Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 03 Lecture Number 11

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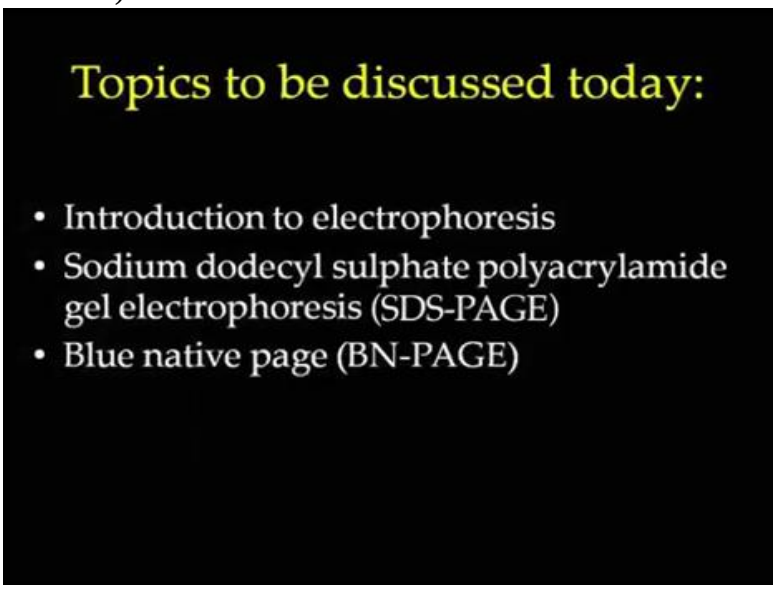

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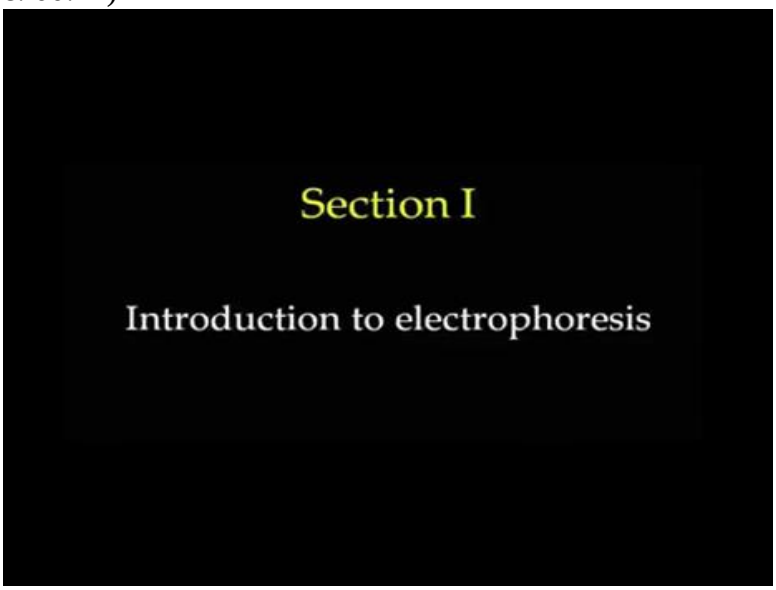

Let me give you the historical perspective of electrophoresis.

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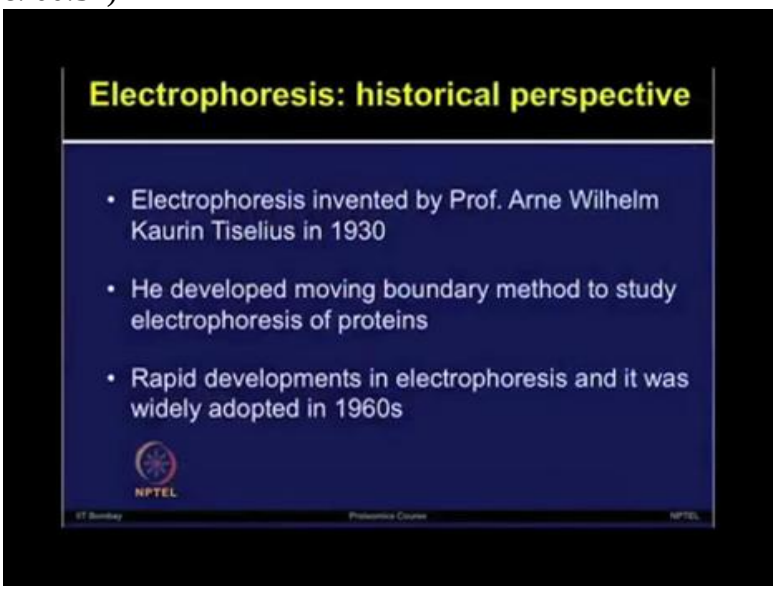

This process was invented by Professor Tiselius in 1930. He developed the moving boundary method to study the electrophoresis of proteins. Since then lot of development happened in the field and during the 1950s and 60s this process was highly adopted in different laboratories.

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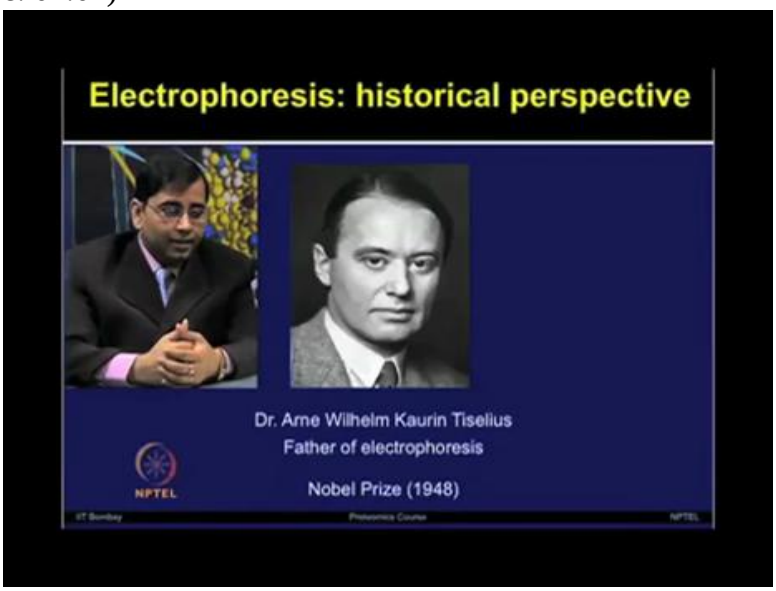

So here is Doctor Tiselius who is also known as the father of electrophoresis. For his great contribution in chemistry, he obtained the Nobel Prize in 1948.

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So now we will describe one dimensional electrophoresis, some of the concepts involved in that.

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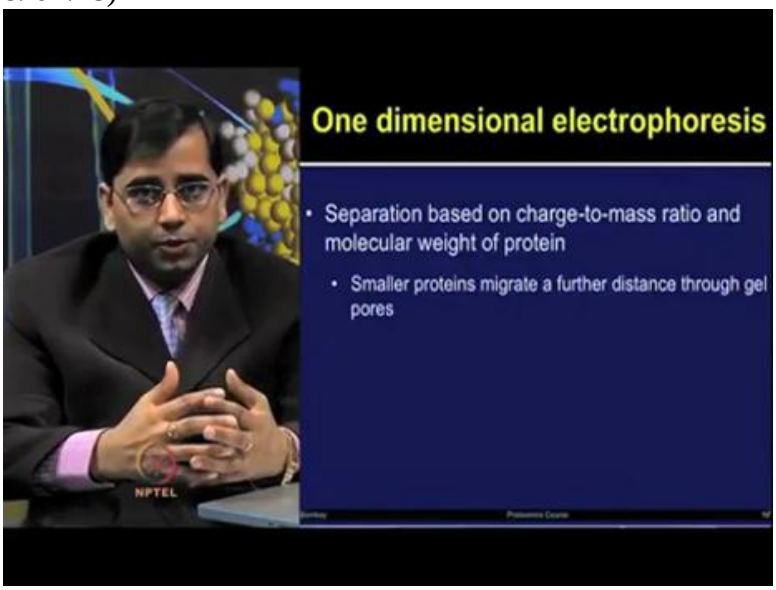

So in the one dimensional electrophoresis, separation is based on the charge to mass ratio and the molecular weight of the protein. Therefore on a given gel, if you have smaller the protein in the electric field, they will move further down and the higher molecular weight proteins, they will remain on the top.

So if you apply some standard molecular weight markers which give you the known molecular weight of the proteins, then for a given unknown protein, you can determine the

molecular weight and it is expected that the higher molecular weight proteins will remain on the top, and the smaller molecular weight proteins will reach towards the bottom of the gel.

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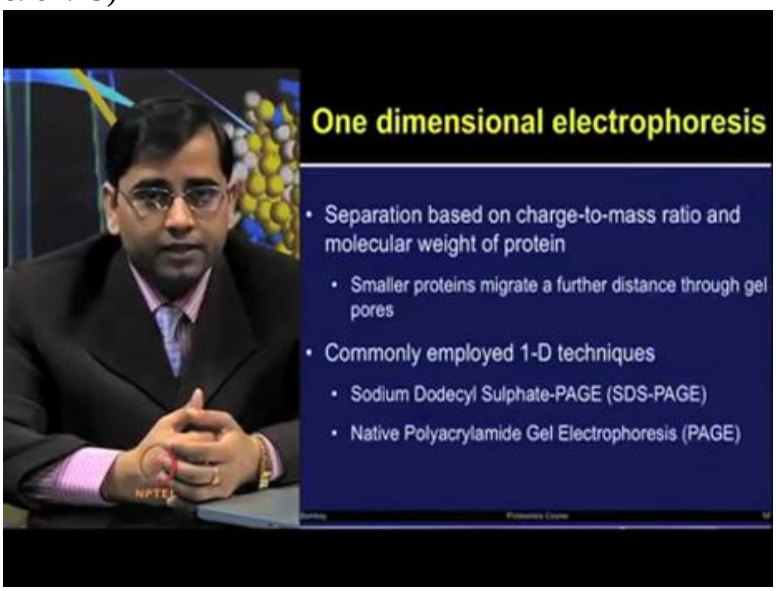

So, commonly implied one-dimensional techniques include the sodium dodecyl sulphate-PAGE SDS PAGE or other technique which is used for studying the protein in the native conformation Native Form which is known as native Polyacrylamide Gel Electrophoresis or PAGE.

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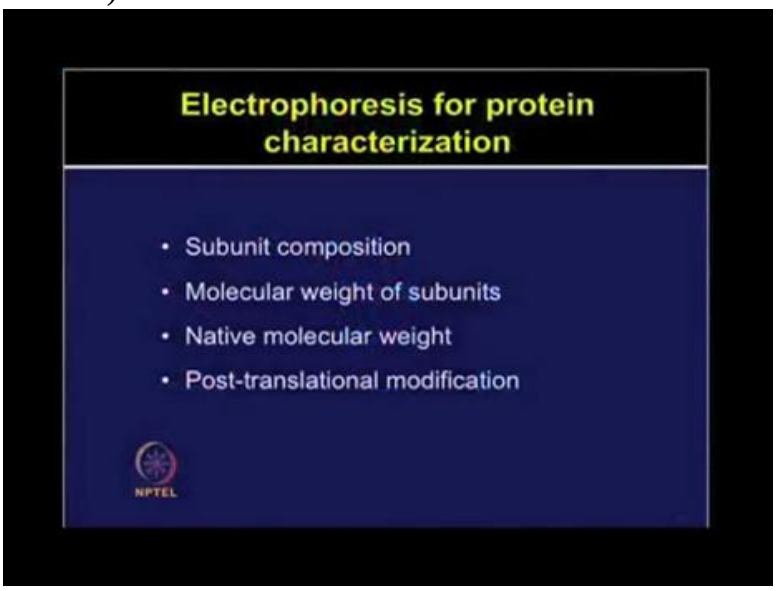

The electrophoretic techniques can be used for the protein characterization to study different properties, for example, the subunit composition of proteins. What is the molecular weight of those subunits? What is the molecular weight of native proteins?

Different types of post-translational modification; again, only one technique may not be able to answer all of these questions so often you have to involve more than one type of electrophoretic method. For example, while comparing native and subunit composition forms, you need both native electrophoresis and SDS PAGE.

If you want to look for post-translational modification, then SDS PAGE would be useful. If you want to compare the proteins based on their Isoelectric Point as well as the separation of isoforms or PTM forms based on the molecular weight, again two-dimensional electrophoresis will be useful.

So one needs to know the concept and the laboratory way of using this technique so that, by applying a combination of the techniques one can study different types of properties.

