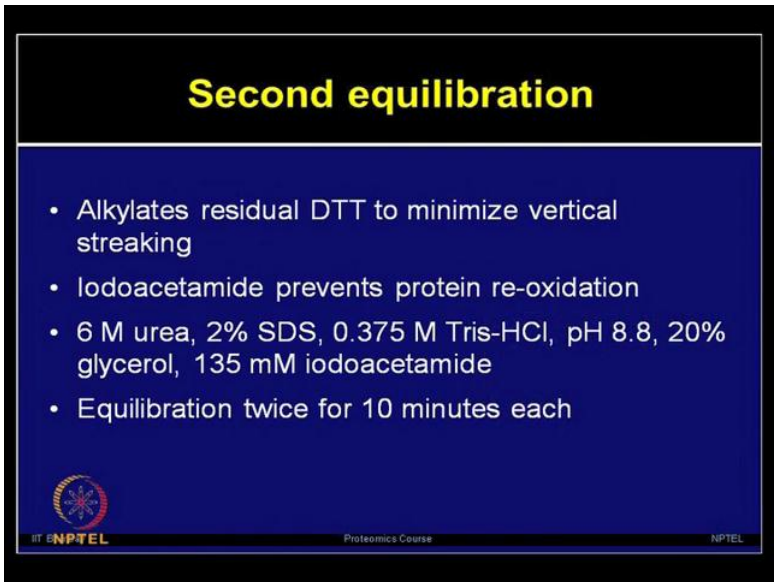


Now, this is very important because i e a can alkylate, the residual DTT and it minimizes the vertical streaking. It also prevents reoxidation. So, you want to really ensure that your protein is denatured again after doing the IES process it is coated with SDS molecule; so that now, you can separate 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 steps are done then you are ready to perform the SDS page.

I will show you in this slide the briefstep 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 train in a well. And then you change that with a second equilibration solution, the DTT reductant will be used for the first step followed by i aaiodoacetamide, which functions as a scavenger of the excess reductant and alkylates and prevent the reoxidation. So here, you can see howdifferent bonds are broken from the disulfide and i a s preventing the reoxidation alkylating these residues.

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After having discussed the equilibration step, now we can talk about how to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS page. As I mentioned when you perform SDS page alone for separating your protein extract then, you need to add both stacking and resolving gel. Now, in this case when you are using SDS page only in the second dimension of two dimensional electrophoresis; then you do not need to add the stacking gel you have already focussed the protein in thegel in the first dimension based on the IEF.

Now, a second dimension separation, you have prepared the strip to be loaded on the SDS page gel, sorest of the step can be uniform or similar to what one can expect in regular SDS page.

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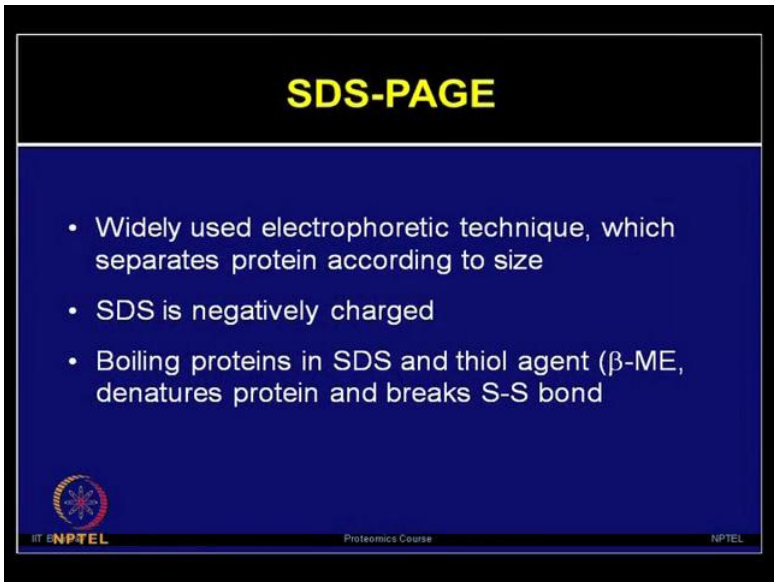


So, SDS page is one of the very widely used electrophoretic technique, which separates protein based on their size or the molecular weight. So, this molecule SDS or sodium dodecyl sulfade which is an anionic detergent it provides negative charge. It binds at a ratio of 1.4 gram of SDS per gram of protein to provide almost similar charge to mass ratio. Therefore, your protein separation will be mainly based on charge in the electric field. As I mentioned there is step required to prepare your protein for denaturation.

So, you need to boil your proteinsin SDS and beta mercaptoethanol and DTT to denature the protein and to remove or break any disulfide bonds. Now, since you cannot do these step boiling step in yourtwo dimensional electrophoresis, where you have already focussed a strip IPG strip in the first dimension, soyou need to do the equilibration step rest of the steps will be very uniform.

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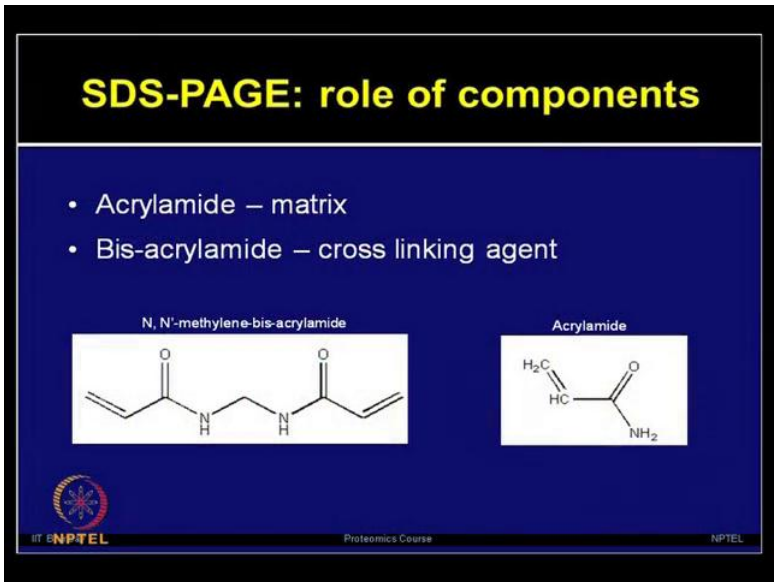


So, let me describe you the role of the components which are being used in SDS page. Acrylamide it provides the matrix or gelling for making the gels. Now, bis-acrylamide that is an cross linking agent, by adding both acrylamide and bis-acrlamide in a fixed ratio, one can make this gel by adding the gelling agent and the cross linking agent.

