Proteins and Gel-Based Proteomics
Professor Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Mod 04 Lecture Number 13

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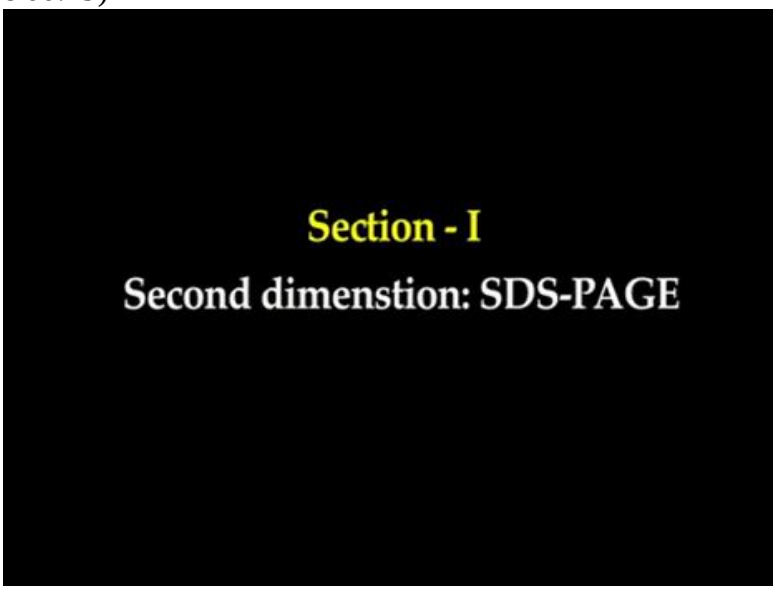

As I mentioned, you have seen the classical SDS PAGE and Blue-Native PAGE, the concept is similar and it can be applied in the two-dimensional electrophoresis. There is a little variation here because now you do not need to add a comb; you have to have a flat comb for making a well to add your IPG strip and you do not need to add the separation for stacking gel.

So in this laboratory demonstration video, I will show you for the two-dimensional electrophoresis, how to make the SDS PAGE

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Protein separation by SDS PAGE; SDS PAGE which constitutes the ….

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… second dimension of two-dimensional gel electrophoresis

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… involves assembly of the gel apparatus, gel casting, equilibration of the IPG strip followed by placement of the IPG strip on the gel and protein separation.

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Assembly of gel apparatus and gel casting

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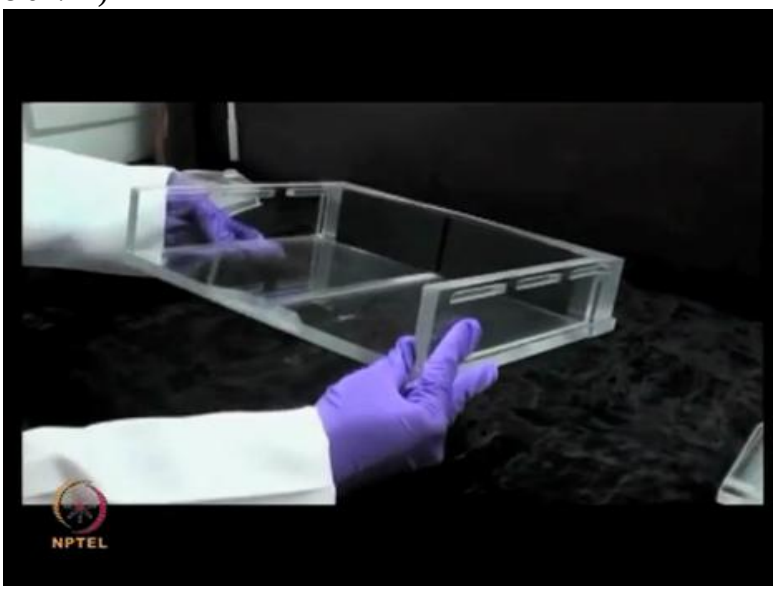

Clean all the components of the gel assembly thoroughly with water …

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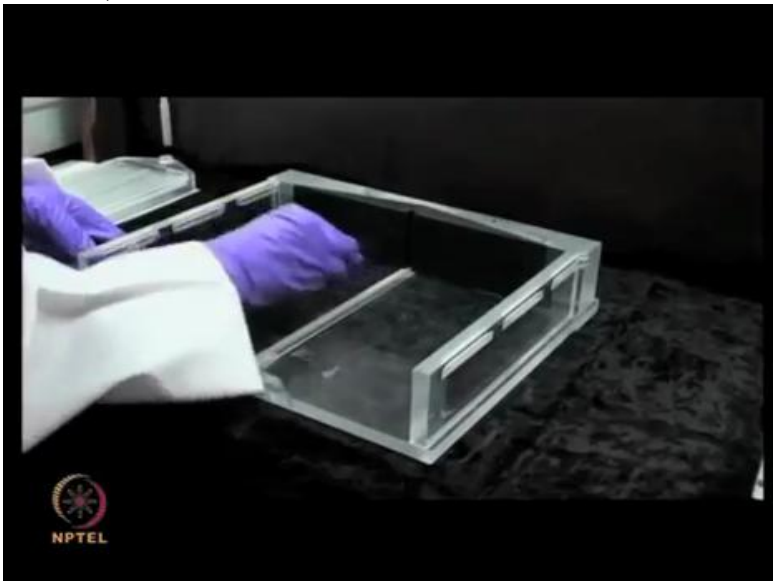

… and dry them with paper towels.

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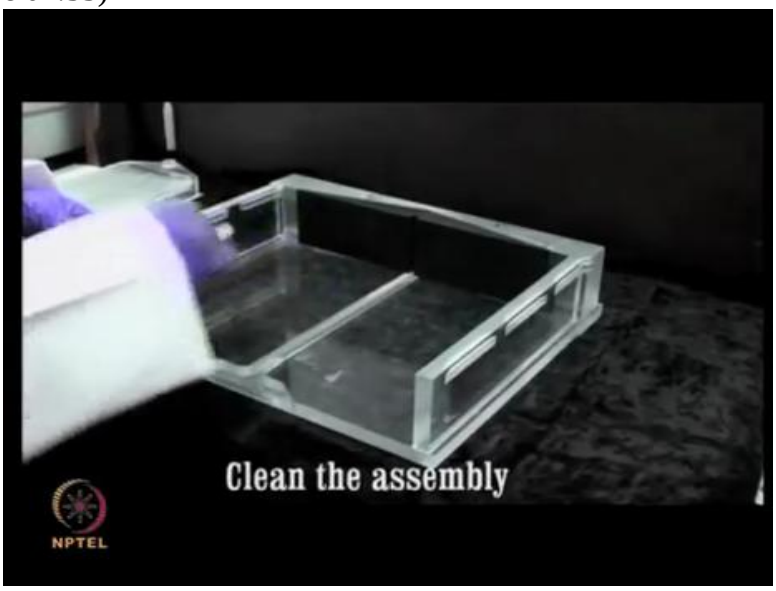

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Carefully arrange the glass plates in the gel casting assembly interspersed with …

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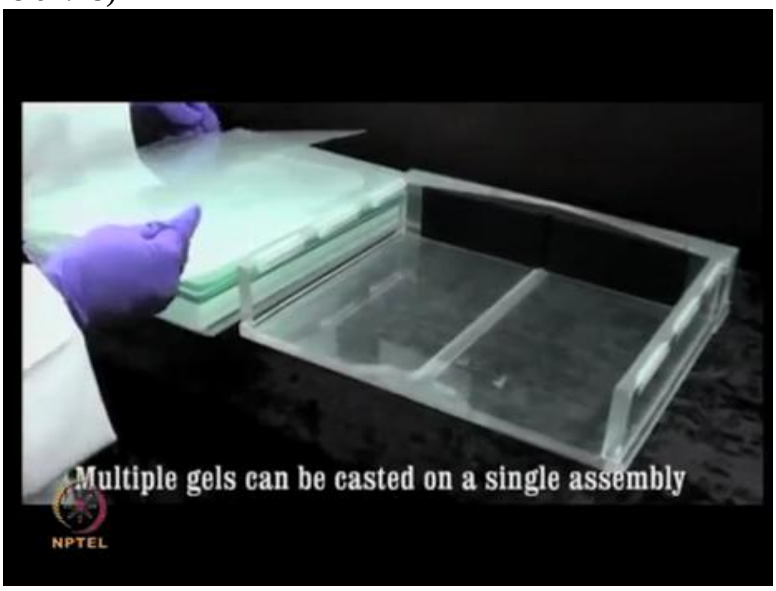