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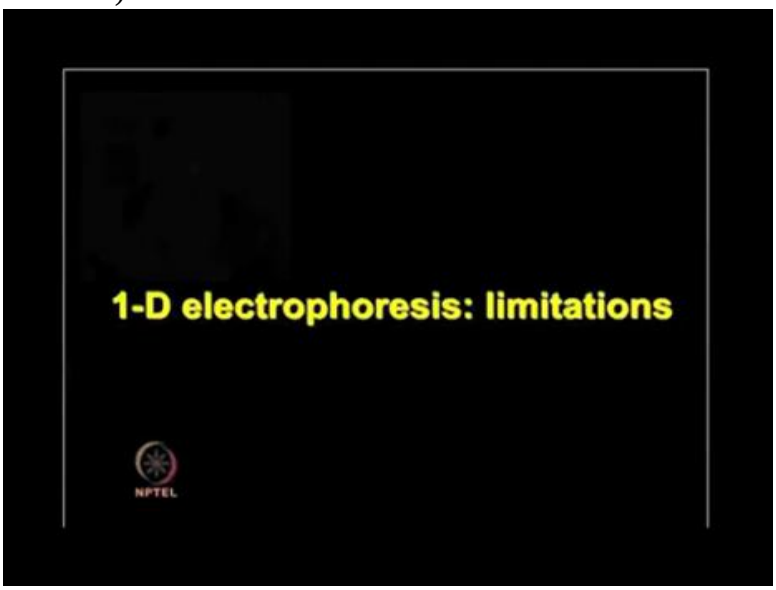

However the one-dimensional electrophoresis has different limitations. You cannot separate your complex proteomes, the complex protein samples. If you have thousands of proteins and you want to study them, then one-dimensional electrophoresis has limitations.

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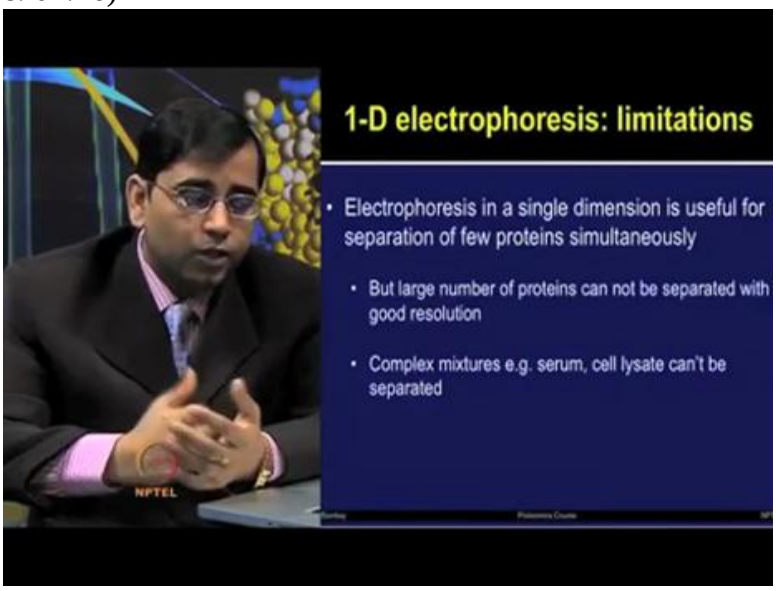

So the one-dimensional electrophoresis is useful to separate few proteins or a simple protein mixture. But when you are studying the complex mixture then two-dimensional electrophoresis or DIGE or different types of mass spectrometry based methods will be more useful.

So if you have very complex samples like cell lysates or you have serum, then separating those and comparing different types of samples on SDS PAGE may not be a very good way. In that case you should apply different methods.

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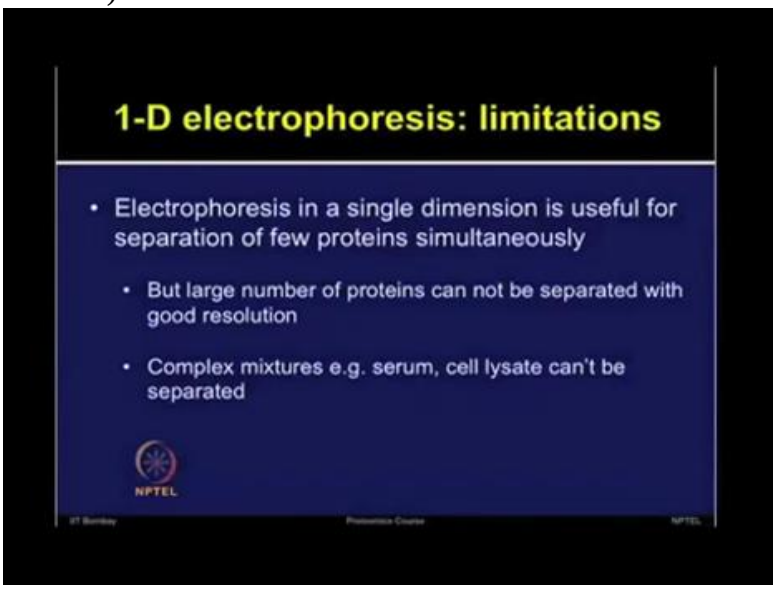

So one-dimensional electrophoresis is very useful and is being used in almost all the laboratories working in the lysis area but it can give you few questions. It can address only

those questions. But if you want to get more characterization, then you have to apply different techniques.

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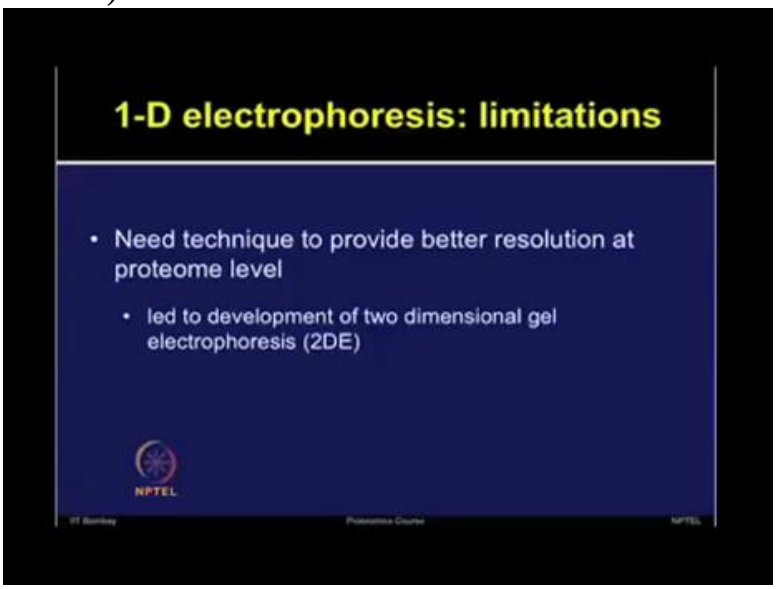

So to overcome these types of limitations and to obtain the better resolution of protein separation, the two-dimensional electrophoresis was applied.

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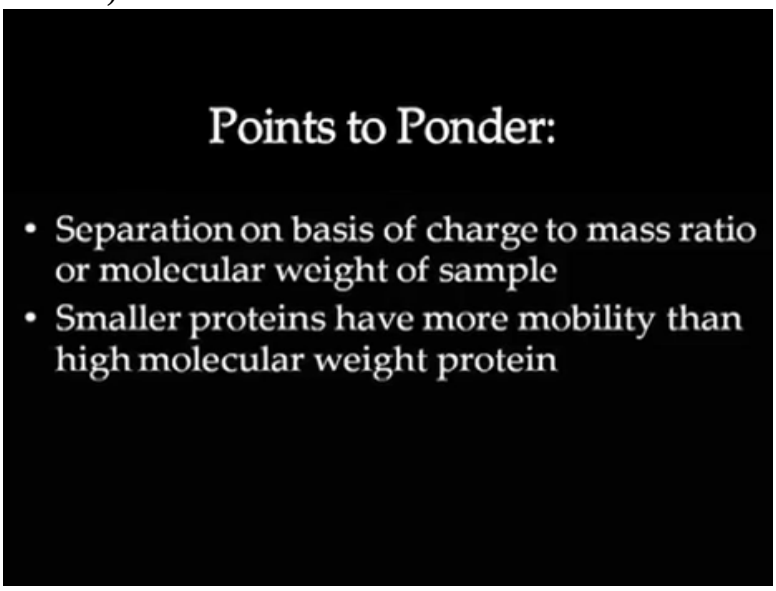

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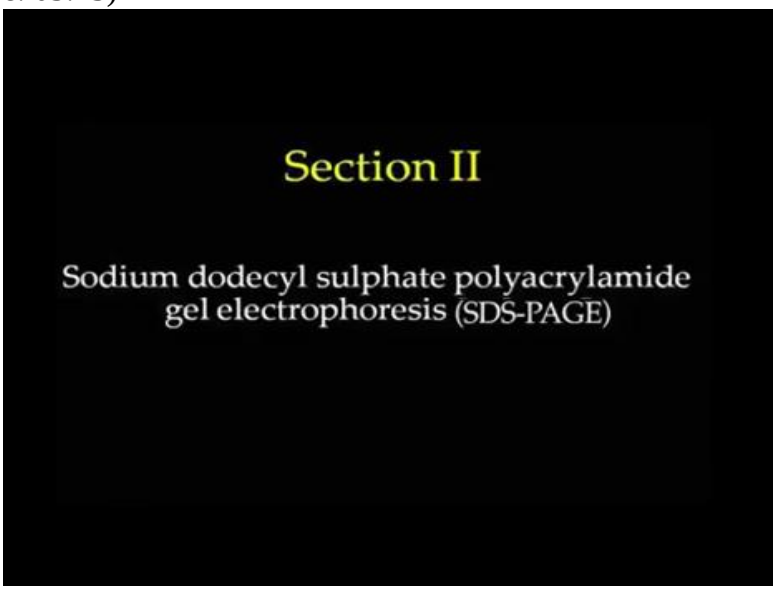

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Now we can talk about how to perform Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis or SDS PAGE.

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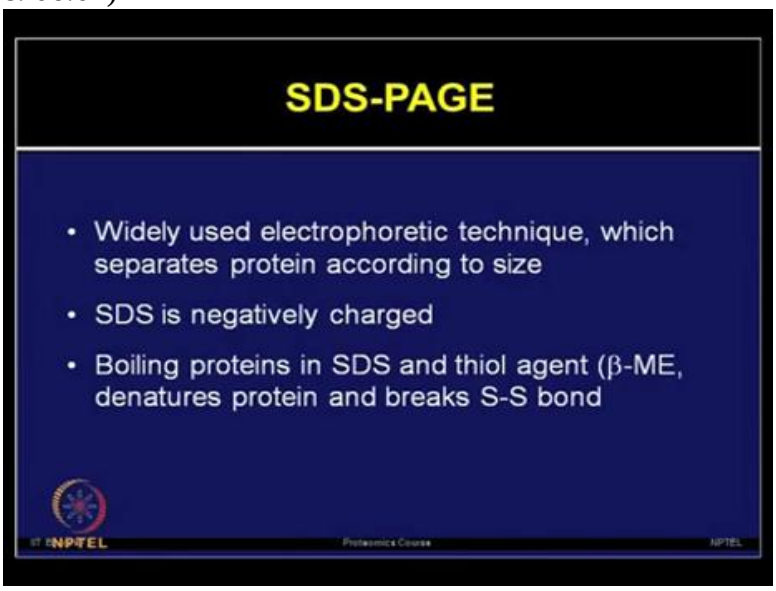

So SDS PAGE is one of the very widely used electrophoretic techniques which separate protein based on their size or their molecular weight. So this molecule SDS or Sodium Dodecyl Sulfate which is an anionic detergent, it provides negative charge. It binds at a ratio of 1.4 gram of SDS per gram of protein to provide almost similar charge to mass ratio.

Therefore your protein separation will be based on the molecular weight in the electric field. As I mentioned, there is step required to prepare your protein for denaturation. So you need to boil your proteins in SDS and beta Mercaptoethanol and DTT to denature the protein and to remove or break any disulfide bonds.

Now, since you cannot do this step, boiling step in your two-dimensional electrophoresis where you have already focused the strip, IPG strip in the first dimension, so you need to do the equilibration step. Rest of the steps will be very uniform. So let me describe you the role of the components which are being used in SDS PAGE.