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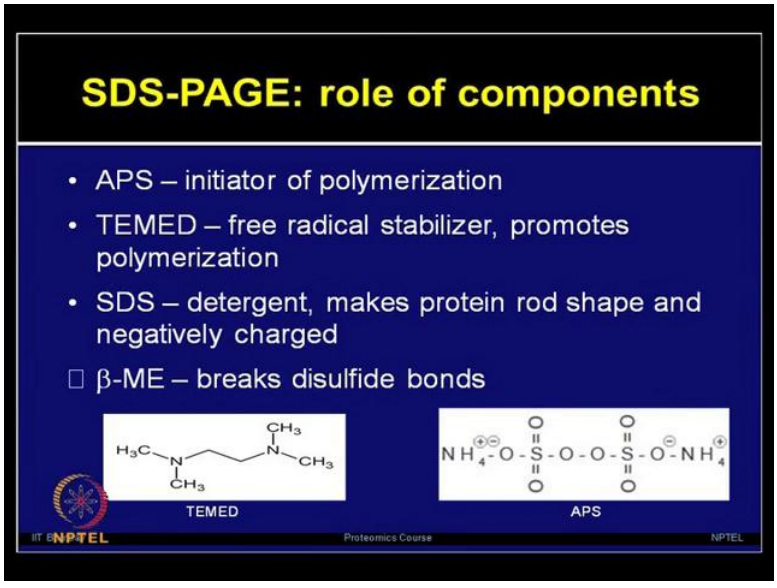


But,that itself is not sufficient to polymerise the gel, you need to add few more reagents which include APS or ammonium persulfate which initiates the polymerisation process, stemed that is a free radical stabilizer it also promotes the polymerisation of the gel process.

So, beta meecaptoethanol is not used in making a gel, but for your protein treatment to break the disulfide bonds. So, I hope you are at least able to understand the role of each of these component, which are being used in SDS page either during the protein preparation of doing the SDS page or making the gel to separate the protein.

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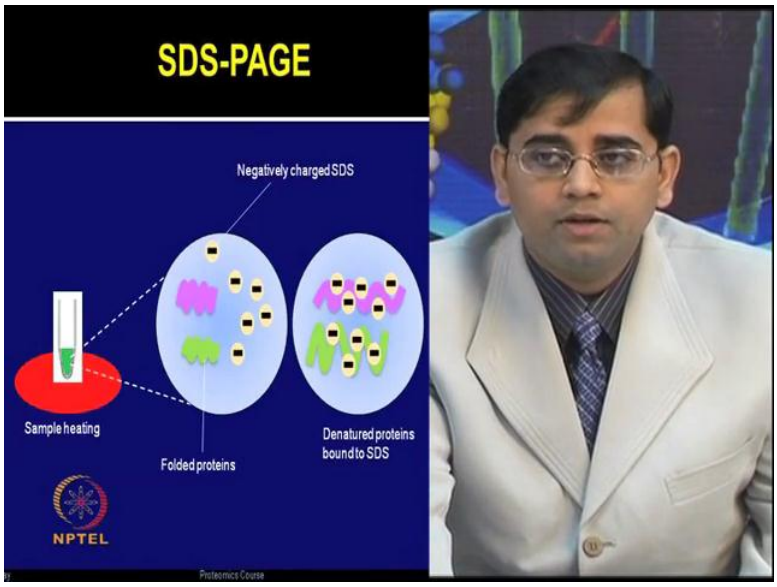


Now, once this step is done you need to boil your protein sample, if you are performing a regular SDS page the negatively charged SDS molecule will bind to the folded proteins; it will denature to the protein and provide the negative charge.

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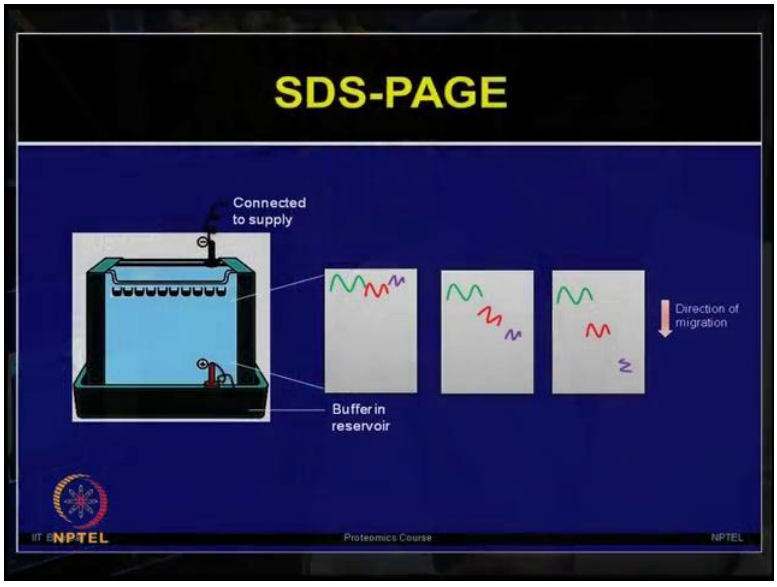

Once this protein preparation is done then you can load this protein on SDS page gel and based on the molecular weight, the lower size proteins will migrate the farthest and the higher molecular weight protein will remain near the top surface of the gel. So, depending upon the protein size you can separate the protein based on the lower and hollow higher molecular weight in this SDS page gel. You can add the standards or known molecular weight proteins to determine your unknown proteins.