… separator sheets depending on the number of gels to be run

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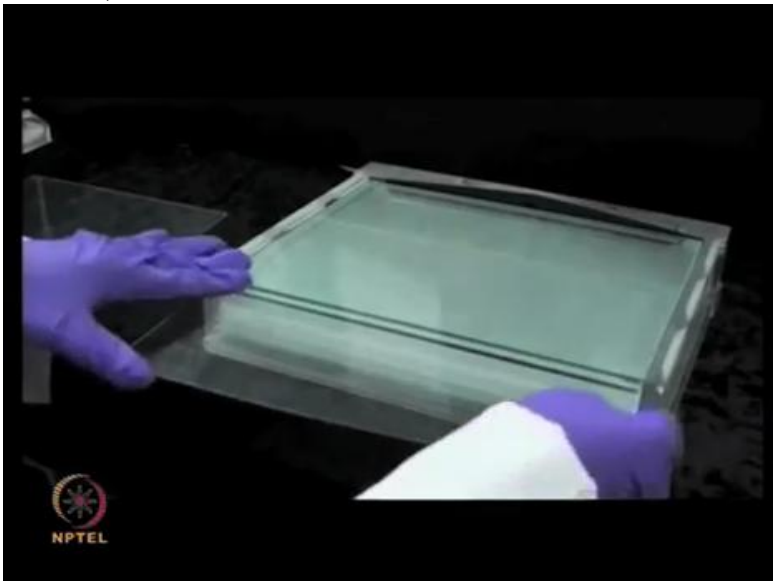

The assembly should be tightly packed such that there are no leaks.

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Close the casting assembly and tighten the screws provided.

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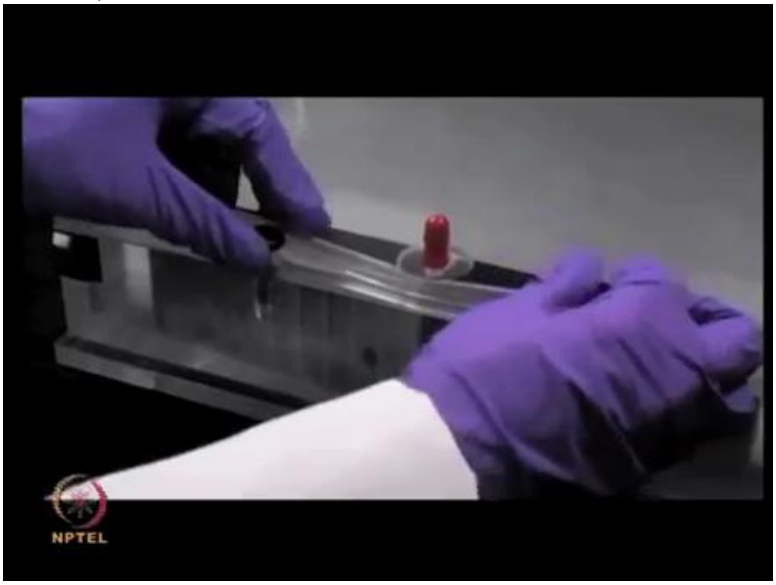

Prepare the gel casting solution consisting …

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… of Acrylamide, bis-Acrylamide, TRIS-chloride, SDS, APS and TEMED and pour it

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with the help of the funnel into

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the central channel of the casting assembly

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APS acts as a free radical generator while TEMED functions as a catalyst for the polymer reaction.

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Ensure that the solution spreads evenly such that the top edge of the gel is uniform. Allow the solution to stand for polymerization to occur

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and spray the solution of SDS across the top

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…to ensure that the gel does not become dry.

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SDS PAGE

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Drain out the excess liquid of the second equilibration solution from the IPG Strip using a tissue paper.

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Soak the strip for few minutes in the same buffer solution that is to be used in the gel tank

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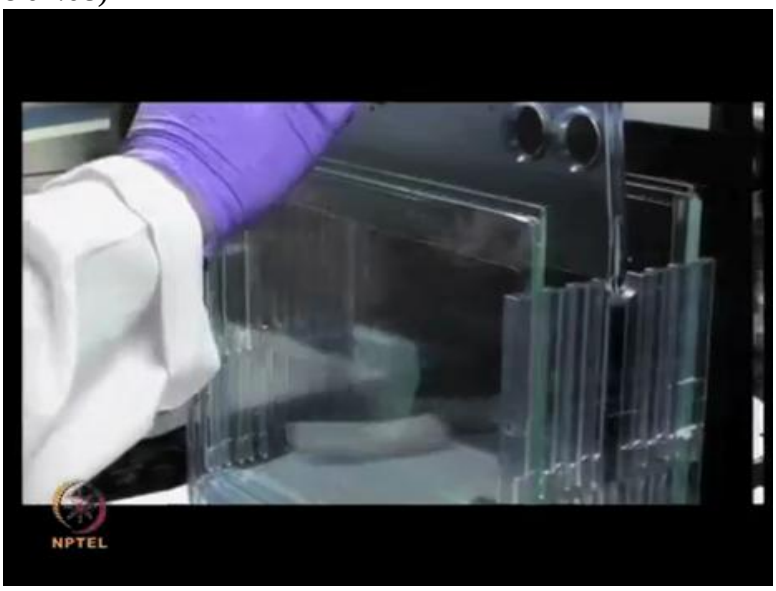

Then carefully place this strip in-between the glass plates and

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… gently push it in until it rests firmly on gel surface without any gaps.

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Place the filter paper containing the molecular weight marker beyond the positive end of the strip.

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Then pour the lukewarm agarose overlay solution over the strip to prevent the gel from drying up.

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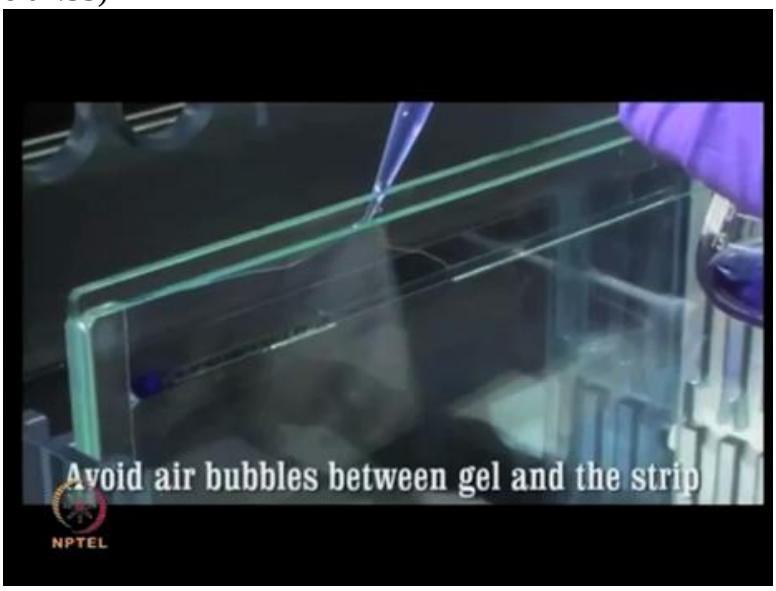

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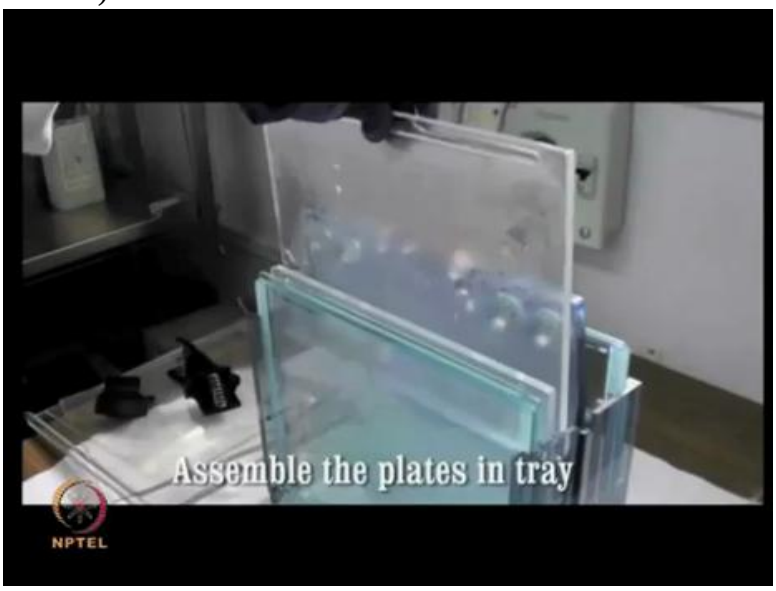

Assemble the remaining plates in the gel assembly and carefully lower the entire apparatus

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into the gel tank containing the buffer solution.