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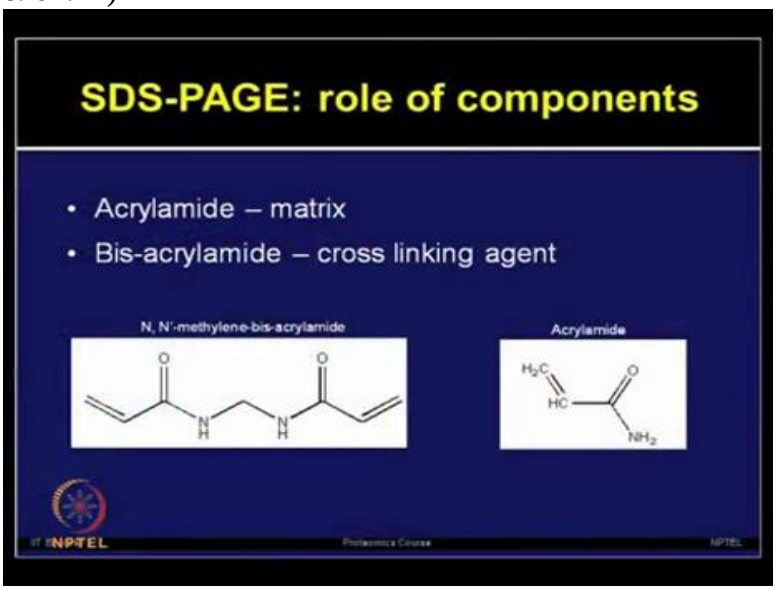

Acrylamide, it provides the matrix or gelling for making the gels. Now Bis-acrylamide, that is a cross-linking agent. By adding both Acrylamide and Bis-acrylamide in a fixed ratio, one can make the gel by adding the gelling agent and the cross-linking agent.

But that itself is not sufficient to polymerize the gel. You need to add few more reagents which include APS or ammonium persulfate which initiates the polymerization process.

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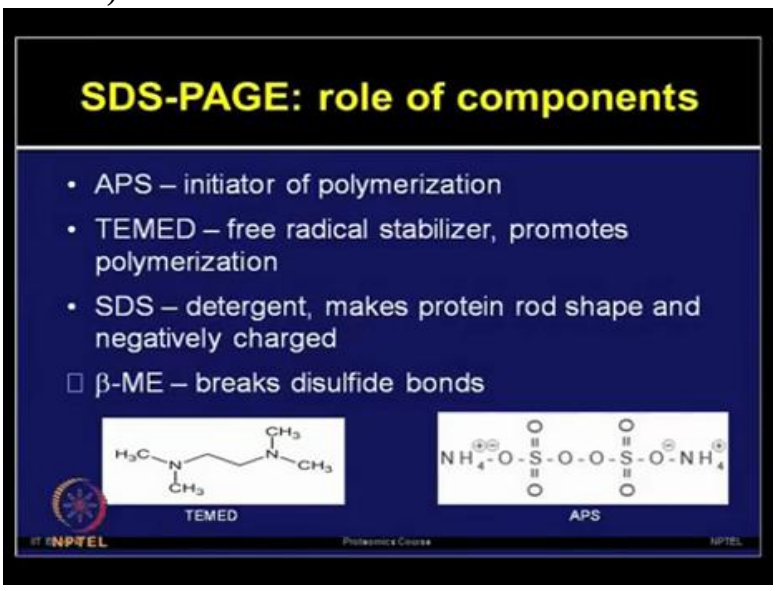

TEMED, that is a free radical stabilizer. It also promotes the polymerization of the gel process. So beta Mercaptoethanol is not used in making the gel, but for your protein treatment to break the disulfide bond.

So I hope you are at least able to understand the role of each of these components which are being used in SDS PAGE, either during doing the protein preparation of doing the SDS PAGE or making the gel to separate the protein.

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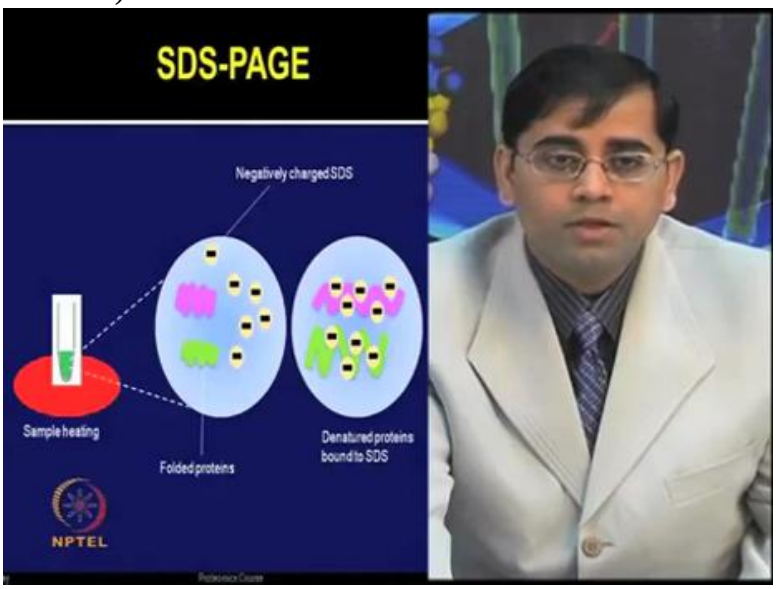

Now once this step is done, you need to boil your protein sample if you are performing a regular SDS PAGE. The negatively charged SDS molecule will bind to the folded proteins. It will denature to the protein and provide the negative charge.

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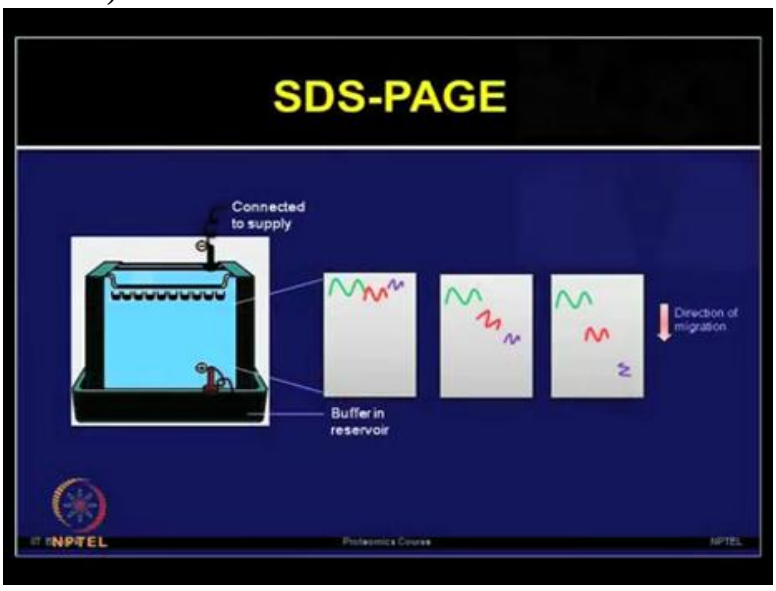

Once this protein preparation is done, then you can load this protein on SDS PAGE gel and based on the molecular weight the lower size proteins will migrate the furthest and the higher molecular weight protein will remain near the top surface of the gel.

So depending upon the protein size you can separate the protein based on the lower and higher molecular weight in this SDS PAGE gel. You can add the standards or known molecular weight proteins to determine your unknown proteins.

I think it is important to clarify that in 2DE 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 comb, 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. The stacking part is not done for the 2DE which has already been done during the first process of IEF.

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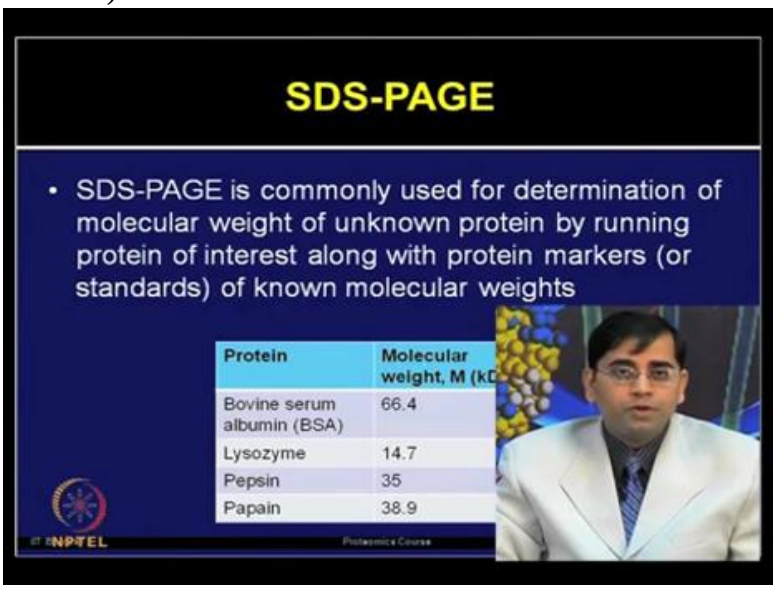

So SDS PAGE is one of the very commonly used methods for determining the molecular weight of protein of your choice or unknown protein which you want to know how much molecular weight it is.

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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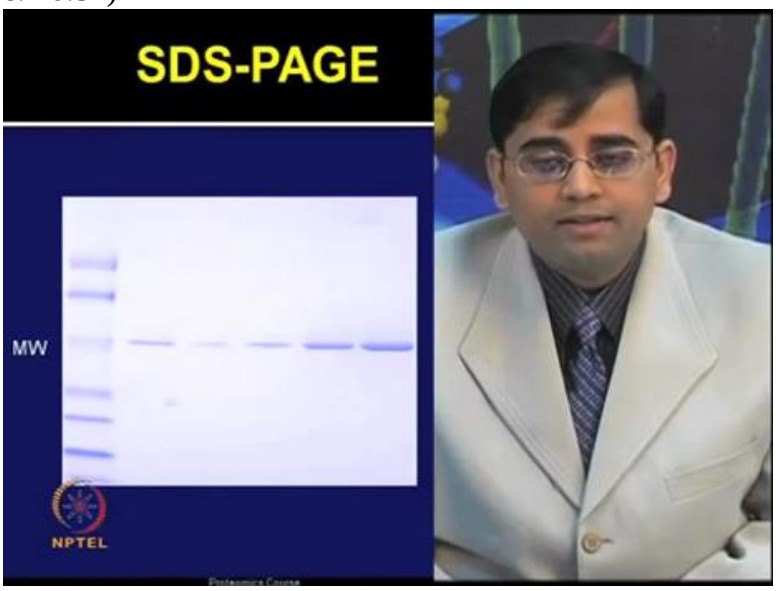

I am showing you one SDS PAGE image in which, from the left to the right, you can see; the first lane is the standard or the known molecular weight. In this well you can see there are different, at least 5 or 6 bands are visible. These are the known molecular weights, sometimes starting from 150 kilodalton to 10 kilodalton 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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I will now describe you the animation and show you the strips involved in SDS PAGE

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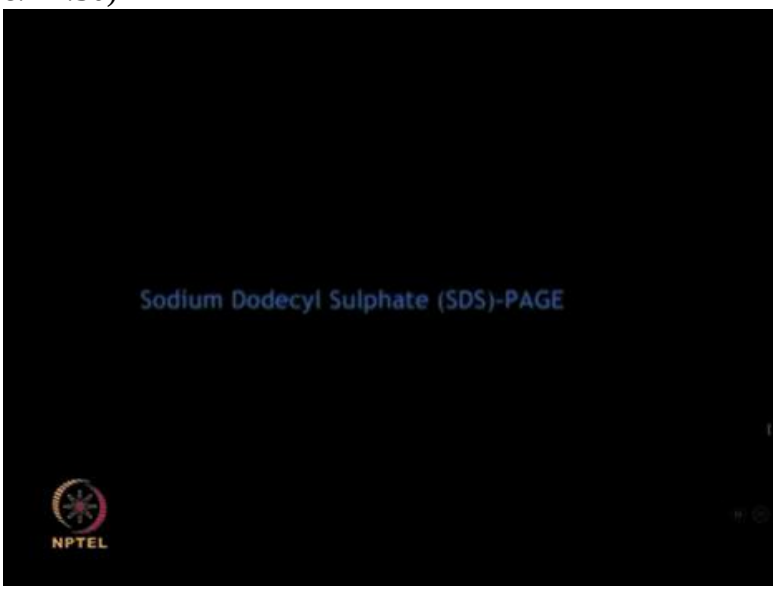

Sodium dodecyl sulphate SDS Polyacrylamide Gel electrophoresis SDS PAGE

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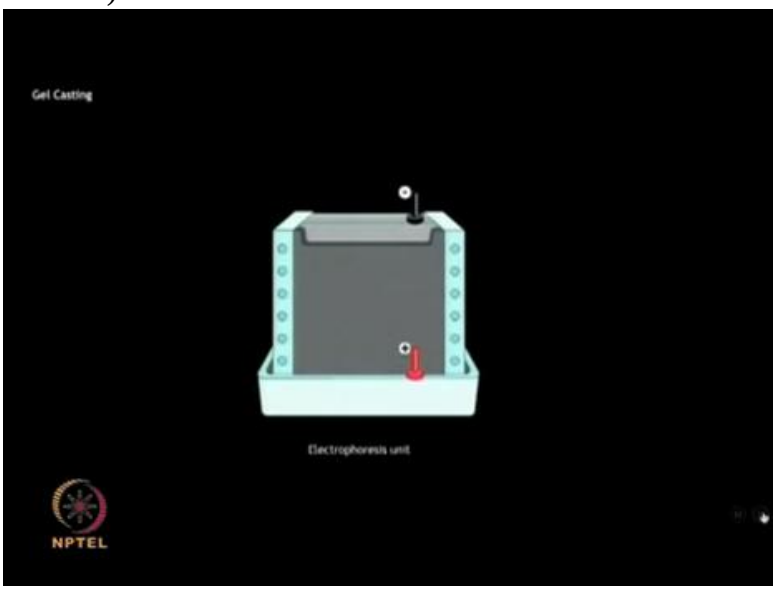