Since, we are talking here about two dimensional gel electrophoresis work flow, I think its important to clarify that in 2 DE, when you use SDS page you do not need to make these wells by adding a comb; because you already have a flat IPG strip which has done the first dimension protein separation. So, in this case you need to make a flat com you need to make a flat well. So, that your IPG strip can be placed on top of the SDS page gel. Again to remind you here, you are only making the resolving gel for better resolution and separation of the protein stacking part is not done for the 2 DE, which has already been done during the first process of IEF.

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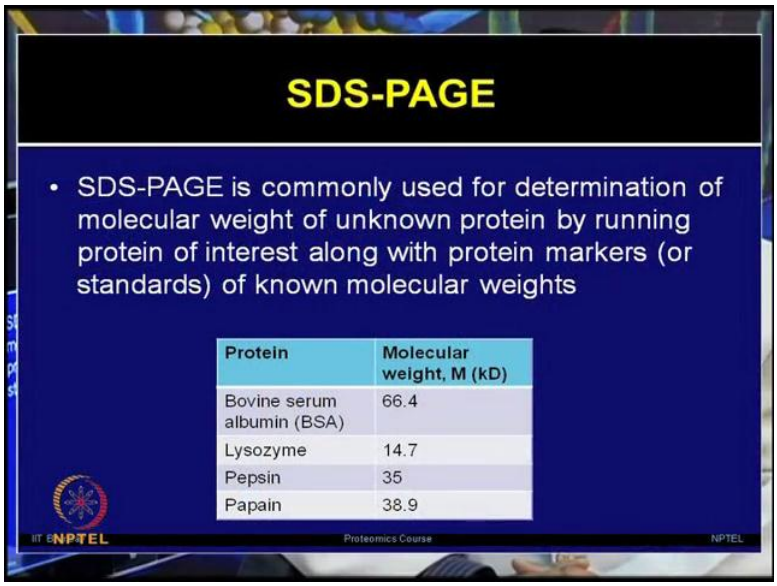


So, SDS page is one of the very commonly used methods for determining the molecular weight of a protein of your choice or unknown protein, which you want to know how much molecular weight it is. So, if you run the standard protein markers along with your unknown protein, you can determine the molecular weight of the unknown proteins.

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I am showing you one SDS page image in which from the left to the right, you can see the first lane is a standard or known molecular weight in this well you can see there are different at least 5 or 6 bands are visible; these are the known molecular weights. Sometimes, starting from 150 kilo Dalton to 10 kilo Dalton of molecular weight range, then we have different wells and in each well unknown protein for which you want to determine the molecular weight can be loaded and after staining with coomassie blue, you can see a pure band here in this case. So, one can determine the molecular weight by performing the SDS page.

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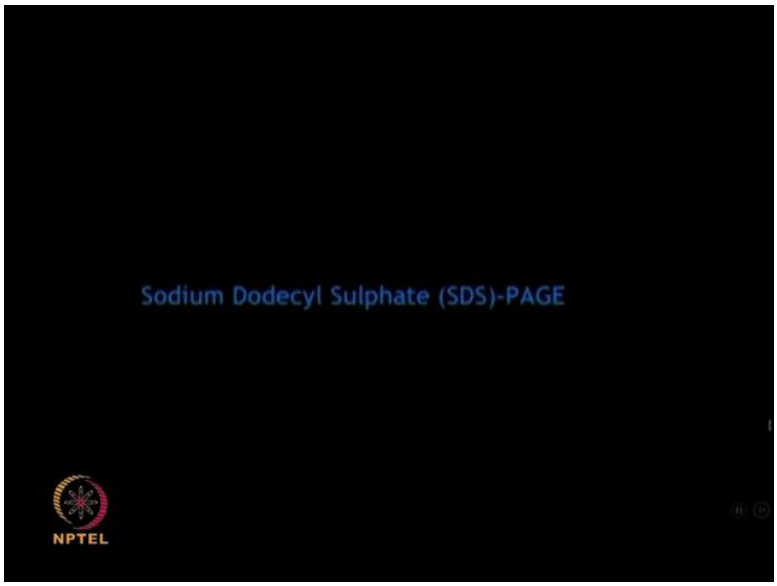


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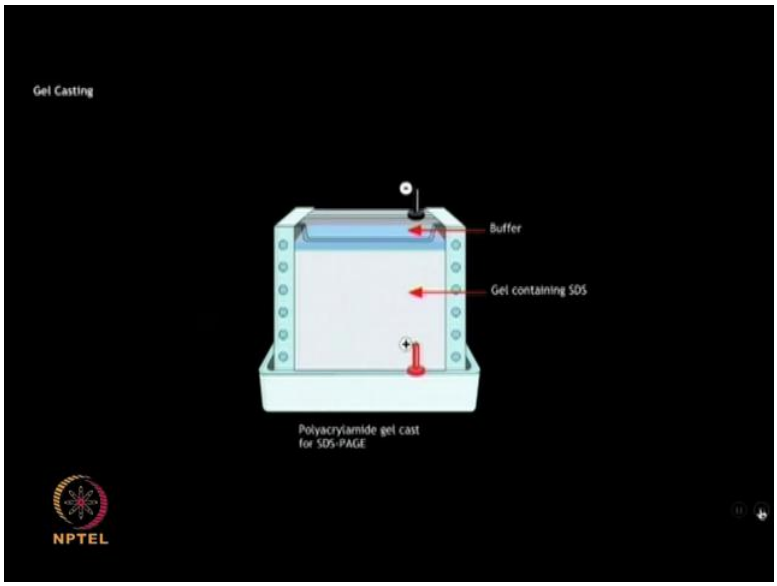


I will now describe you the animation and show you the steps involved in SDS page sodium dodecyl sulphate SDS polyacrylamide gel electrophoresis SDS page in this animation, you will see various step involved in separating proteins based on their molecular weight. First of all polyacrylamide gel containing SDS is cast between the glass plates as a vertical slab in the same buffer, that is used for electrophoresis. The molecular dimensions of the port can be controlled by varying the amount of n n methylenebisacrylamide with free radical cross linking being facilitated by ammonium persulphate or APS and timid.