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Ensure that the buffer level does not rise above the mark indicated.

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Place the separating chamber above this and pour the buffer solution into the upper chamber.

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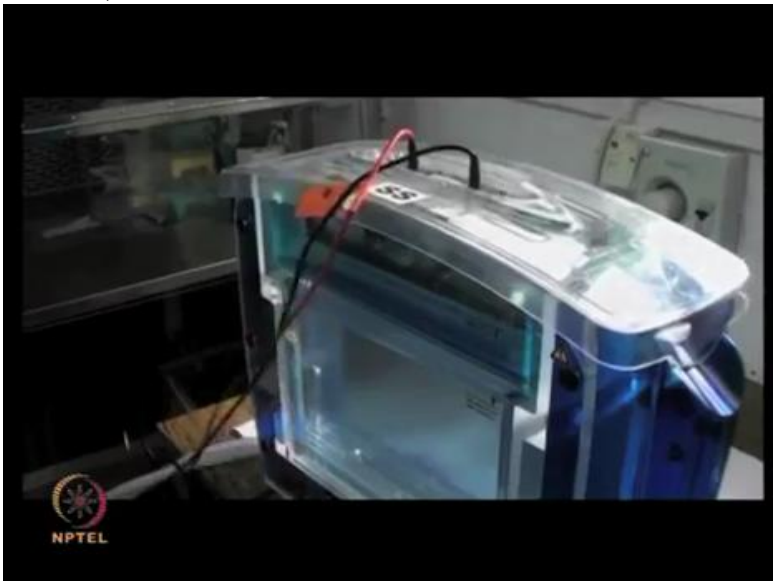

Close the gel assembly and connect the apparatus to the electricity supply. Set the appropriate voltage and begin the run.

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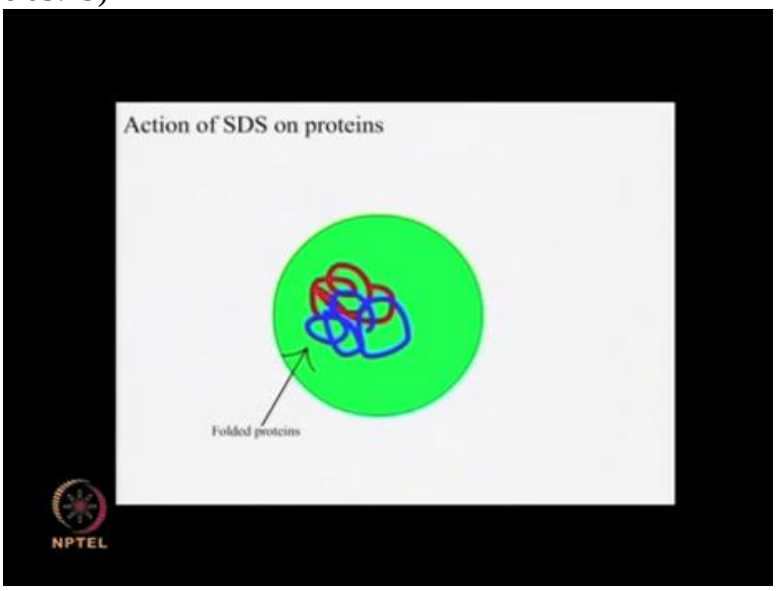

All the proteins present on the IPG Strip carry a large negative charge

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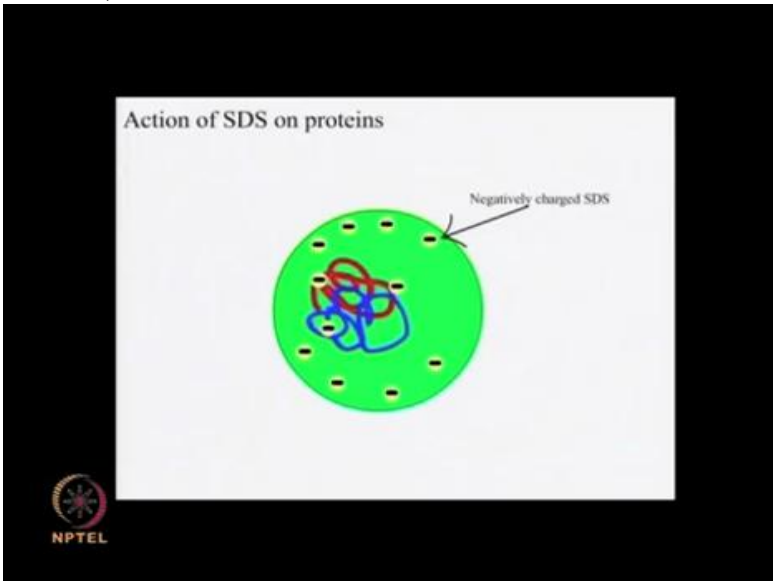

due to action of SDS which denatures the protein

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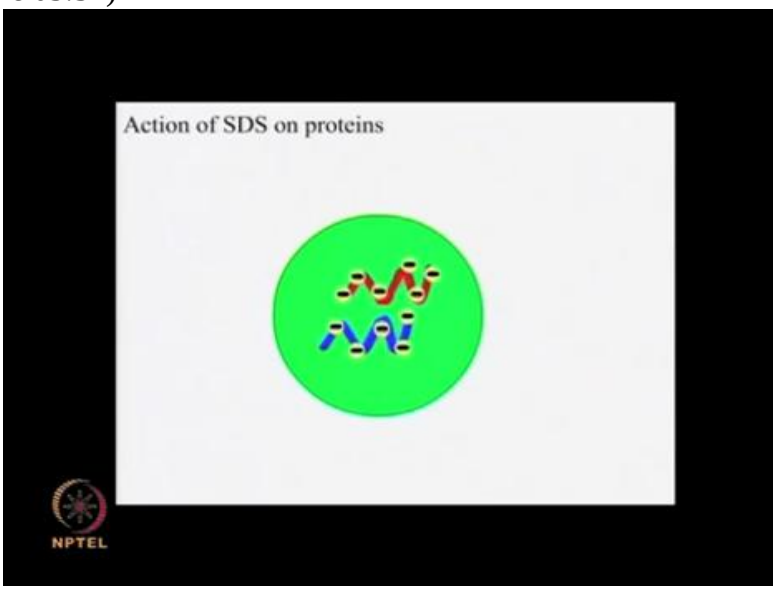

and binds to the polypeptide backbone at a constant weight ratio. This ensures that the protein present gets separated exclusively on the basis of the molecular weight rather than their mass to charge ratios as in Native PAGE.