In this animation, you will see various steps…

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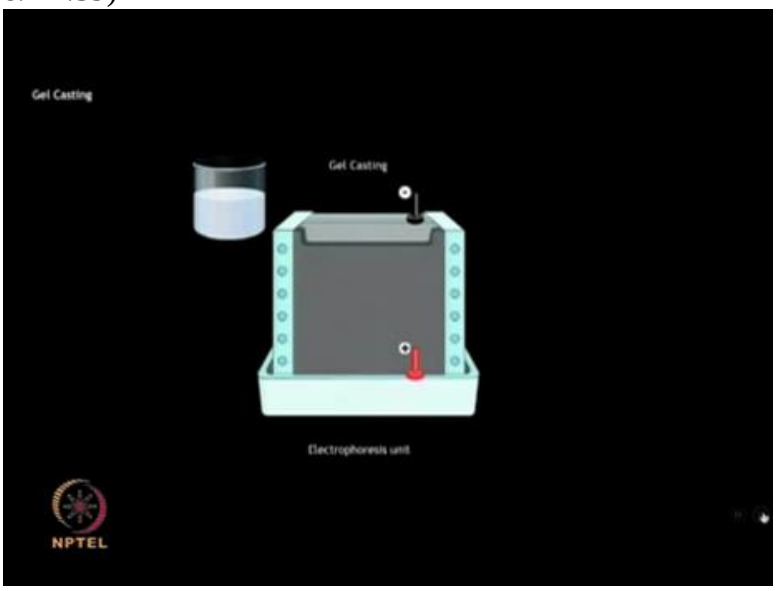

… involved in separating proteins…

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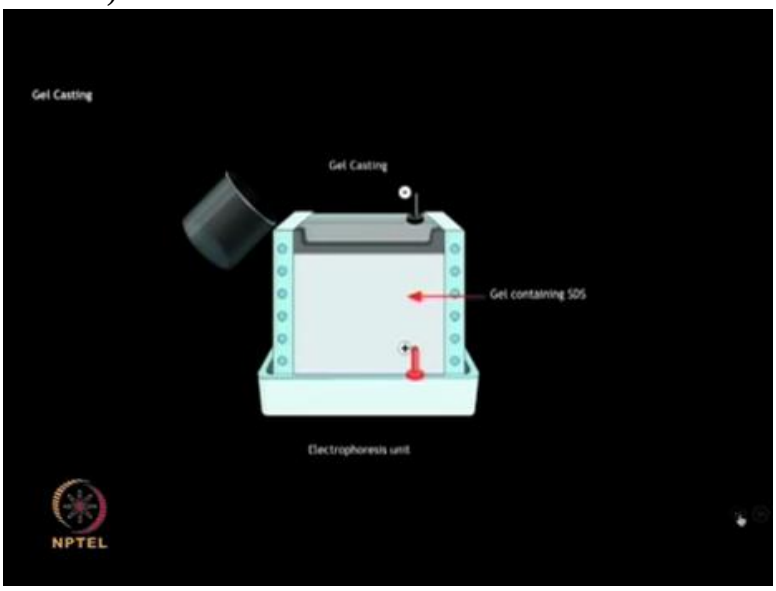

…based on their molecular weight. First of all, the Polyacrylamide Gel containing SDS is cast between the glass plates as a vertical slab in the same buffer that is used for electrophoresis The molecular dimensions of the pour can be controlled by varying the amount of n n'-methylenebisacrylamide with free radical cross-linking being facilitated…

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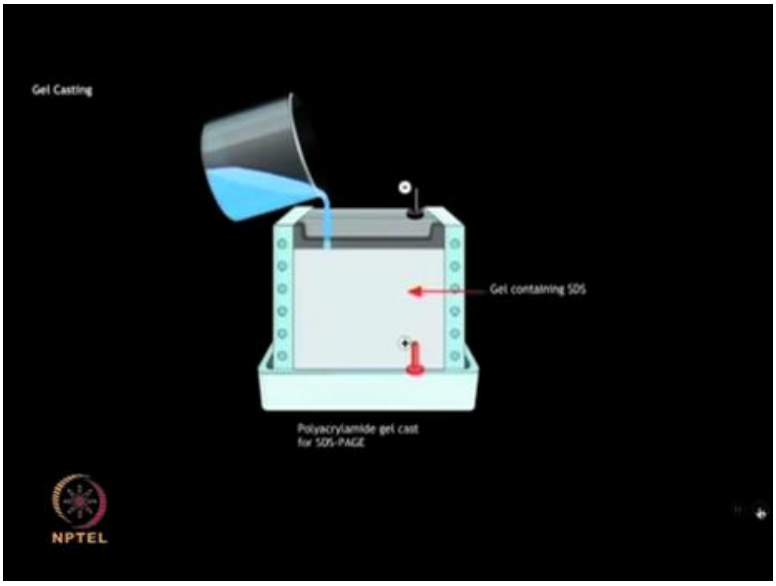

…by ammonium persulfate or APS and TEMED Next step is to create the wells on these gels.

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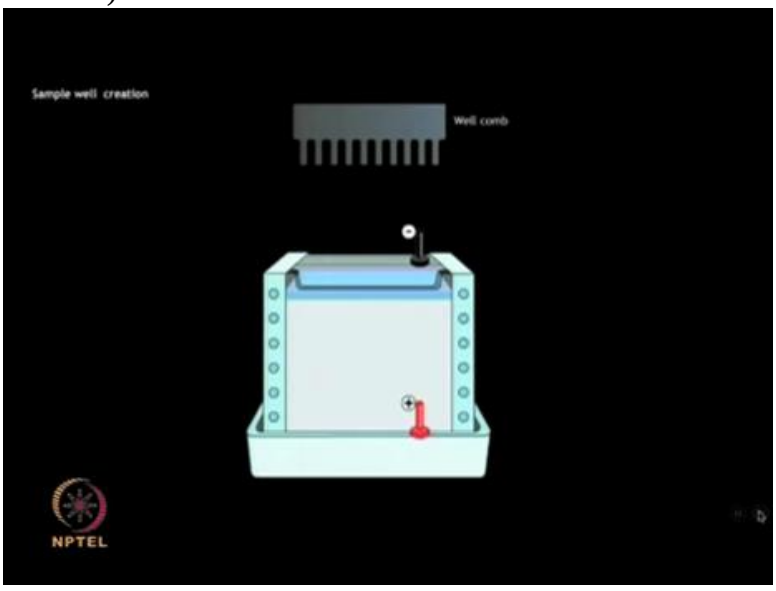

The sample wells of uniform size, shape and separation are made using a comb…

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… which is placed in the gel as soon as it has been poured

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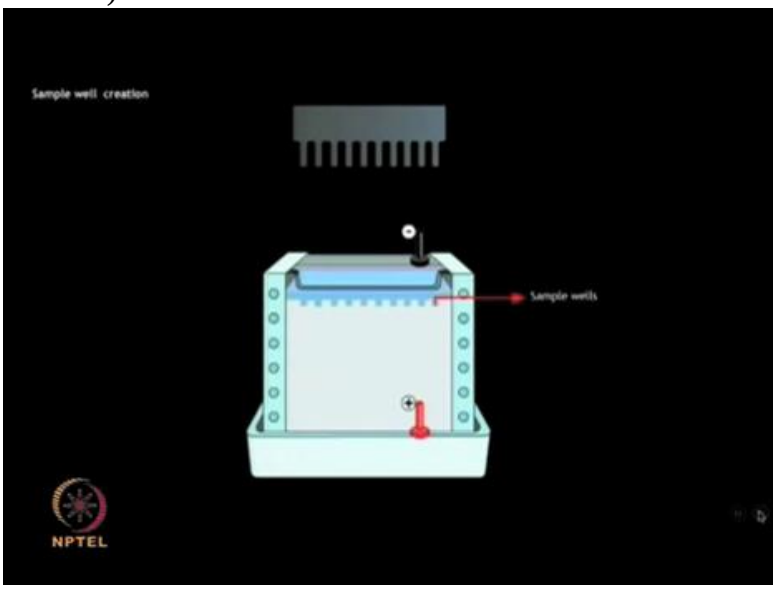

After the gel has polymerized the comb is removed…

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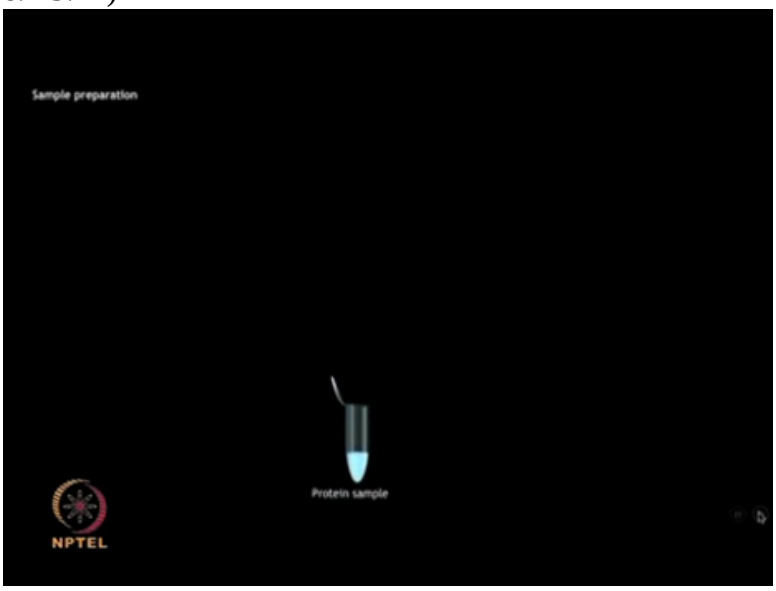

… to provide the gel ready to start the process.

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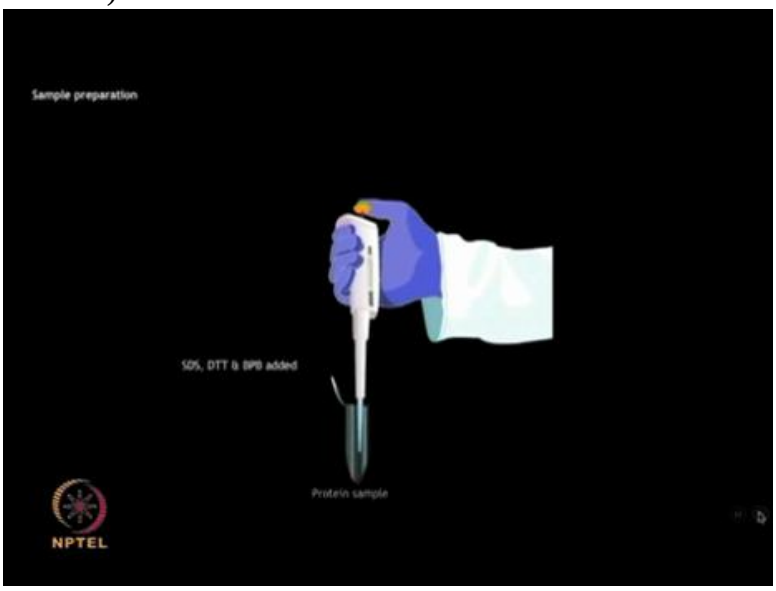

Now you can prepare the sample…

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…to load on SDS PAGE gel. SDS is a negatively charged anionic detergent that binds to protein molecules and causes them to denature.