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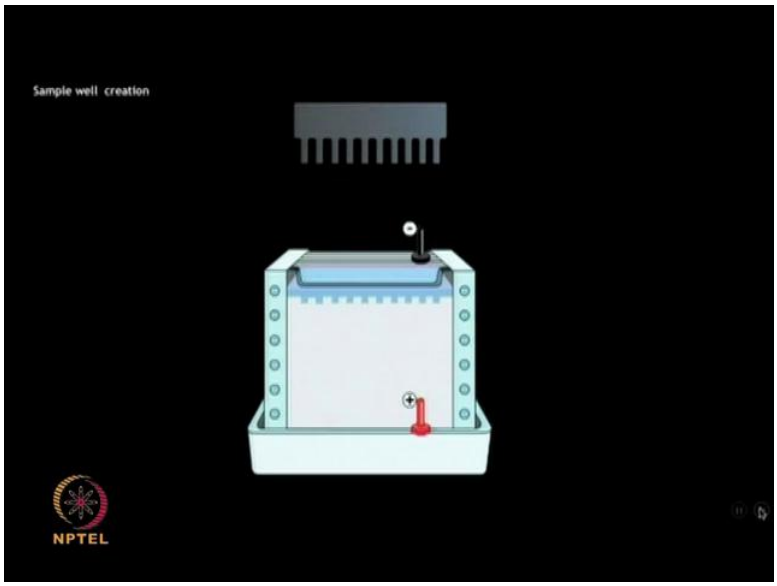


Next step is to create the wells on these gels a sample wells of uniform size, shape and separation are made using a comb, which is placed in the gel (( )), it has been poured. After the gel has polymerized the comb is removed to provide the gel ready to start the process.

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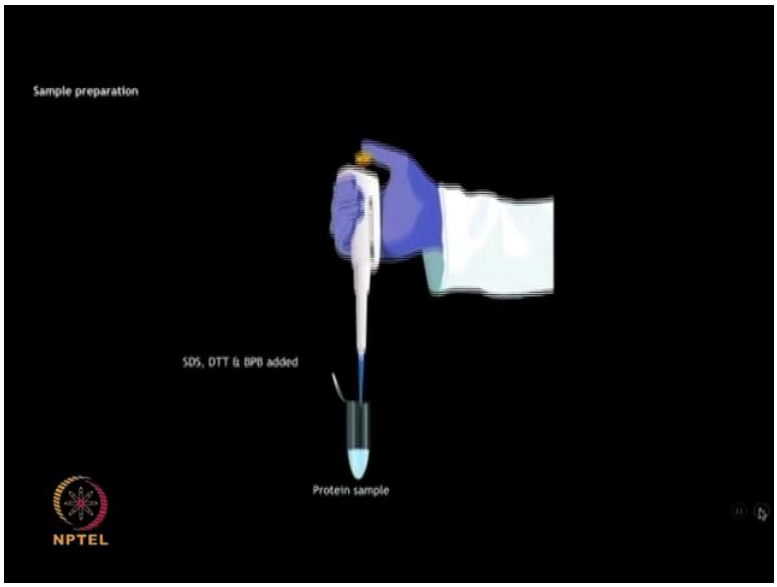


Now, you can prepare the sample to load on a SDS page gel.SDS is a negatively charged an ionic detergent that binds to protein molecules and causes them to denature.

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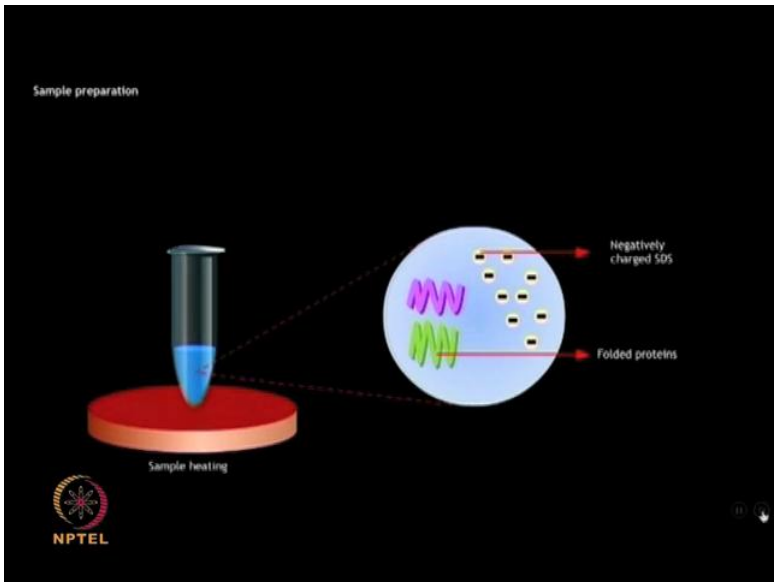


The DTT used breaks any disulphide linkages that may be present during the process, you boil or heat your protein samples to denature the protein. Now, the negatively charged SDS molecule will bind to the proteins and denature them.