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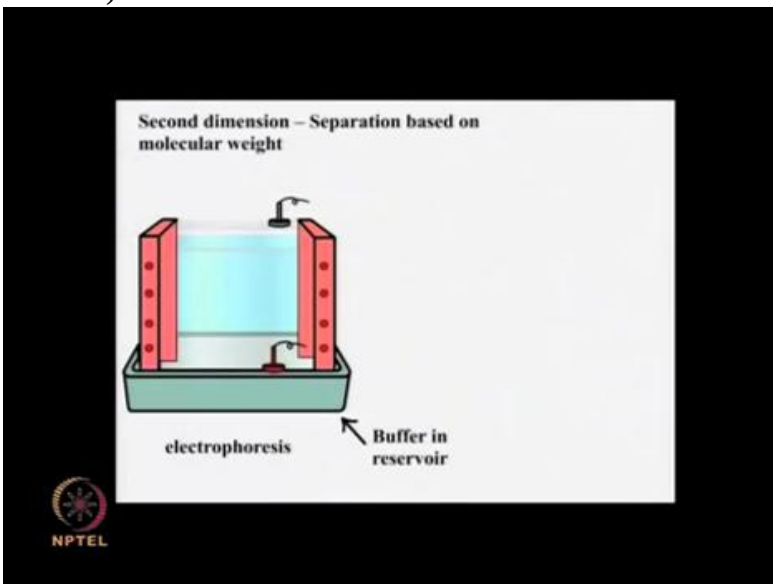

The proteins having lower molecular weight have high mobility

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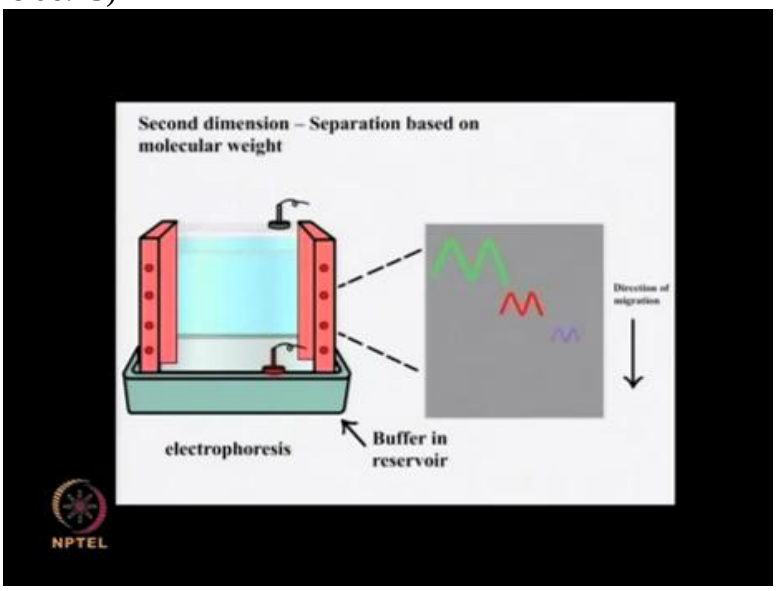

and migrate further through the gel pores while the high molecular weight proteins remain closer to the point of application. This allows efficient separation of the proteins in the second dimension.

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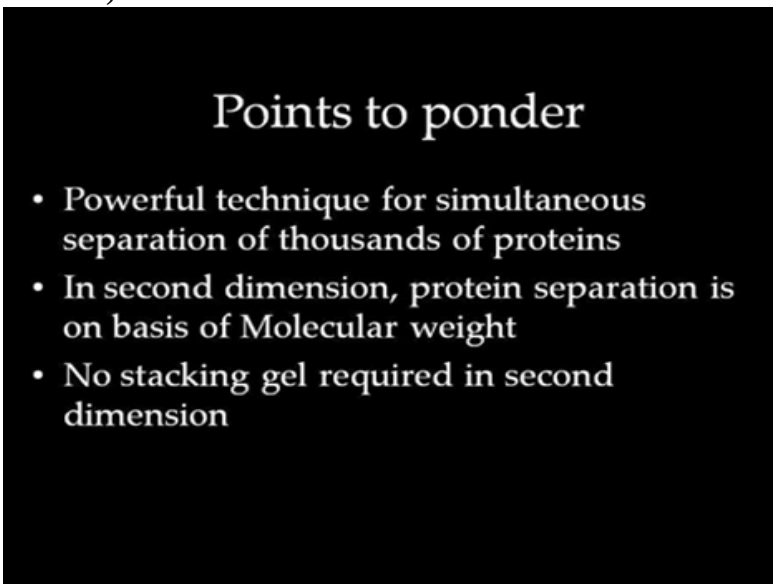

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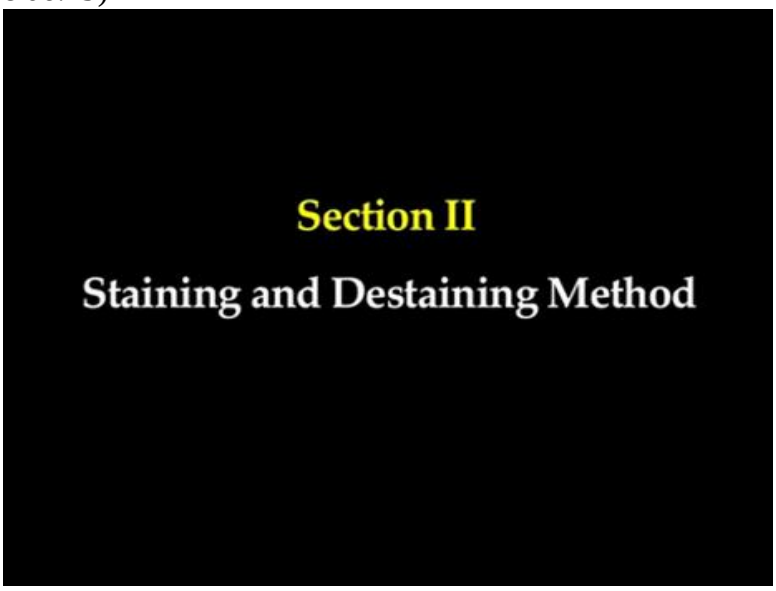

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So now we will talk about how to stain the gel and visualize the protein spot. Because so far, now you are able to separate the protein based on their pI, based on their molecular weight and now everything is on a transparent gel. You do not know how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

So different types of staining methods have been developed and those can be applied to visualize your protein bands or your protein spots depending on your what type of gel you are using.

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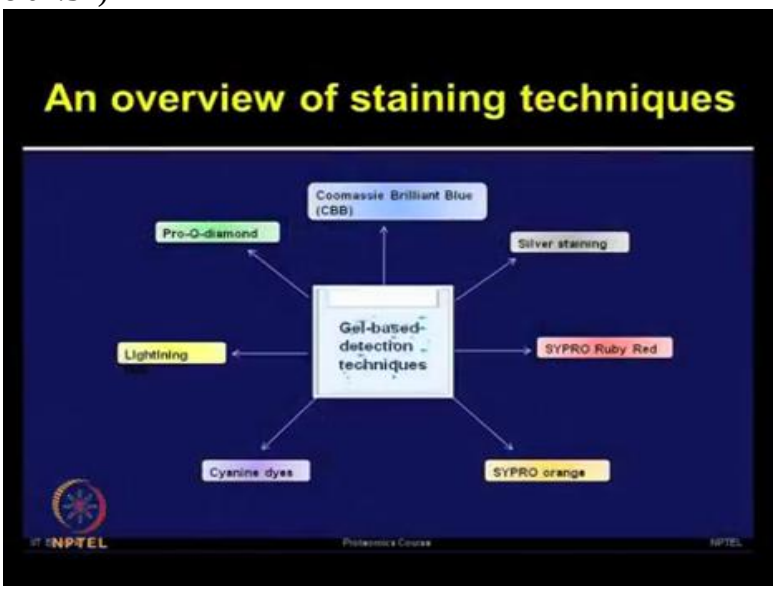

After giving you an overview of staining techniques, let me describe you few staining methods in more detail. Let's start with Coomassie blue staining. This is one of the most commonly used stains for protein detection in polyacrylamide gels.