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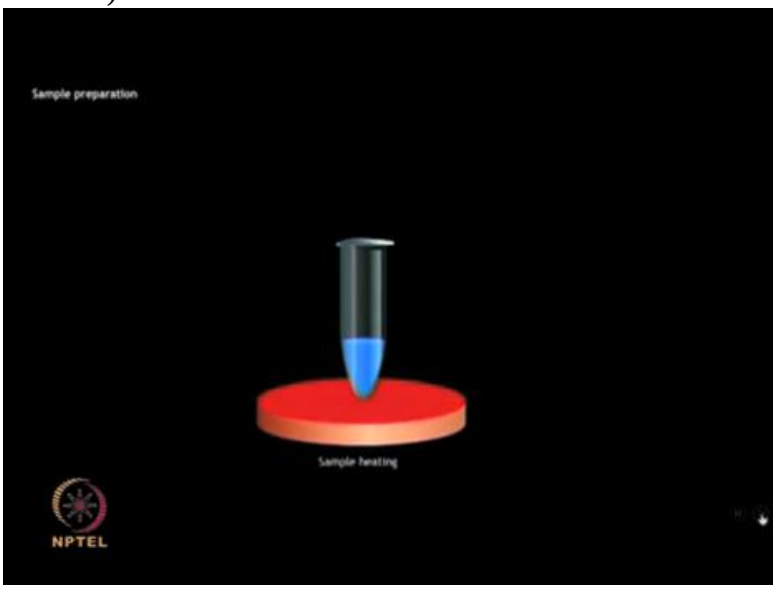

The DTT used breaks any disulfide linkages that may be present during the process you boil or heat your protein samples…

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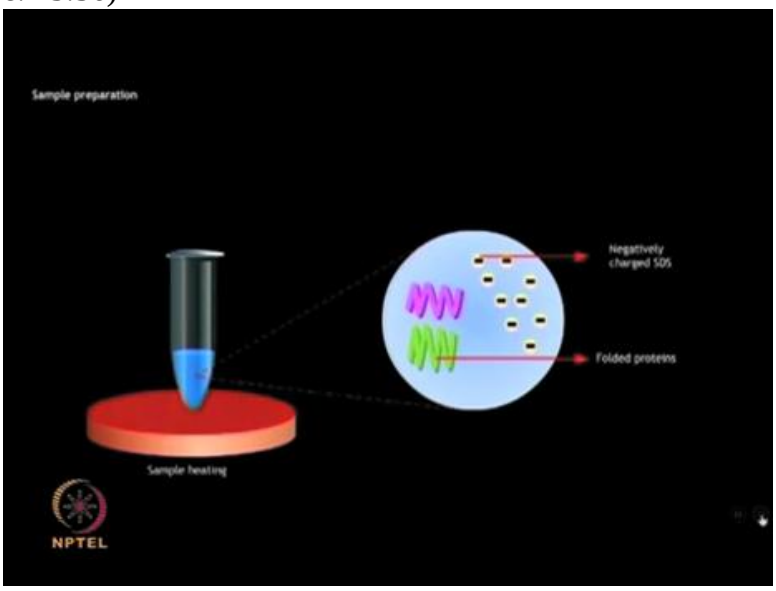

…to denature the protein Now the negatively charged SDS molecule will bind to the proteins

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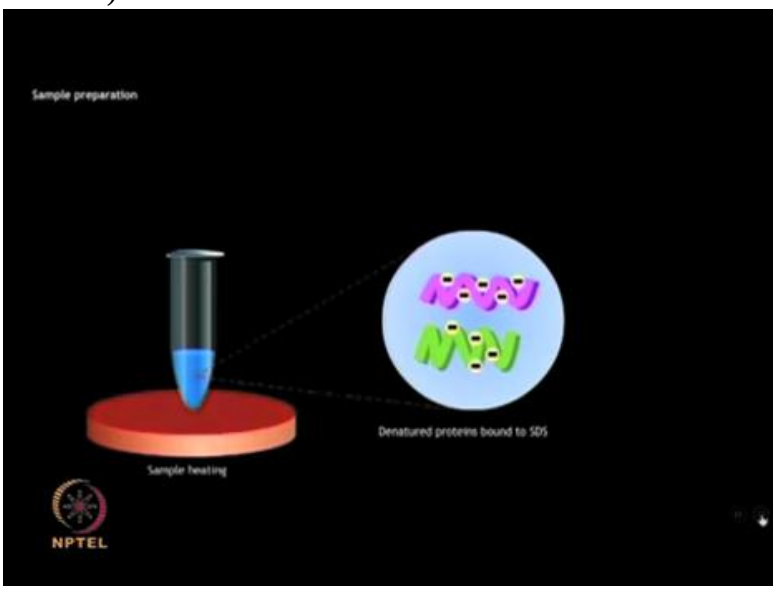

…and denature them.

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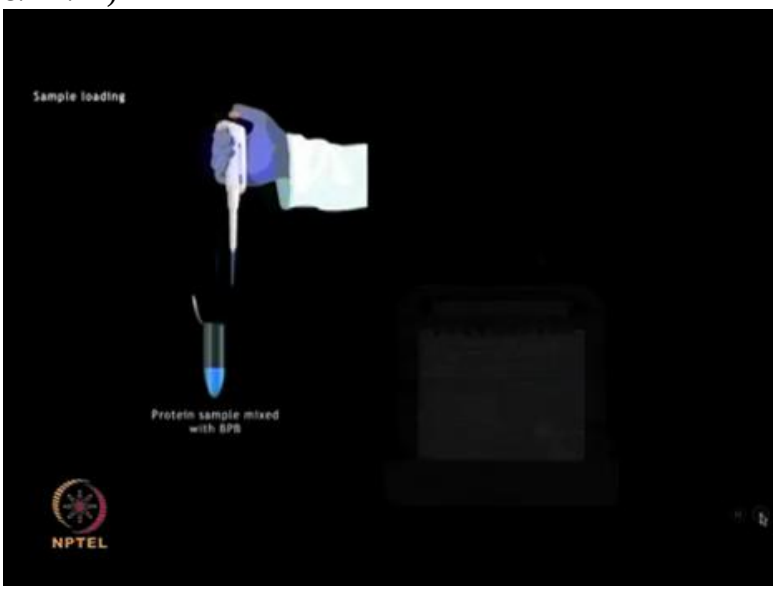

The binding of SDS causes the proteins to have uniform charge to mass ratio thereby allowing the separation purely on the basis of molecular weight.

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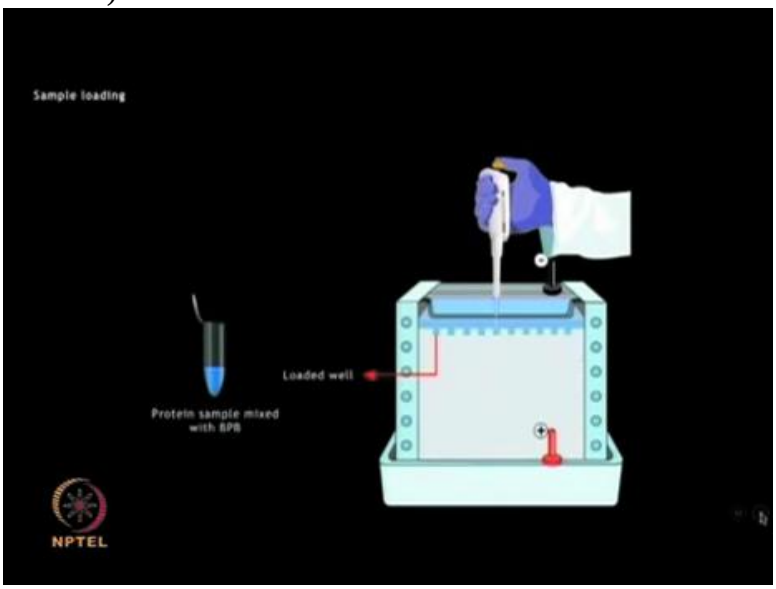

The protein samples can be loaded on the wells by using a micropipette. As you can see in the animation you can use different wells to load different types of samples. Once all the samples are loaded, then you can take this unit and move into electrophoretic apparatus.

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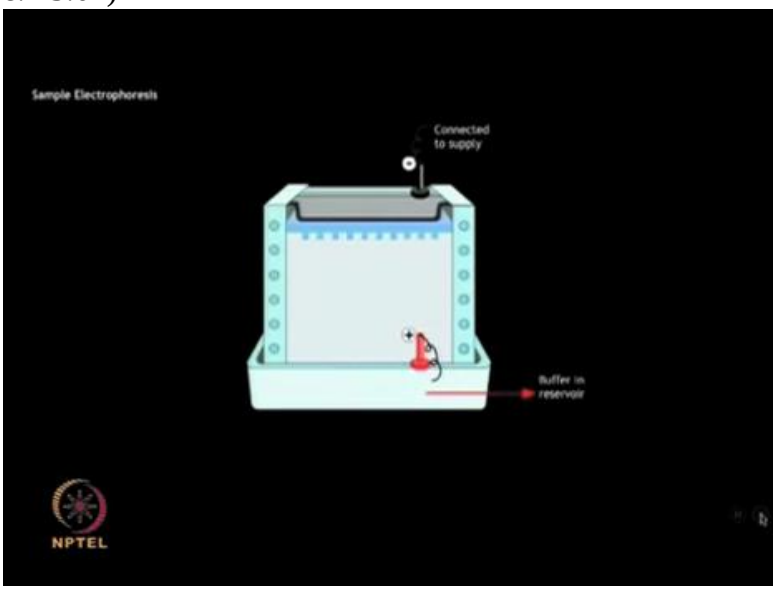

A direct current supply between 100 to 350 Volts is passed depending upon the size of gel…

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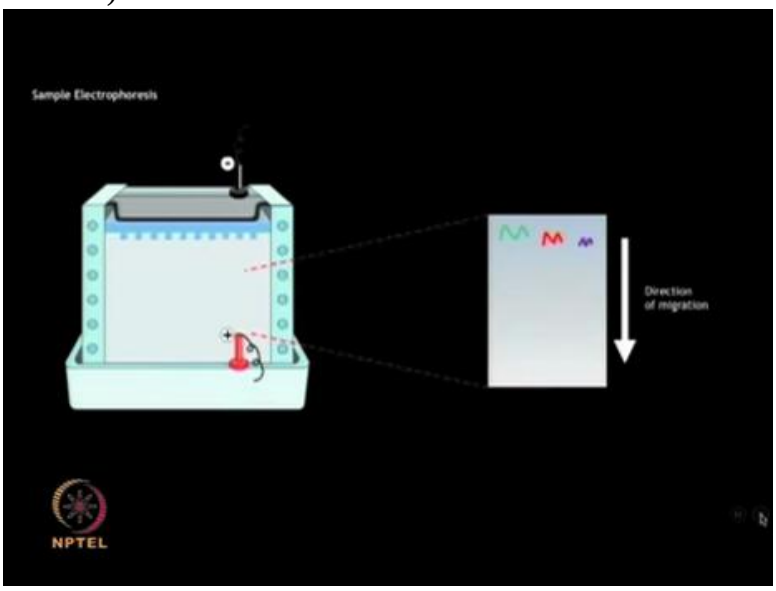

… for a time sufficient to separate the protein mixture into discrete bands …

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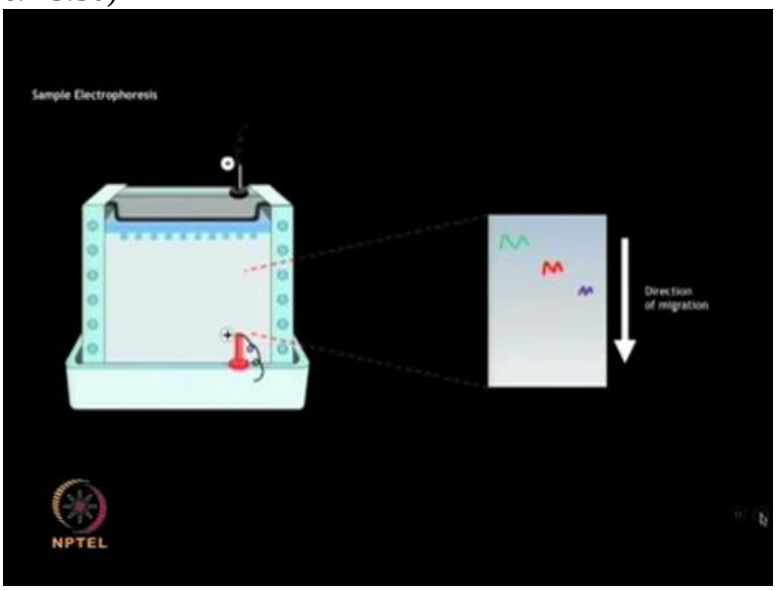

… based on their molecular weight. The progress of electrophoresis can be observed with the help of tracking dye.

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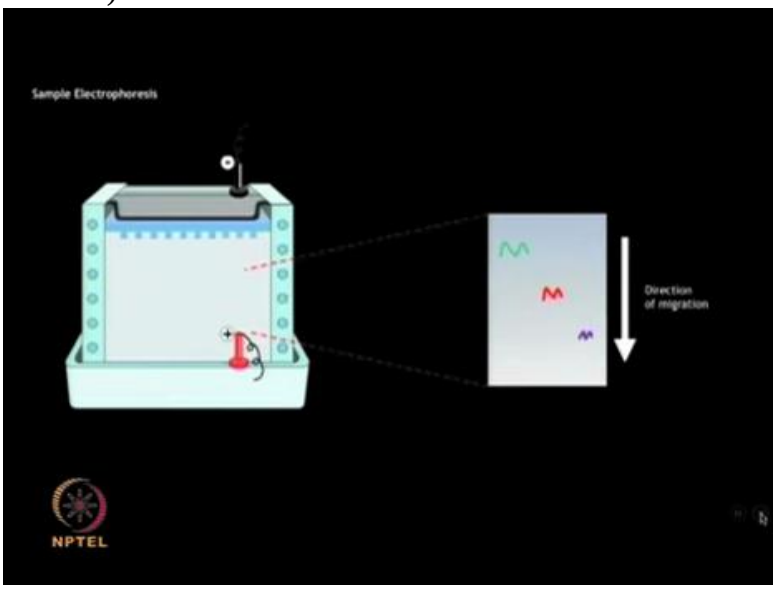

The larger proteins are retarded in the gel and remain close to the point of application while the smaller proteins migrate further along the gel.