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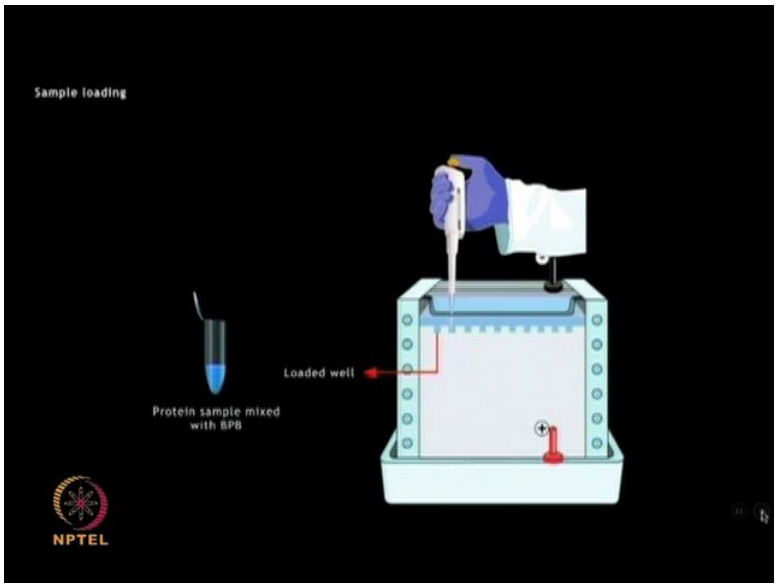


The binding of SDS causes the proteins to have uniform charge to mass ratio there by allowing the separation purely on the bases of molecular weight. The protein samples can be loaded on the wells by a using a micropipette. As you can see in the animation you can

use different wells to load different type of samples. Once all the samples are loaded then you can take this unit and move into an electrophoretic operators.

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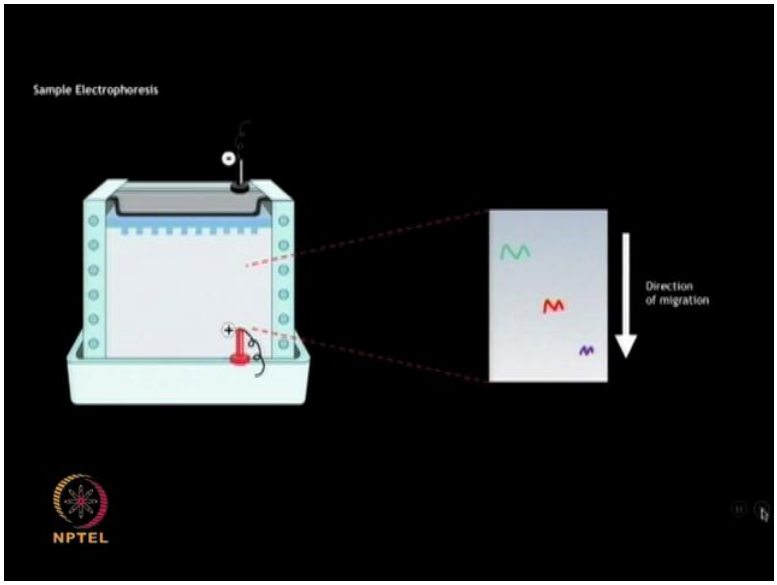


A direct current supply between 100 to 350 volt is passed depending upon the size of gel for a time sufficient to separate the protein mixture into discrete bands based n their molecular weight.

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The progress of electrophoresis can be observed with the help of cracking dye. The larger proteins are retarded in the gel and remain close to the point of application while the smaller

proteins migrate further along the gel. The gel can bethen stained with either coomassie or silver stain and viewed to observe the various discrete band discrete protein bands present in each of the sample. For example, the left image here is silver stained gel and the right image is of coomassie stained gel. You can load the molecular weight marker or the standells as shown in the first and the last and the 10th well and then unknown protein sample for which you want to determine the molecular weight can be separated in different wells.

So, I hope after looking at this animation now you are clear, how to make a gel to separate the proteins based on their molecular weight. And I will show you later on onelaboratory demonstration, how to use SDS page gelfor separating proteins in two dimensional electrophoresis.

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So, let us now move to a variation of SDS page known as blue native page blue native polyacrylamide gel electrophoresis. Now, in SDS page we are providing a denaturing environment; we want to denature the protein where as in the blue native page we want to separate the proteins in their native condition. And by applying these two techniques together by using SDS page and blue native page, one can obtain very unique and different information often related to the isoforms, subunit composition, molecular weight and different type of post transitional modification.

This information can be obtained either alone from SDS page or from blue native page or by combining these two together toget the integrated information. So, I will briefly describe

you here the blue native page. Please keep in mind this is not going to be used for 2 DE, but often people also use the native form for the 2 DE separation.

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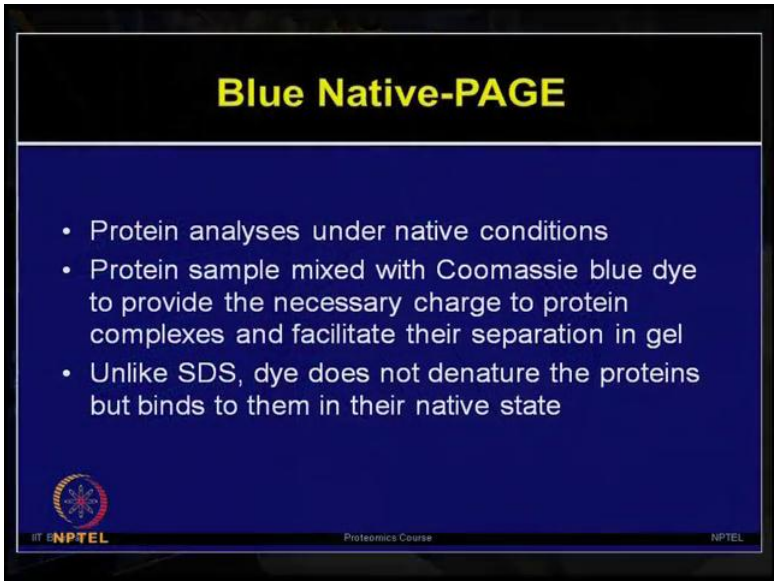


So, in the blue native page the protein analysis is performed under the native condition. Here, you are not going to boil your protein sample with SDS and providing different type of denaturing condition. So, you need to add a sample with coomassie dye which provides the necessary charge required for protein complexes to separate in the gel. But, as I mentioned unlike SDS this dye will not denature the proteins, but it will bind in their native state itself.