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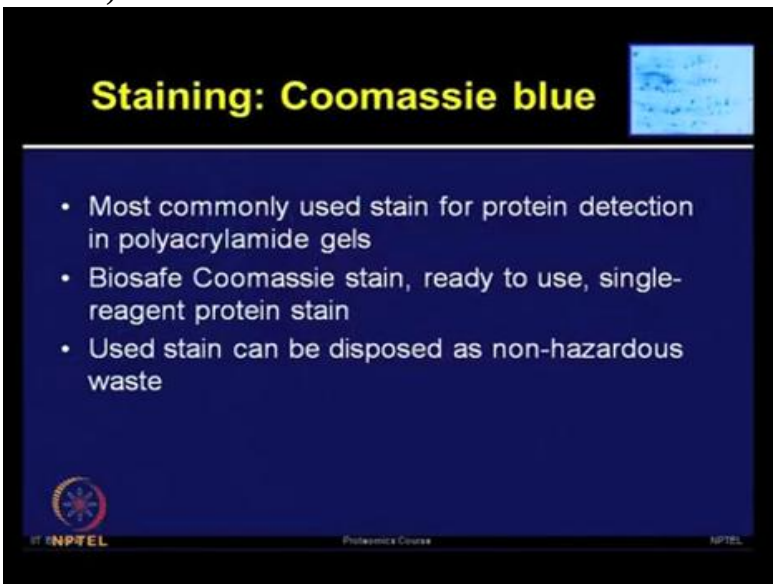

The sensitivity is good but not very sensitive as compared to the silver or SYPRO Ruby but its ease of performing the staining method, the stability, the cost very less and good compatibility with mass spectrometry, all of this make Coomassie blue as a stain of choice for most of the laboratories world-wide.

Now there are some advanced forms of Coomassie stains being developed including Biosafe Coomassie stains which are non-hazardous, made keeping in mind the environmental scenario. So it can be disposed as non-hazardous waste. The Biosafe Coomassie is also ready-to-use stain and a single reagent protein stain.

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So I will show 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.

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The completed electrophoresis gel is placed in a tray containing Coomassie blue staining solution…

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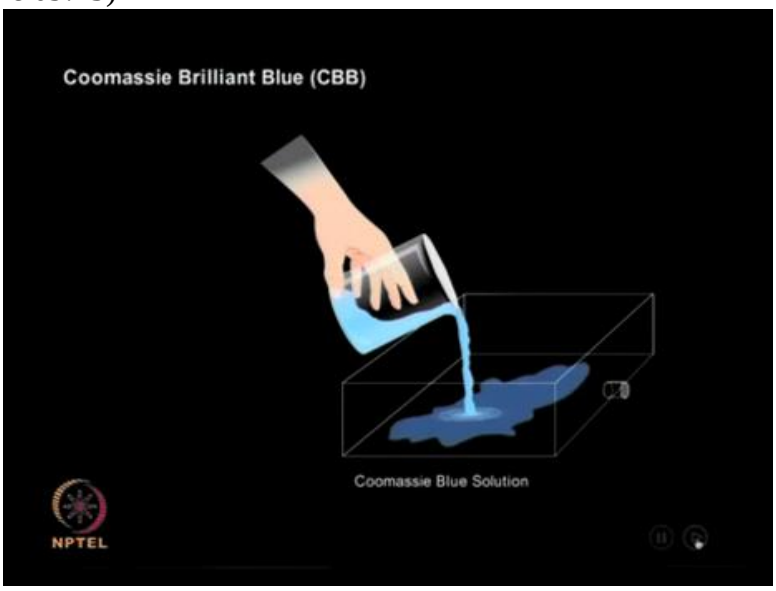

, typically R250 that has been dissolved in an aqueous solution of methanol and acetic acid.

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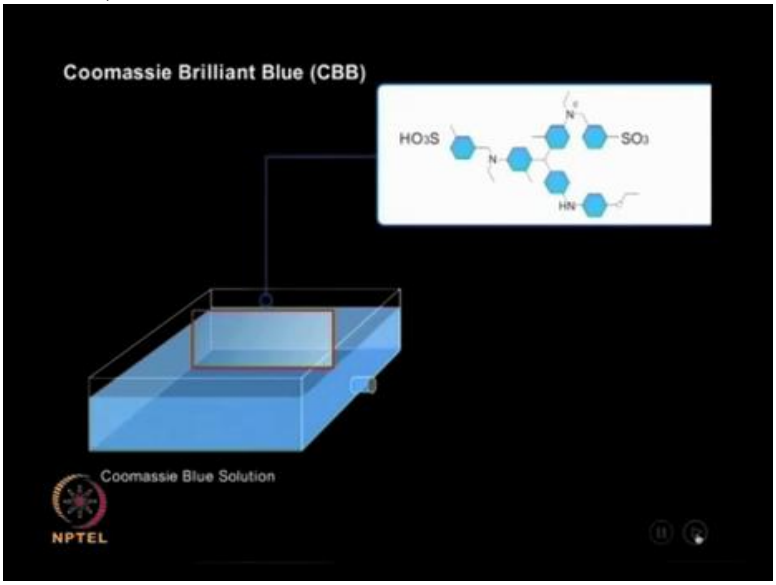

The negatively charged Coomassie dye …

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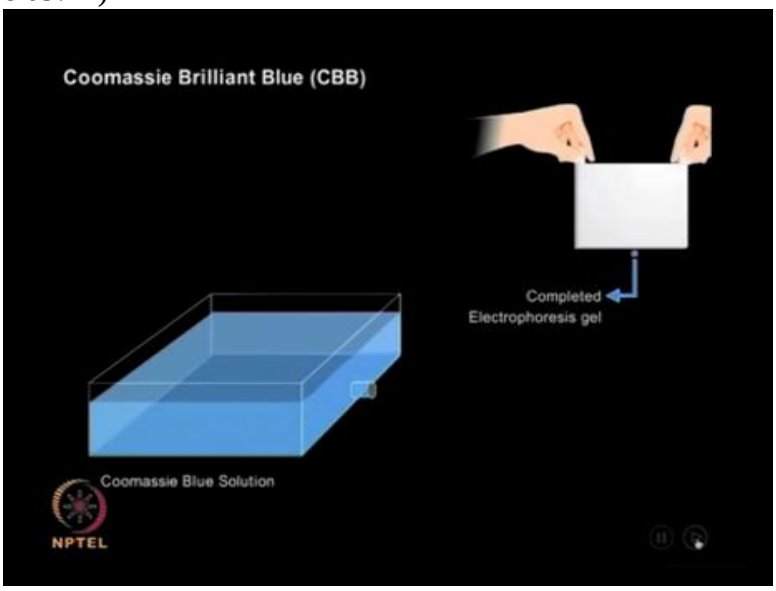

interacts with protein through ionic and non-covalent interactions

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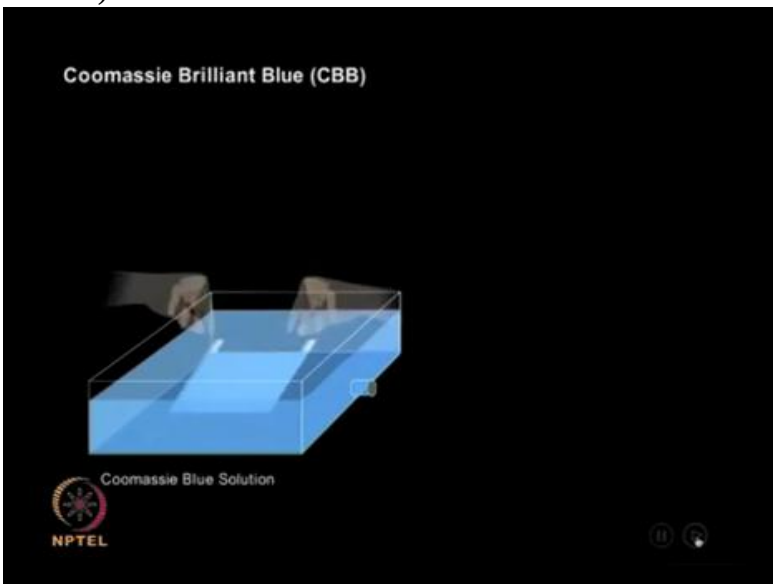