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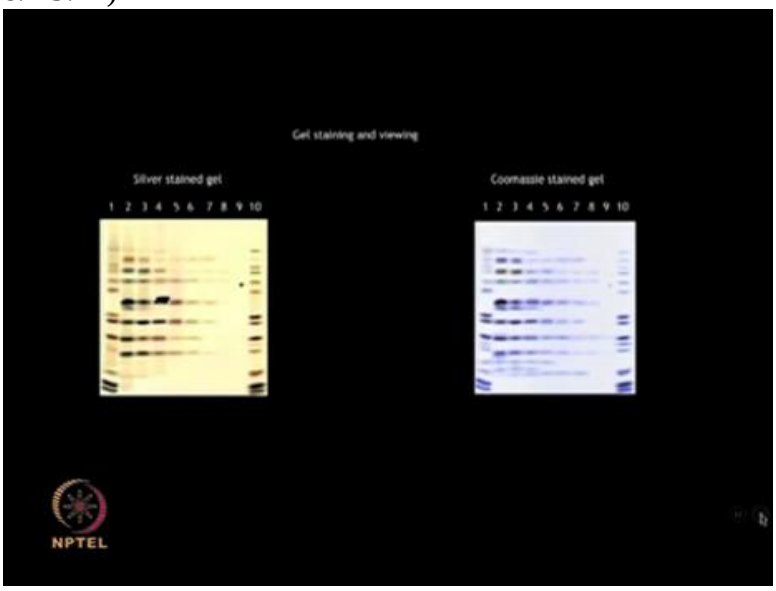

The gel can be then stained with either Coomassie or silver stain and viewed to observe the various discrete bands, discrete protein bands present in each of the sample. For example the left image here is silver stained gel and the right image is of Coomassie stained gel.

You can load the molecular weight marker or the standards as shown in the first and the last and the tenth 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 one laboratory demonstration how to use SDS PAGE gel for separating proteins in two-dimensional electrophoresis.

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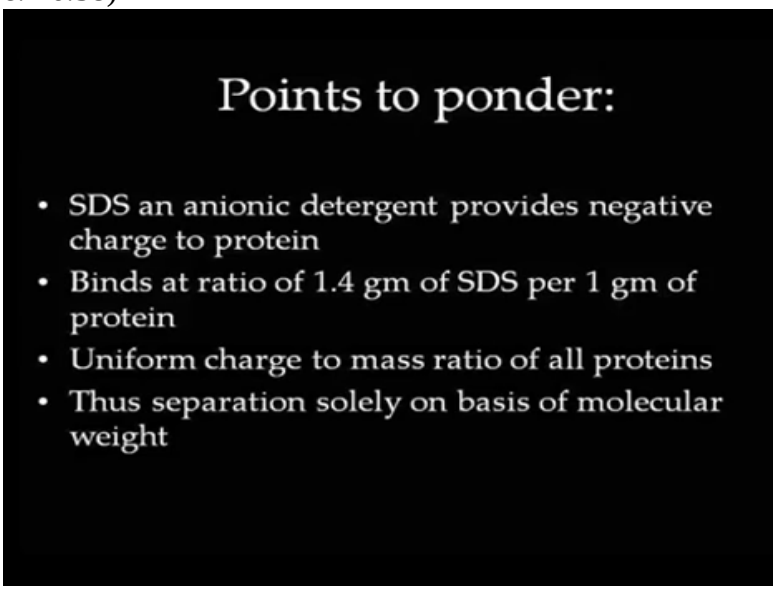

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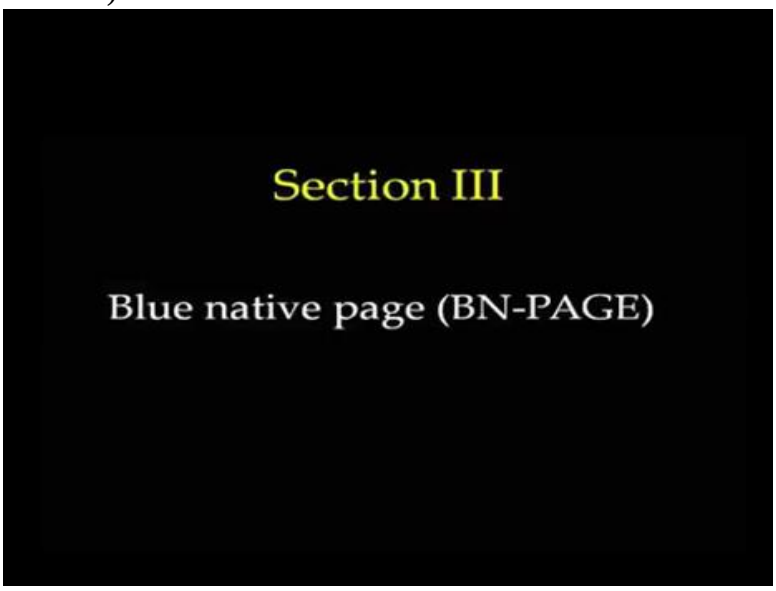


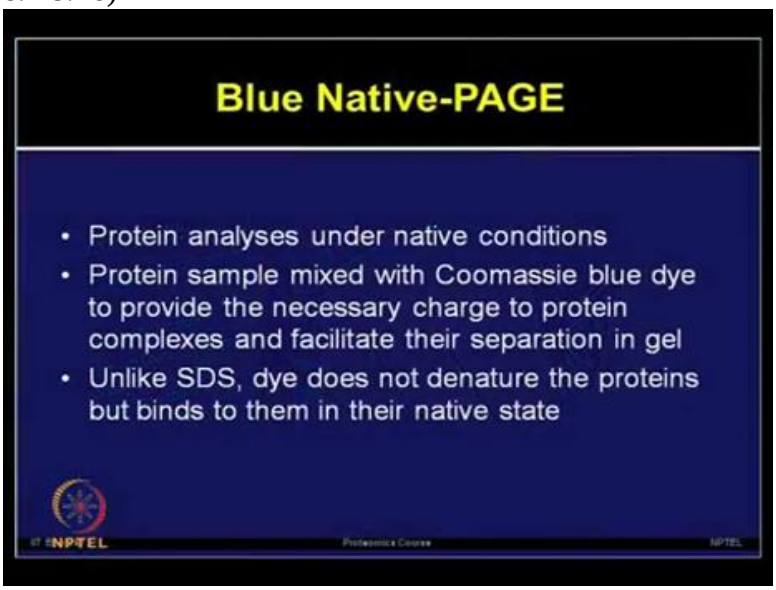
So let's now move to a variation of SDS PAGE known as Blue-Native PAGE, Blue-Native PolyAcrylamide Gel Electrophoresis. Now in SDS PAGE, we are providing a denaturing environment. We want to denature the protein where as in the Blue-Native PAGE; we want to separate the proteins in their native condition.

And by applying these 2 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 types of post-translational modification.

This information can be obtained either alone from SDS PAGE or from Blue Native PAGE or from combining these two together to get 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 2DE but often people also use the Native Form for the 2DE separation.
So in the Blue-Native PAGE, the protein analysis is performed under the native condition.

(Refer Slide Time: 18:26)



Here you are not going to boil your protein sample with SDS and providing the different type of denaturing condition.

So you need the 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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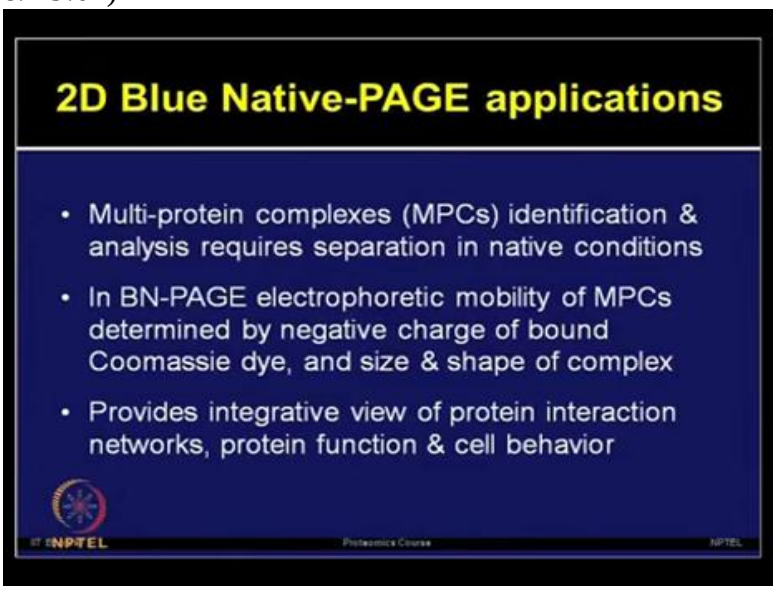

People apply the 2D Native PAGE for various applications such as studying about the Multi-Protein Complexes or MPCs. So identification of multiple protein complexes is not possible using the SDS PAGE or denaturing environment. So MPCs can be identified by using the Blue-Native PAGE.

In Blue-Native PAGE, the electrophoretic mobility of MPCs or Multi-Protein complexes determined by the negative charge of bound Coomassie dye and size and shape of complexes. So this technique can provide the integrated view of protein function.

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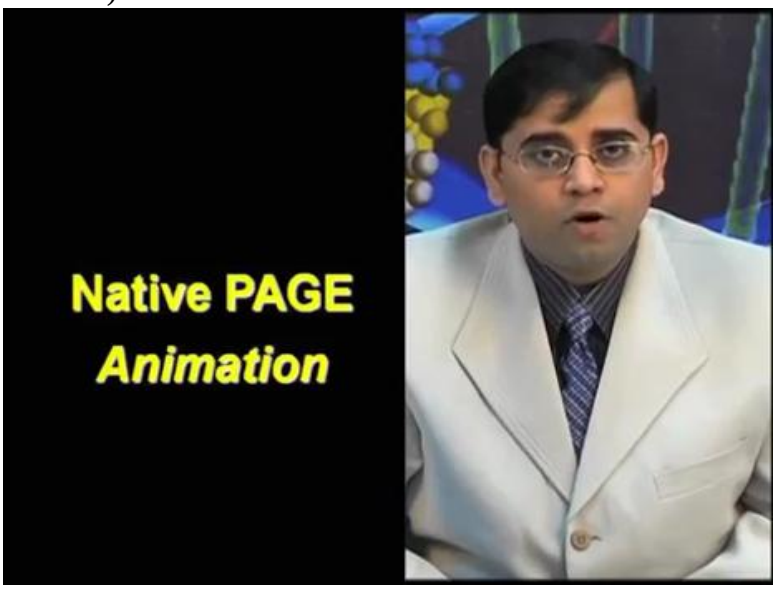

I will now show an animation how to perform the Native Polyacrylamide Gel electrophoresis.

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Blue Native PAGE or BN PAGE

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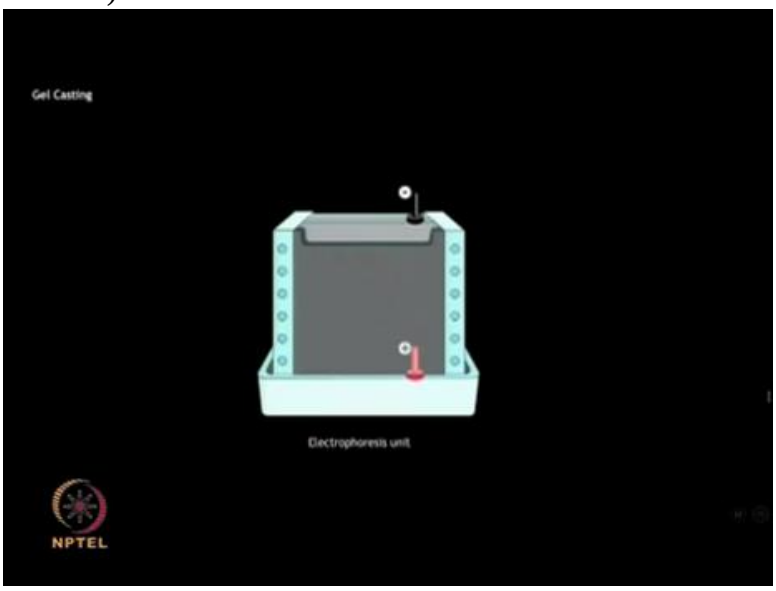

The polyacrylamide gel is cast between the glass plates …

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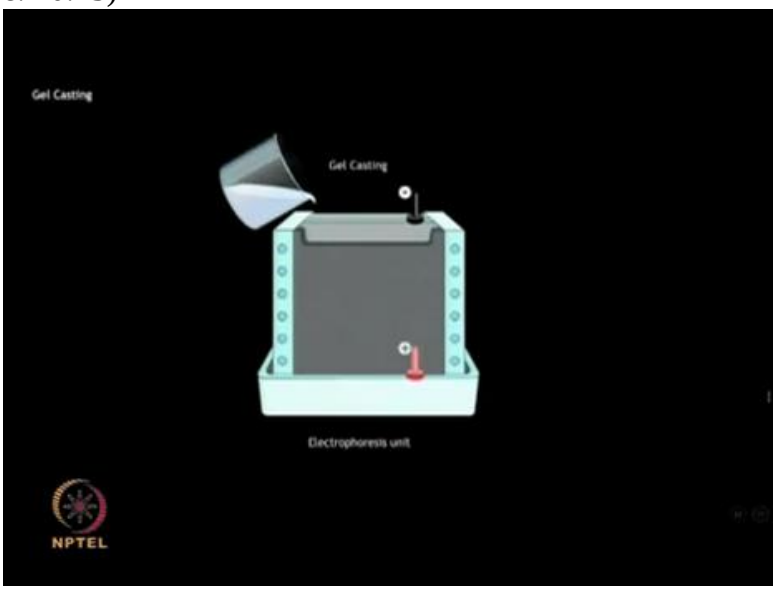

… as a vertical slab in the same buffer that is used for electrophoresis.