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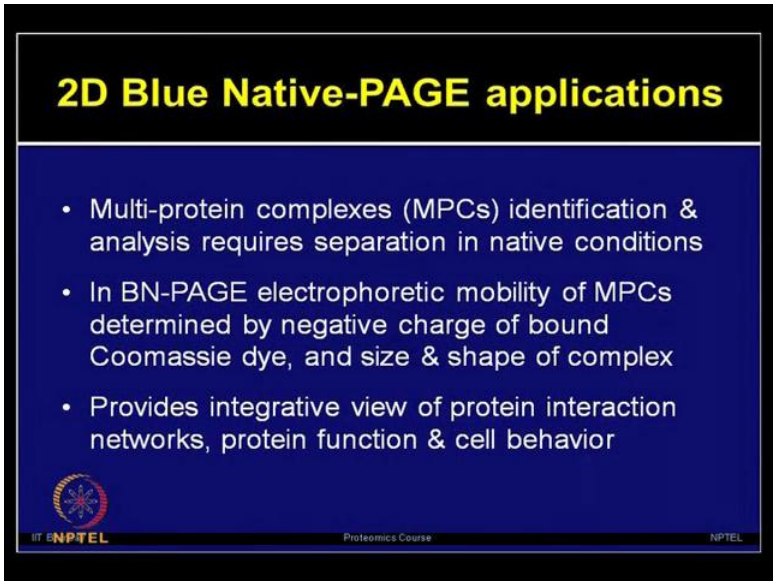


People apply the 2 D blue native page for various application, such as studying about the multiprotein complexes or MPC's. So, identification of multiple protein complexes is not possible by using the SDS page or denaturing environment. So, MPC's can be identified by using the blue native page. In blue native page, the electrophoretic mobility of MPC's or multiprotein complexes determined by the negative charge of bound coomassie dye and size and the shape of complexes.

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So, this technique can provide the integrated view of protein function. I will now show you an animation, how to perform the native polyacrylamide gel electrophoresis.

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Blue Native page or BN page.

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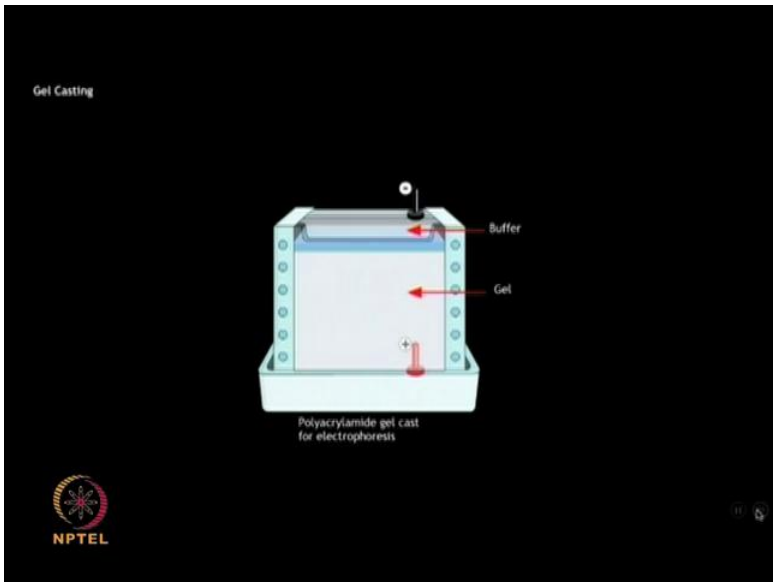


The polyacrylamide gel is cast between the glass plates as a vertical slab in the same buffer, that is used for electrophoresis. The gel is prepared by free radical induced polymerization of acrylamide and bisacrylamide in a suitable buffer ammonium persulphate and timid are added to facilitate the generation of free radicals and cross linking. The molecular dimensions of the ports can be controlled by varying the amount of n n methylenebisacrylamide.

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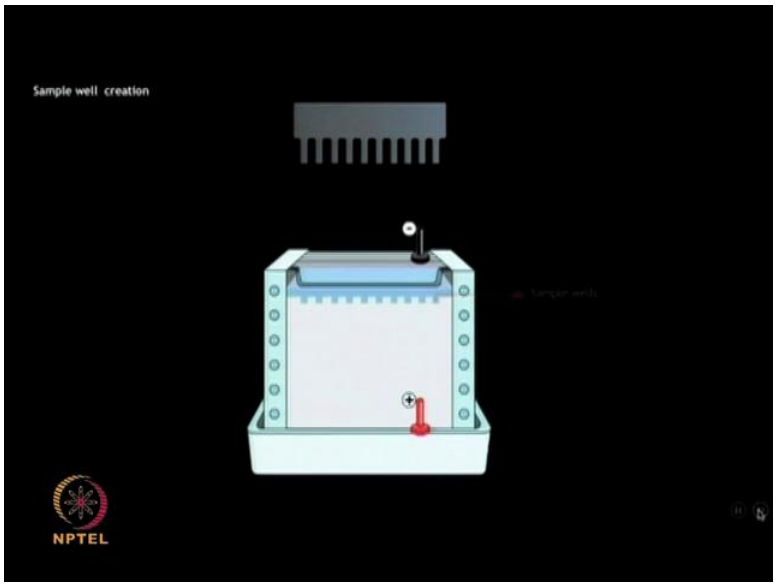


Once the solution is poured then sample wells can be created, the sample wells of uniform size shape and separation are made using a comb, which is placed in the gel as soon as it has been poured. After the gel has polymerized the comb can be removed to provide a gel ready for the process.

Sample preparation, the protein sample present in a suitable buffer is mixed with coomassie blue, which provides the necessary charge to the protein complexes there by facilitating their separation in the gel, unlike SDS a dye does not denature the protein, but binds to them in the native state. The protein samples are then loaded into the wells with the help of a micropipette. As it is shown in the animation, you have different wells to load different protein samples. You can use one of the wells to add known or standard markers and other wells can be used to separate the unknown proteins.