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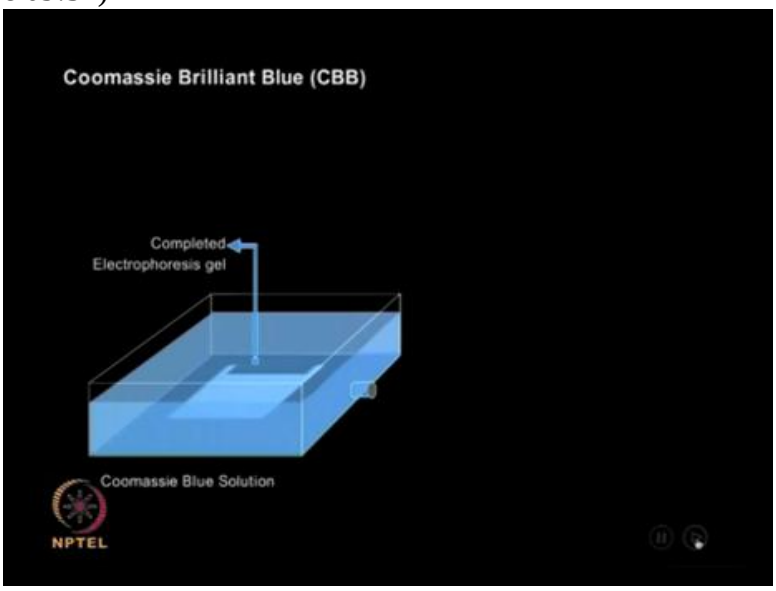

After adding the stain

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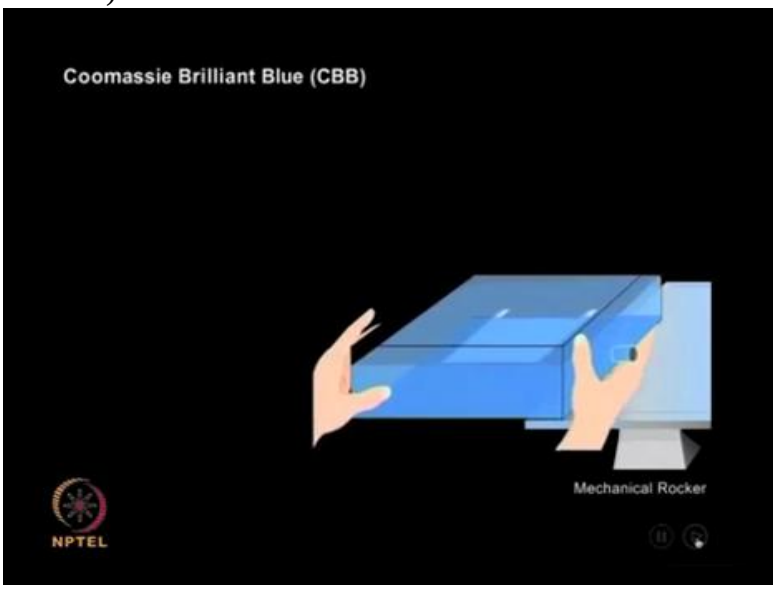

, the tray is then placed on a mechanical rocker which allows for

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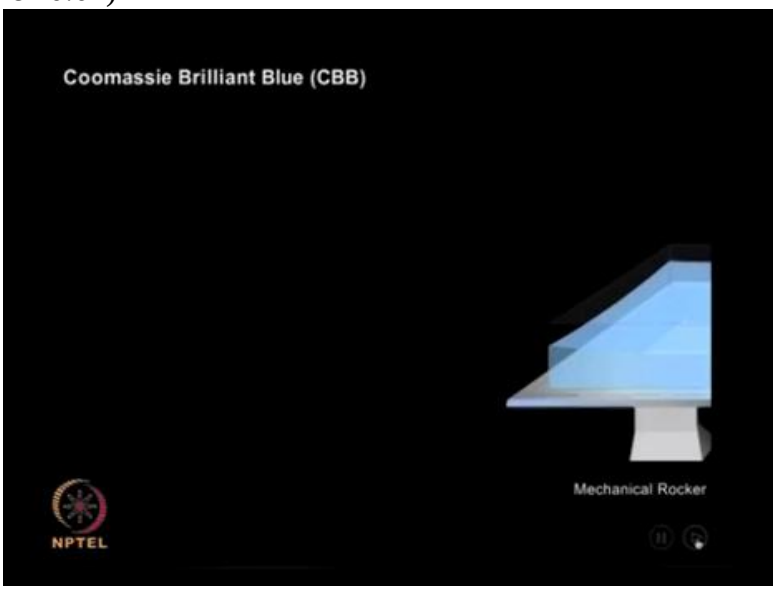

uniform contact of the gel with the solution by means of gentle rocking.

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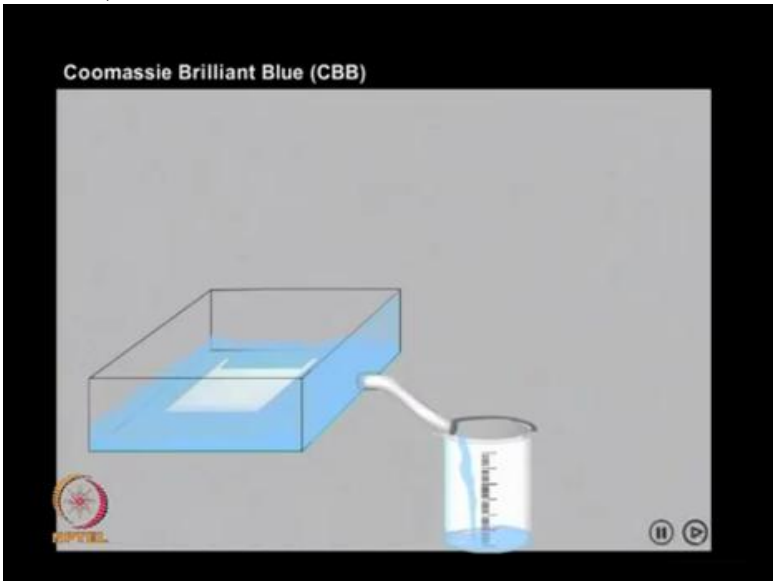

After overnight stain, you have to drain out the staining solution.

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The stained gel is then placed in the destaining solution which consists of 50% methanol and 10% acetic acid to remove any excess dye bound to the gel.

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Again this process involves overnight steps or

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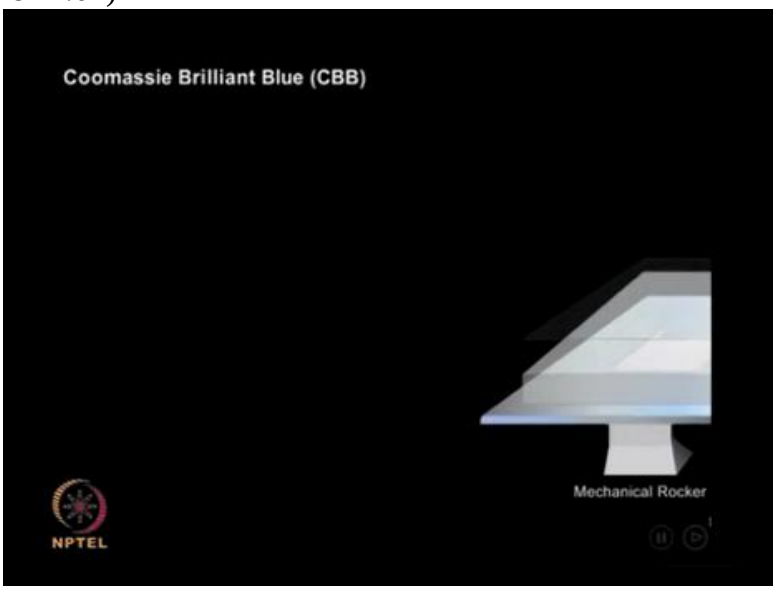

at least of 10 to 12 hours of gentle shaking on the rocker.