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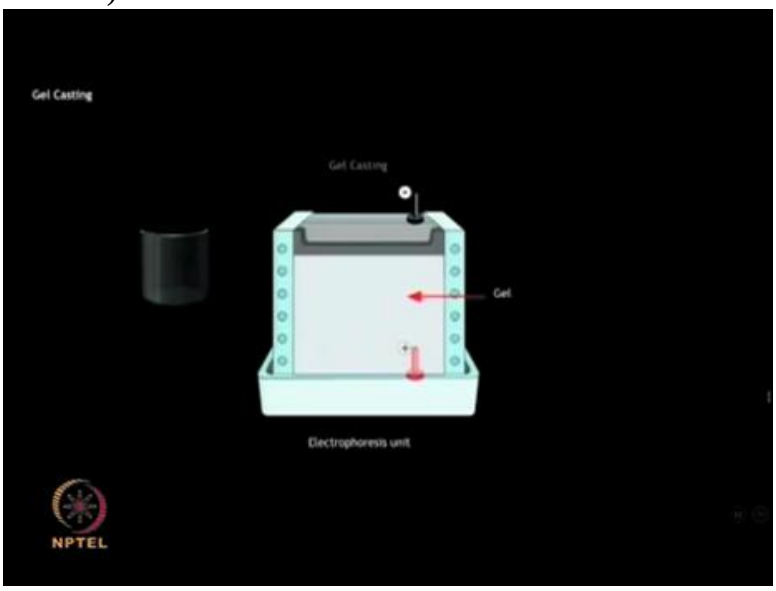

The gel is prepared by free radical induced polymerization of acrylamide and bis-acrylamide in a suitable buffer.

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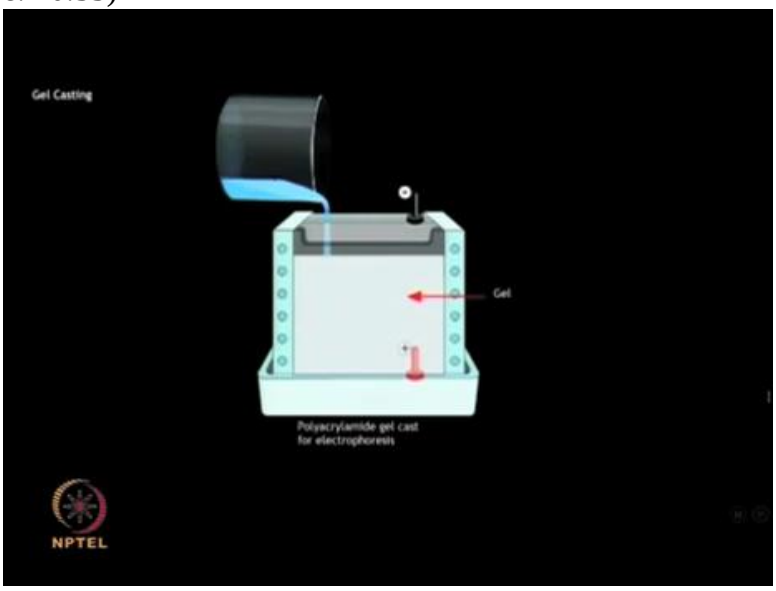

Ammonium persulfate and TEMED are added to facilitate the generation of free radicals and cross-linking.

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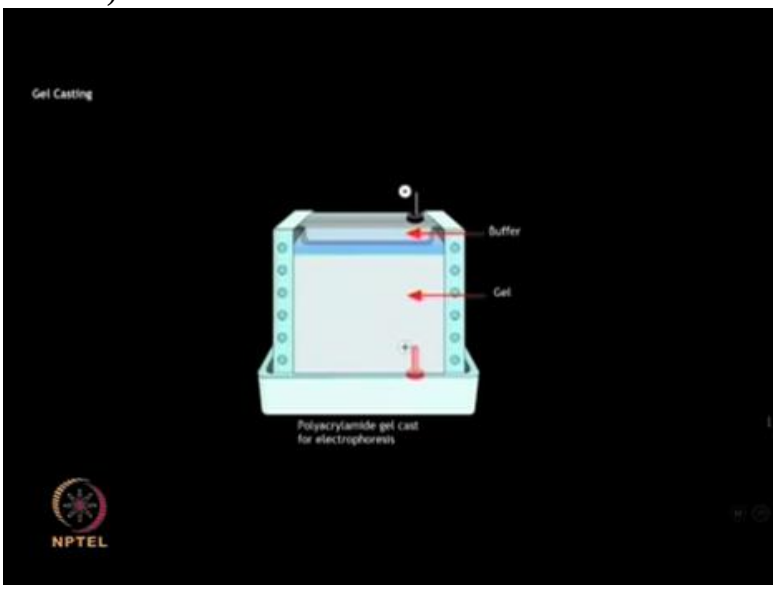

The molecular dimensions of the pores can be controlled by varying the amount of n n'-methylenebisacrylamide. Once the solution is poured, then the sample wells can be created.

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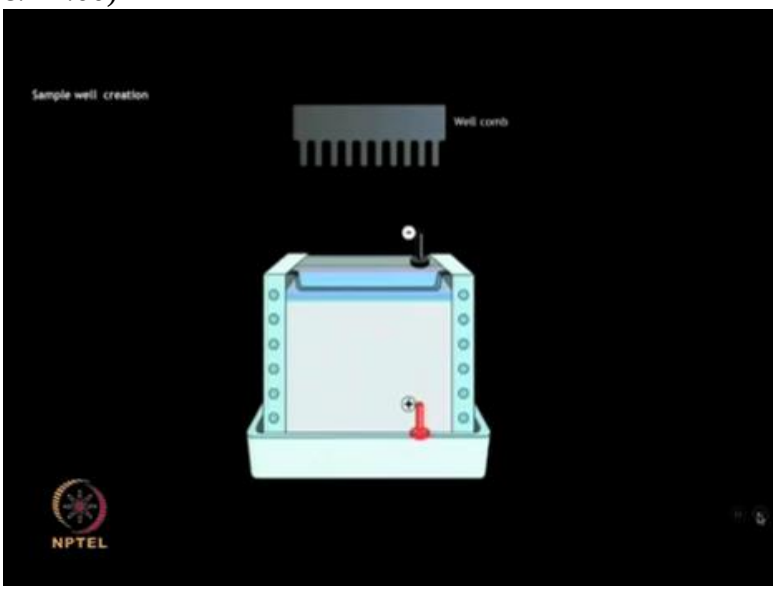

The sample wells of uniform size, shape and separation are made using a comb….

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… which is placed in the gel, as soon as it has been poured.

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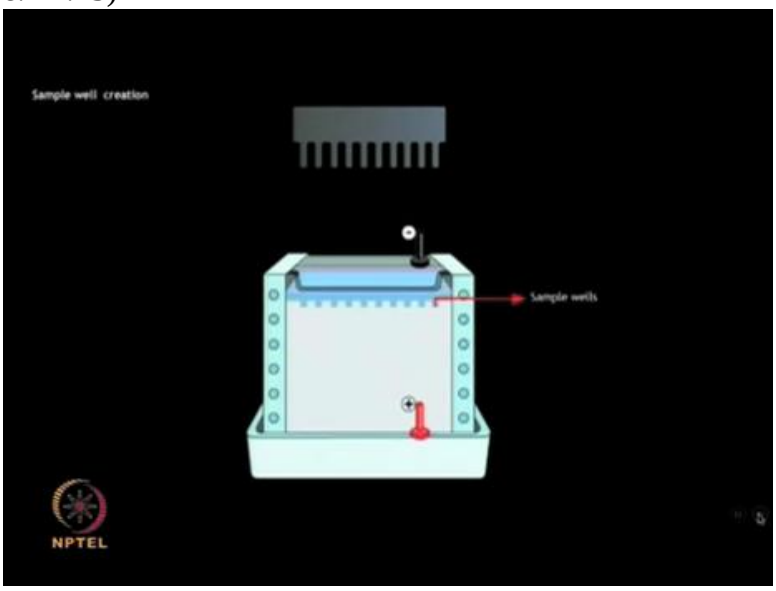

After the gel has polymerized, the comb can be removed…

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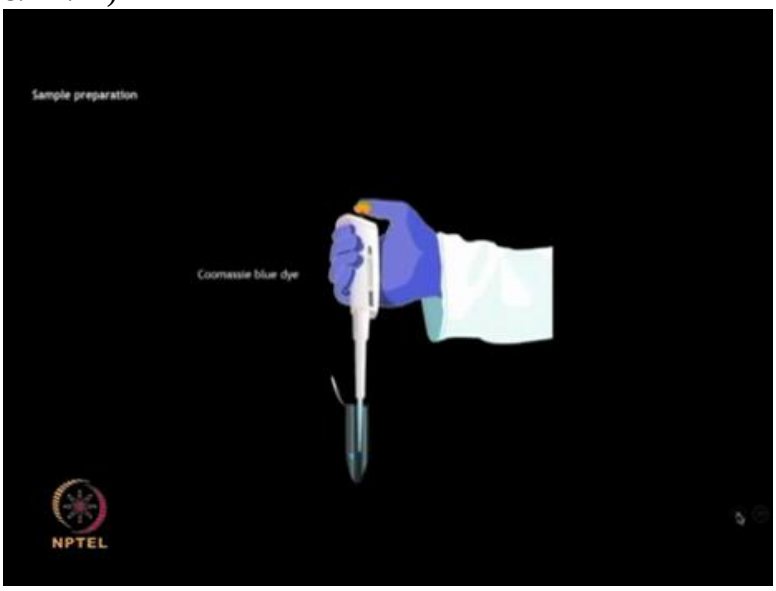
… to provide the gel ready for the process.

(Refer Slide Time: 21:24)



Sample preparation

The protein sample present in the suitable buffer is mixed with Coomassie Blue dye…

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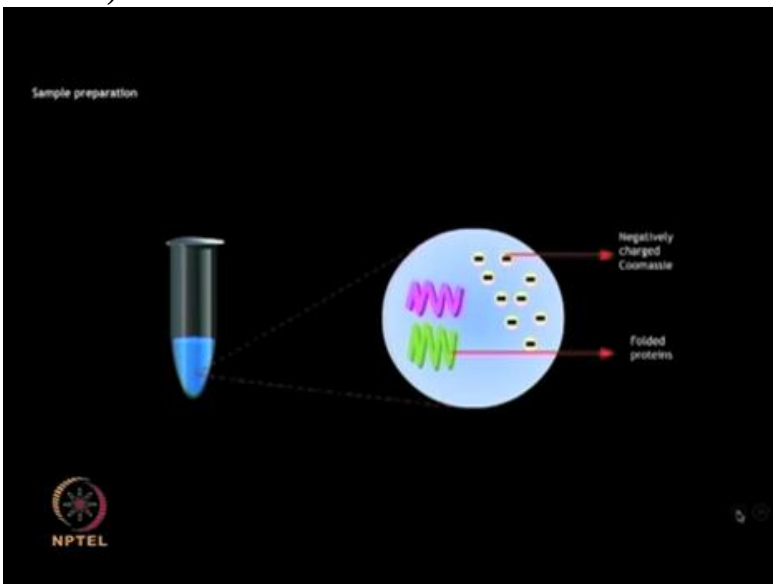

… which provides the necessary charge to the protein complexes thereby facilitating their separation in the gel

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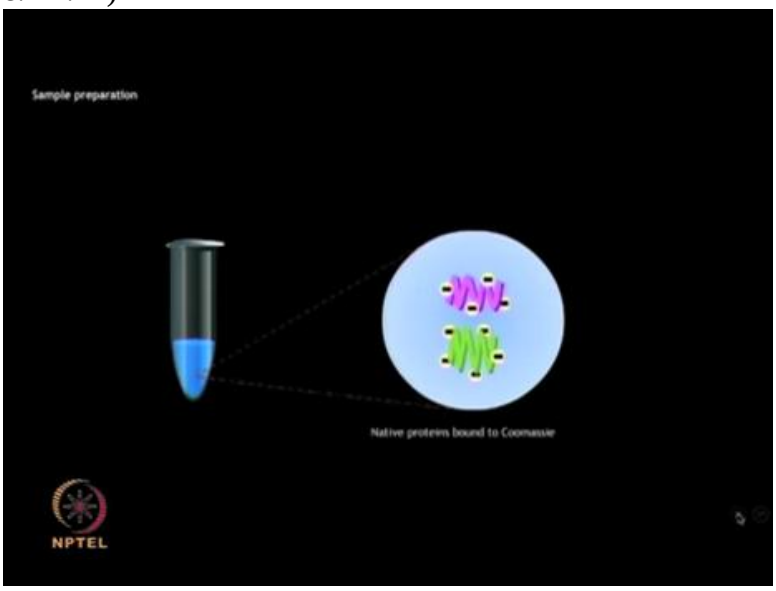

Unlike SDS, the dye does not denature the protein but binds to them in the native state.