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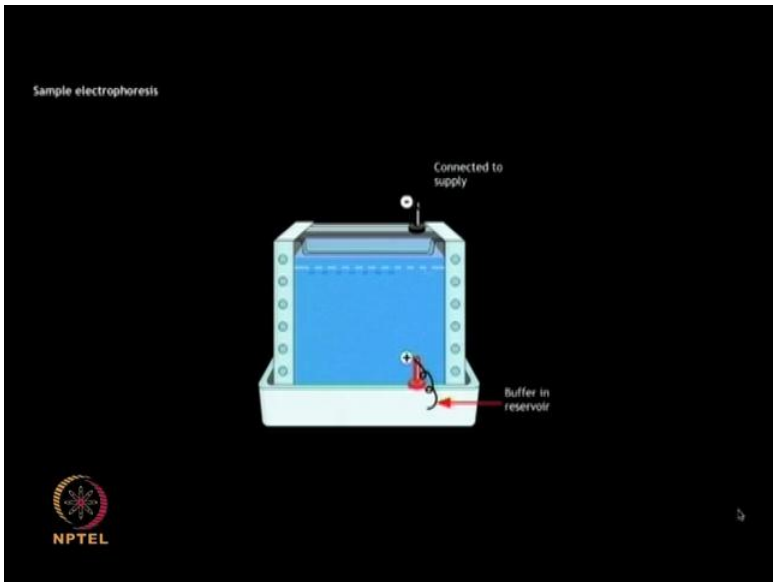


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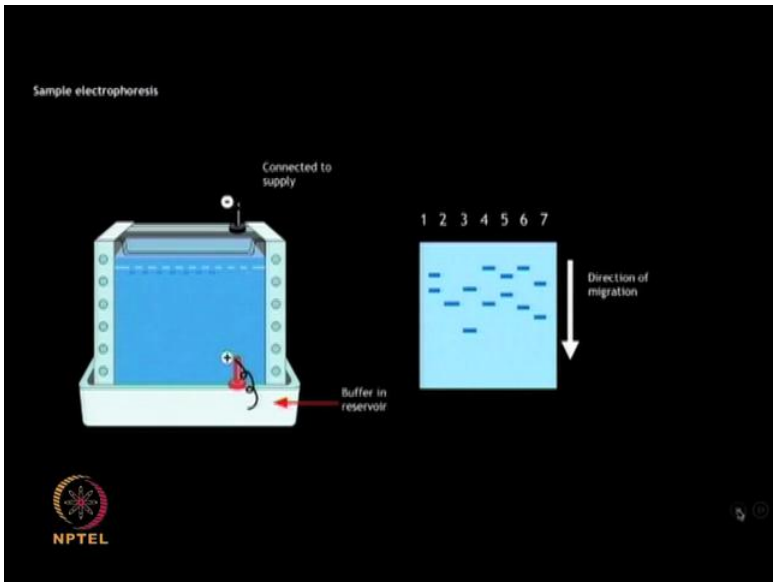


Once all the sample are loaded then this unit can be placed into electrophoretic unit and a direct current supply of around 100 to 350 volt can be passed depending upon the size of the gel for a time sufficient to separate the protein mixture into discrete band based on their mass, charge to mass ratio. The progress of electrophoresis can be observed with the help of tracking dye.

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The larger proteins are (( )) in the gel and remain closed to the point of application while the smaller proteins migrate further along the gel. The gel can be then stained with coomassie brilliant blue and viewed to observe various discrete protein bands. After watching this animation, I hope you are able to understand how to perform native polyacrylamide gel electrophoresis and these concepts can be applied for using the 2 DE as well.

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So, now you are familiar that how you can apply both SDS page and blue native page and you can obtain unique formation about sub unit composition and different type of isoforms from different proteins; and you need to apply both the techniques together. Now, you are also familiar how to determine the molecular weight of an unknown protein.

So, as I mentioned you have seen the classical SDS page and blue native page, the concept is similar and it can be applied in the two dimensional electrophoresis there are little variation here; because now you do not need to add comb you have to have a flat comb for making a well to add yours IPG strip and you do not need to add the separation for a stacking gel.

So, in this laboratory demonstration video, I will show you further two dimensional electrophoresis, how to make the SDS page. So, equilibrium process will be demonstrated as well as how to run the SDS page for 2 DE.

Protein separation by SDS page, SDS page which constitutes the second dimension of two dimensional gel electrophoresis involves assembly of the gel apparatus gel castingequilibration of the IPG strip followed by placement of the IPG strip on the gel and protein separation. Assembly of gel apparatus and gel casting, clean all the components of the gel assembly thoroughly with water and dry them with paper towels. Carefully arrange the glass plates in the gel casting assembly interspersed with separator sheets depending onthe number of gels to be run.

These assembly should be tightly packed such that there are no leaks.Close the casting assembly and tighten the screws provided, prepare the gel casting solution consisting of acrylamide, bisacrylamide, tris chloride, STS,APS and timid and pour it with a help of a funnel into the central channel of the casting assembly.APS acts as a free radical generator while timid functions as a catalyst for the polymerization reaction.Ensure that the solution spreads evenly such that the top edge of the gel is uniform, allow the solution to stand for polymerization to occurand spray a solution of STS across the top that the gel does not become dry.

Equilibration of IPG strips, place the thawed 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 DTT. Place the assembly on

mechanical shaker for 10 minutes the dithiothreitol enables cleavage of all disulfide bonds present in the proteins on the IPG strip.Next, add the second equilibration solution to another wellof the tray,this solution has the same composition as the first, except that DTT is replace by iodoacetamide.

Transfer the IPG strip into this solution after draining off any access liquid from the first .Again place the assembly on the shaker for 10 minutes iodoacetamide prevents reformation of the disulfide bonds by binding to the free sulfidal groups that were obtained, after DTT treatment.