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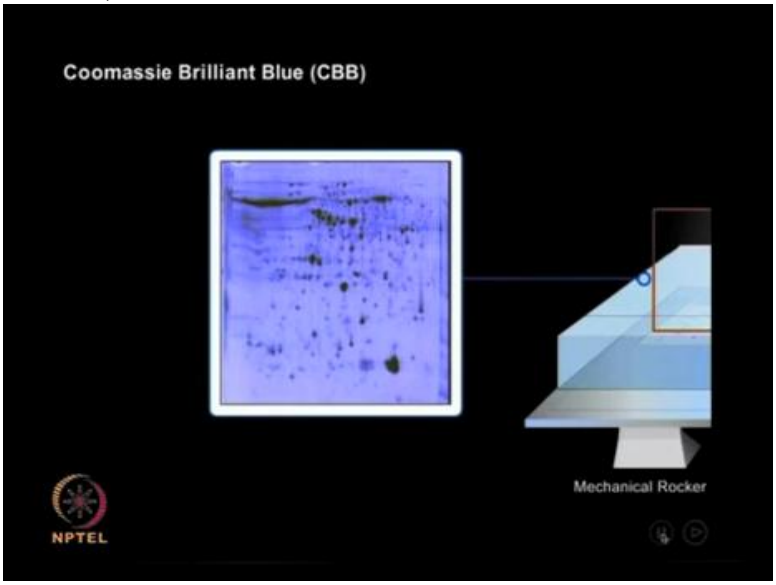

The stained gel can finally be viewed as shown in this image here showing a 2D gel stained with Coomassie Brilliant blue and it can be scanned by using a scanner and these protein spots can be easily viewed.

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So, in this animation you are able to see after performing the 2D 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 spots can be now used further for image analysis purpose.

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 skills, still you are unable to obtain a very good protein quantity. So, how to move forward?

You want to still do the separation of 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 will describe you how to do silver staining.

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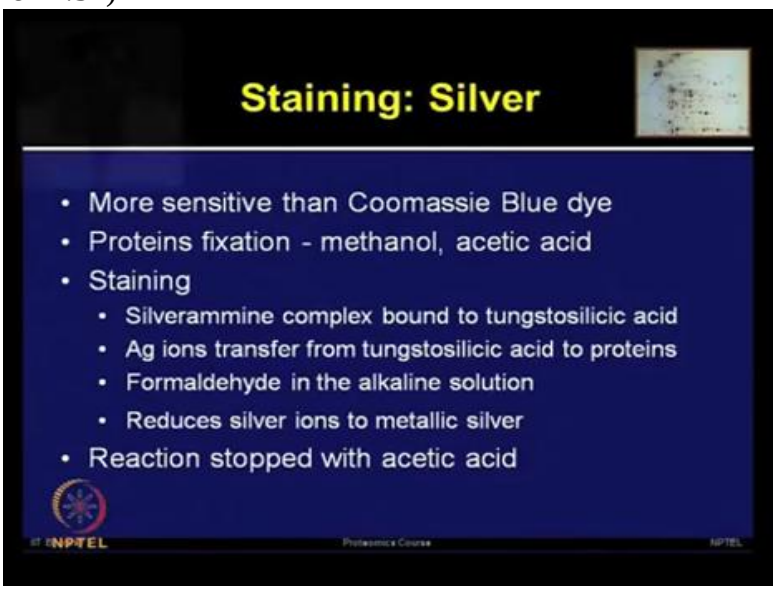

The silver staining is more sensitive than Coomassie blue dye. First of all, when you have run your 2DE gel, you need to do the protein fixation in the gel by adding methanol and acetic acid.

The staining procedure involves silveramine 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 this reaction to occur continuously, you will see a very dark background on the silver stained gel. So you need to stop the reaction by adding acetic acid.

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I will now show you this animation how to perform the silver staining procedure.

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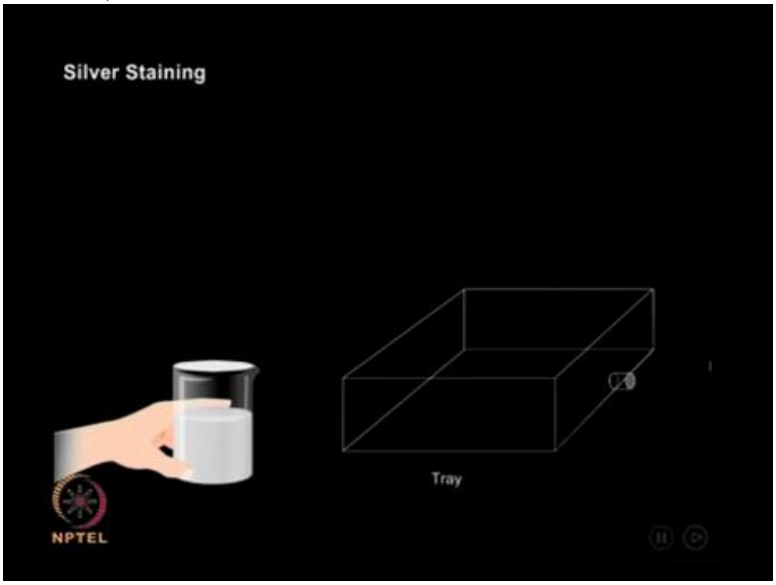

In this animation of silver staining, I will describe you the procedure how to stain the gel by using silver stain.

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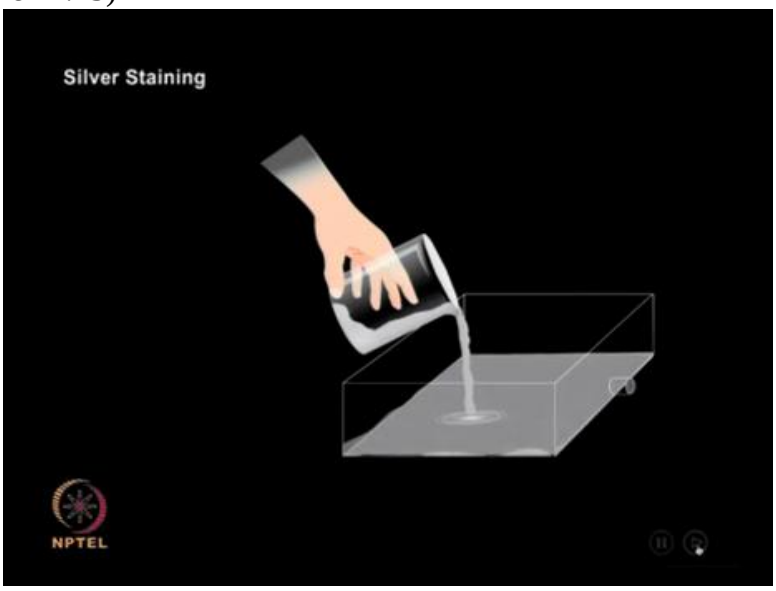

The completed gel is first placed in the fixing solution of methanol and acetic acid that fixes the protein bands in the cell and minimizes any diffusion.

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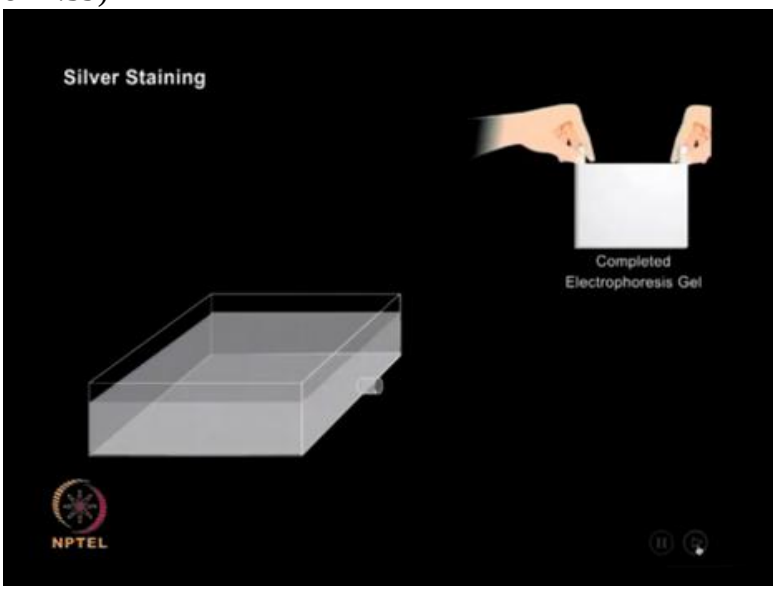