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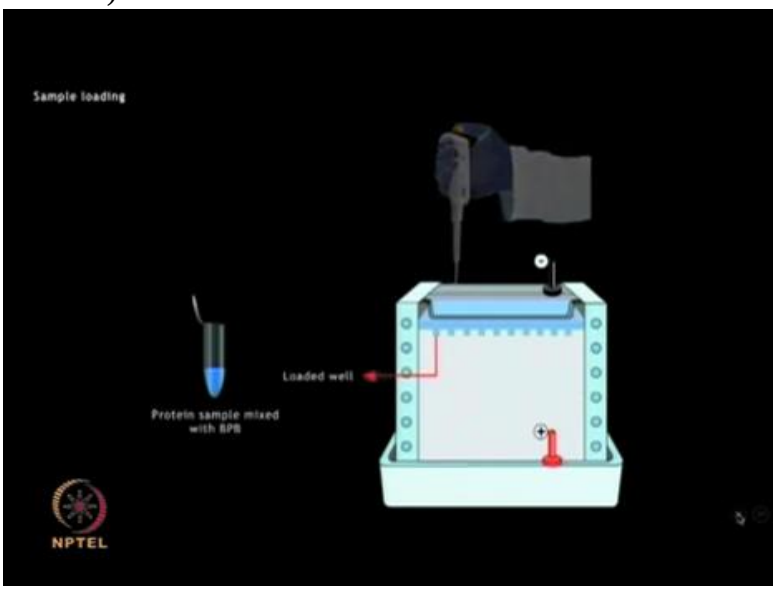

The proteins are then loaded into the wells with the help of the micro-pipette.

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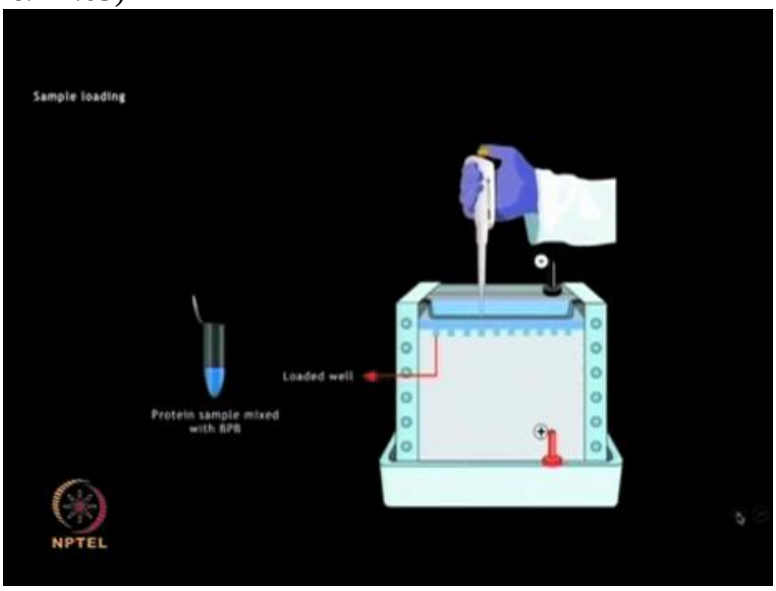

As it is shown in the animation, you have different wells to load different protein samples. You can use one of the wells to add known or standard markers and other wells can be used to separate the unknown proteins.

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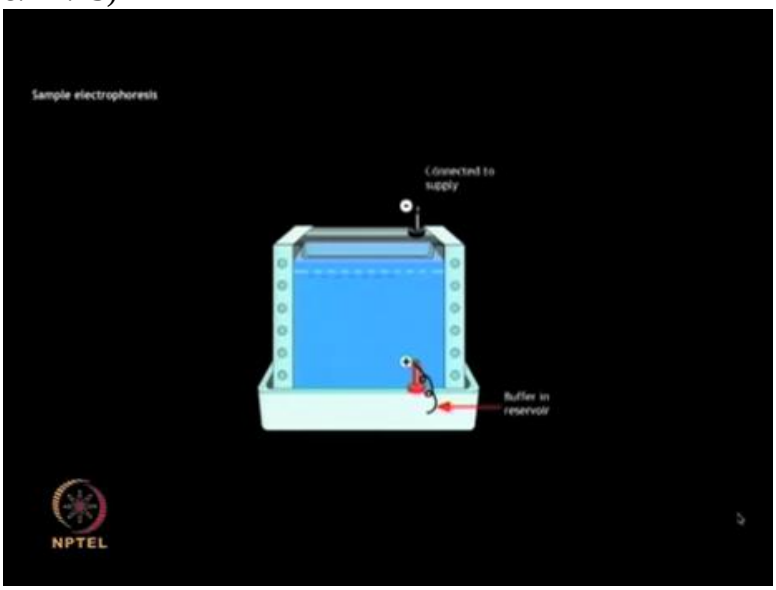

Once all the samples are loaded, then this unit can be placed into electrophoretic unit and a direct current supply of around 100 to 350 Volt can be passed depending upon the size of the gel for a time sufficient…

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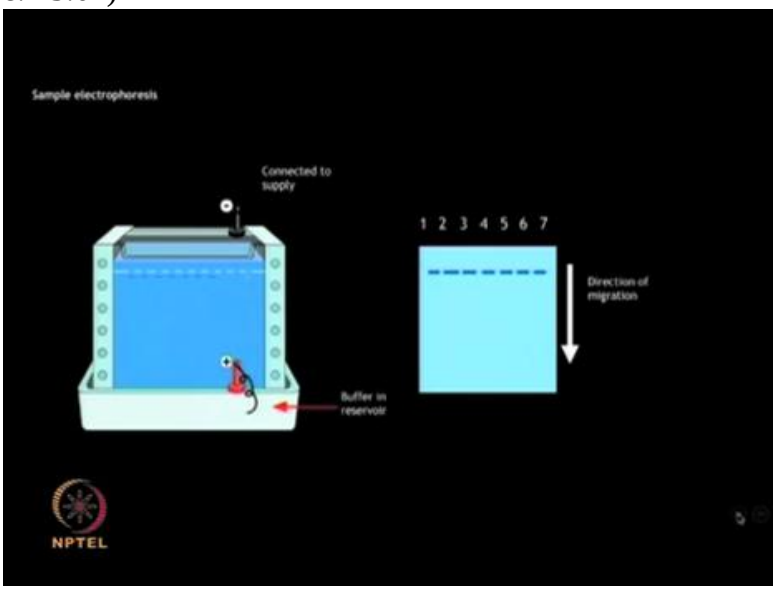

… to separate the protein mixture into discrete bands based on their mass, charge to mass ratio.

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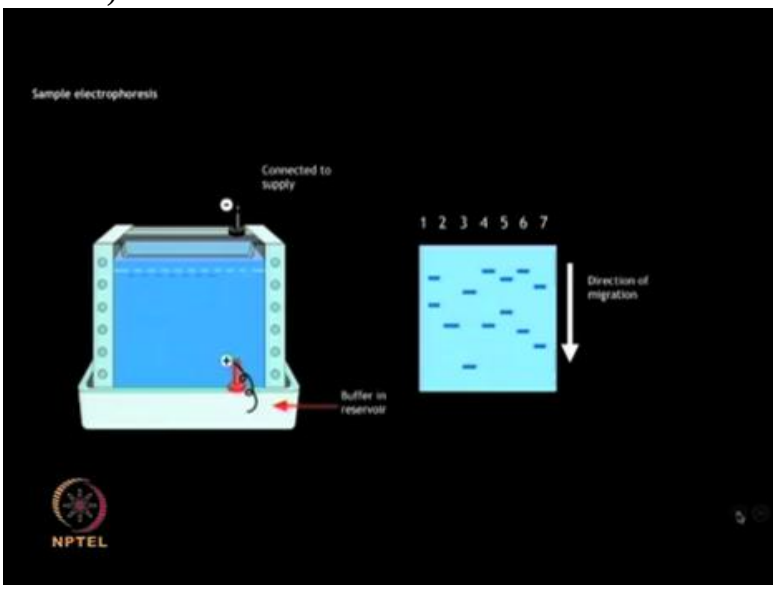

The progress of electrophoresis can be observed with the help of tracking dye.

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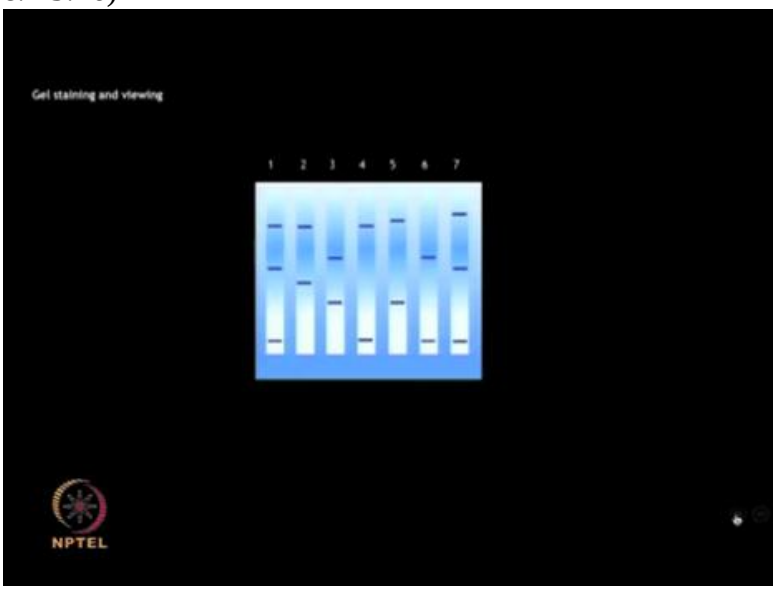

The larger proteins are retarded in the gel and remain close to the point of application while the smaller proteins migrate further along the gel. The gel can then be stained with Coomassie brilliant blue and viewed to observe various discrete protein bands.

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 2DE as well.

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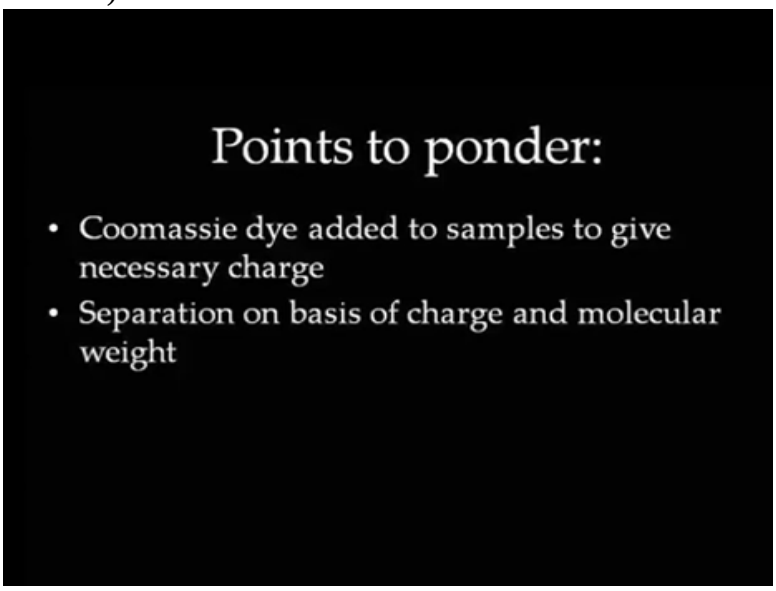

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