SDS page, drain out the excess liquid of the second equilibration solution from the IPG strip using a tissue paper. Soak the strip for few minutes in the same buffer solution, that is to be used in the gel tank. Then, carefully place the strip in between the glass plates and gently push it in until it rests firmly on the gel surface without any gaps.

Place the filter paper containing the molecular weight marker, beyond the positive end of the strip. Then, pour the luke warm agarose overlay solution over the strip to prevent the gel from drying up. Assemble the remaining plates in the gel assembly and carefully lower the apparatus into the gel tank containing the buffer solution. Ensure, that buffer level does not raise above the mark indicated. Place the separating chamber above this and pour the buffer solution into the upper chamber, close the gel assembly and connect the apparatus to the electricity supply; set the appropriate voltage and begin the run.

All the proteins present on the IPG strip carry a large negative charge due to the action of SDS, which denatures the protein and binds to the polypeptide backbone at a constant weight ratio. This this ensures that the proteins present get separated exclusively on the bases of their molecular weights rather than their mass to charge ratio, as in a native page. The proteins having lower molecular weights have high mobility and migrate further through the gel pores.while the high molecular weight proteins remain closer to the point of application. This allows efficient separation of the proteins in the second dimension.

So, now we will talk about how to satin the gel and visualize the protein spot. Because so far now, you are able to separate the protein based on their p i based on their molecular weight; and now everything is on a transparent gel you do not know how well your separation has occurred, so now, you need to visualize your spots. So, different type of

straining methods have been developed and those can be applied to visualize your protein bands or your protein spot depending upon what type of gel you are using. So, after giving you an overview of staining techniques let me describe you few staining methods in more detail.

Let us start with coomassie blue staining this is one of the most commonly used stain for protein detection in polyacrylamide gels. The sensitivity is good,, but not very sensitive as compared to the silver or sypro ruby, but it is ease of performing the staining method stability the cost very less; and good compatibility with mass spectrometry all of this make coomassie blue as a stain of choice for most of the laboratories worldwide.

Now, there are some advanced from of coomassie stains being developed including bio safe coomassie stain, which is non hazardous made keeping mind the environmental scenario, soit can be disposed as non hazardous waste. The bio safe coomassie is also ready to stain and a single reagent protein satin. So, I will shown you one animation, how to perform the coomassie blue staining. In this animation, I will show you how the staining can be performed by using coomassie brilliant blue stain.

The completed electrophoresis gel is placed in a tray containing the coomassie blue staining solution typically are 250 that has been dissolved in an aqueous solution of methanol and acetic acid. The negatively charged coomassie dye interacts with protein through ionic and non covalent interactions. After adding the stain the tray is then placedon a mechanical rocker which allows for uniform contact of the gel with the solution by means of gentle rocking after overnight stain you have to grain out the staining solution.

The stained gel is then placed in a destaining solution, which consist of 50 percent methanol and 10 percent acetic acid to remove any access dye that may be bound to the gel. Again this processinvolves overnight steps or at least ten to twelve hours of gentle shaking on the rocker. This stained gel can finally, be viewed as shown this image here showing a 2 D gel stain with coomassie brilliant blue and it can be scanned by using a scanner and this protein spots can be easily viewed. So, in this animation you are able to see after performing the2 D electrophoresis, how to add the coomassie stain on your gel do the staining and destaining procedure. So that, you can visualize your blue spots on

the transparent background. Each of the spot can be nowused further for image analysis purpose.

So, most of the time, if you are lucky and if your protein extraction protocol is good you have good protein yield, but often your samples are very challenging despite your good extraction protocol and your good laboratory skill still you are unable to obtain a very good protein quantity. So, how to move forward you want to still do the separation of the protein, but coomassie stain may not be sufficient to visualize the spot on the gel. Now, you need few more sensitive stains such as silver stain, so I describe you how to do silver staining the silver staining is more sensitive than the coomassie blue dye.

First of allwhen you have run your 2 DE gel you need to do the protein fixation in the gel by adding methanol and acetic acid.The staining procedure involves silver mine complex, which is bound to the tungstosilicic acid. The silver ions are transferred from the tungstosilicic acid to the proteins. Now, by providing alkaline solution with formaldehyde it can reduce the silver ions to the metallic silver form; and the brownish tinge you can see on the spot which can be further analyzed, but if you allow the reaction to occur continuously, you will see a very dark background on the silver stained gel, soyou need to stop the reaction by adding acetic acid.

I will now show youthis animation, how to perform the silver staining procedure. In this animation of silver staining I will describe you the procedure how to stain the gel by using silver stain. The completed gel is first placed in a fixing solution of methanol and acetic acid that fixes the protein bands in the gel and minimizes any diffusion.

This step must be subjected to gentle shacking for around 30 minutes after which the silver stain solution is added. After adding the silver solution, the gels are rubbed gently in order to allow for proper and uniform staining. The silver stained gels are first washed to remove any access stain and then place in a developing solution, where the silver ions get reduced to metallic silver.

Formaldehyde in an alkaline solution in the presence of sodium carbonate or other alkaline buffers are commonly used for this process. Again a gentle rocking will allow for uniform staining pattern. Finally, the gel can be viewed as dark bands or dark spots against a light background, as you can see in this 2 D gel image, the spots having the dark background are stained with silver stain.

So, let us see what we have learnt today in summary, we started with two dimensional electrophoresis workflow. The first step the equilibration we discussed today isoelectric focusing was discussed earlier in the previous lecture. Then, we talked about how to perform the separation in the second dimension during that course we talked about SDS page and blue native page. Then finally, we talked about different type of staining methods available, we looked at in more detail about coomassie staining and silver staining.

In the next lecture, we will continue with this work flow of performing two dimensional electrophoresis, we will talk about few more sensitive stains available, how to use those then how to analyze the images by suing different software; and then to perform the statistical analysis of those. So, that we can identify the interesting protein spots for a biological question. I hope now you are able to follow the workflow of 2 DE and we will continue in the next class, thank you.