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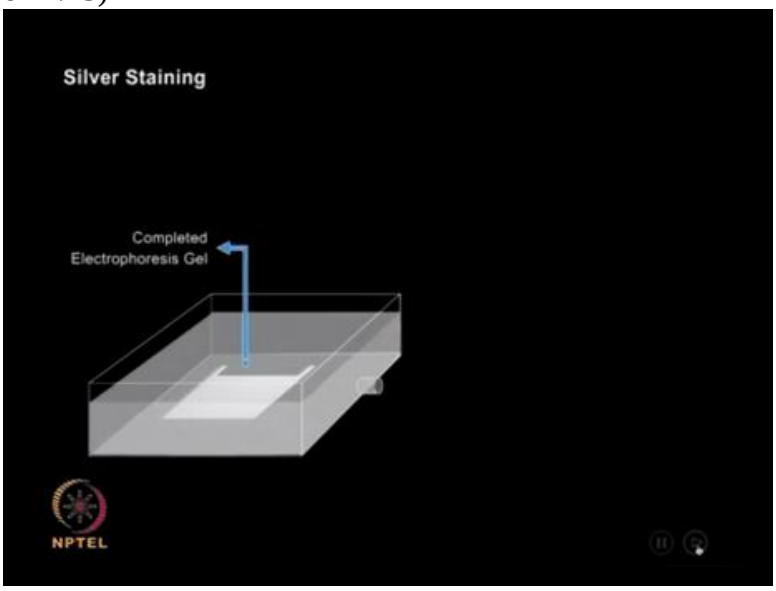

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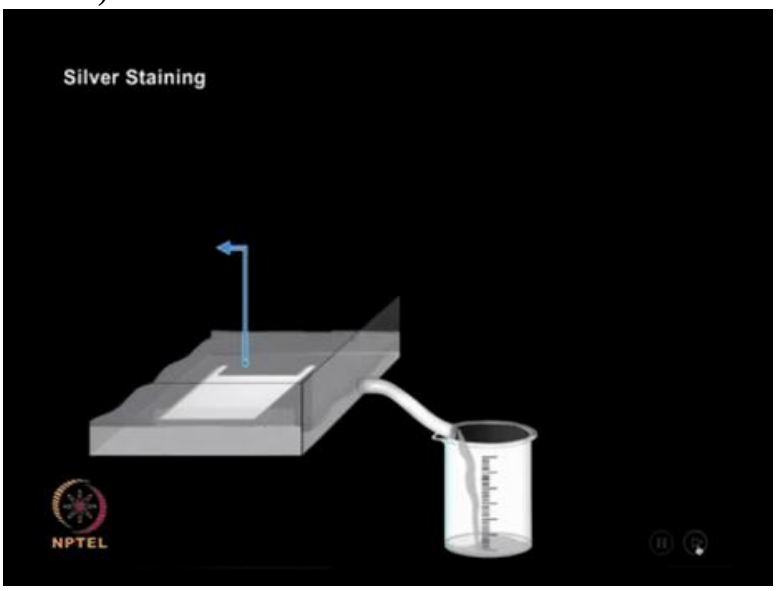

This step must be subjected to gentle shaking of around 30 minutes after

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which the silver stain solution is added.

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After adding the silver solution, the gels are rocked gently in order to allow for proper and uniform staining.

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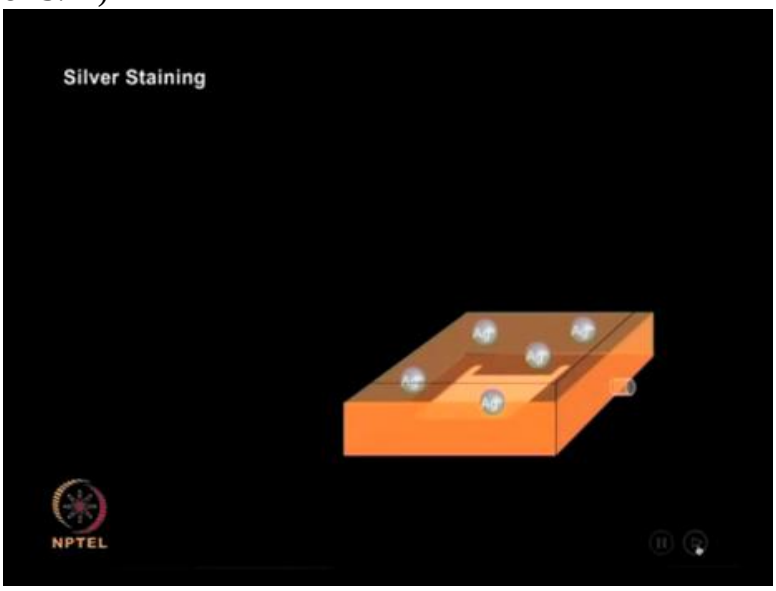

The silver stained gels are first washed to remove any excessive stain and then placed in a developing solution where the silver ions get reduced to metallic silver.

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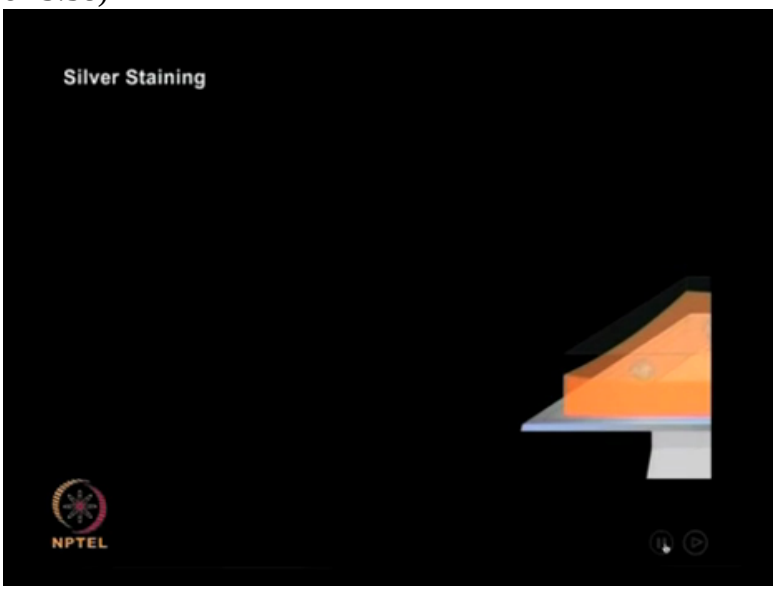

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Formaldehyde in alkaline solution

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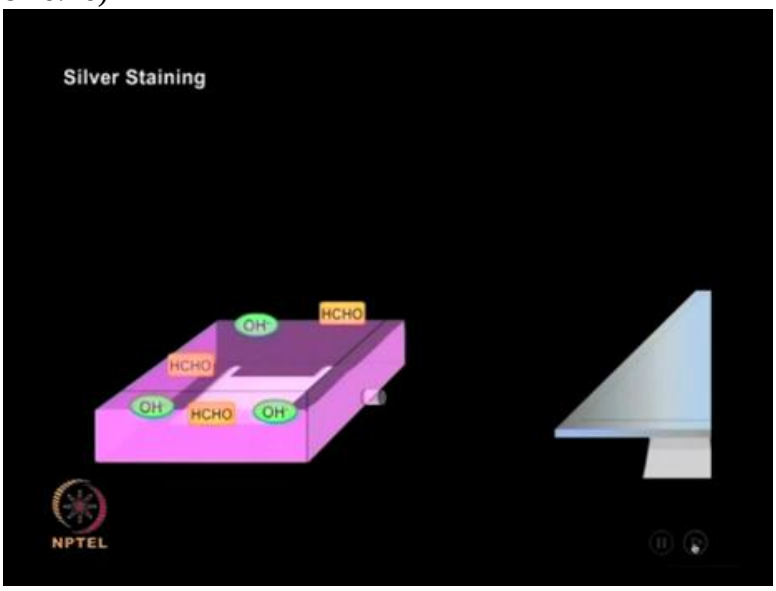

in the presence of sodium carbonate or other alkaline buffers are commonly used for this process.

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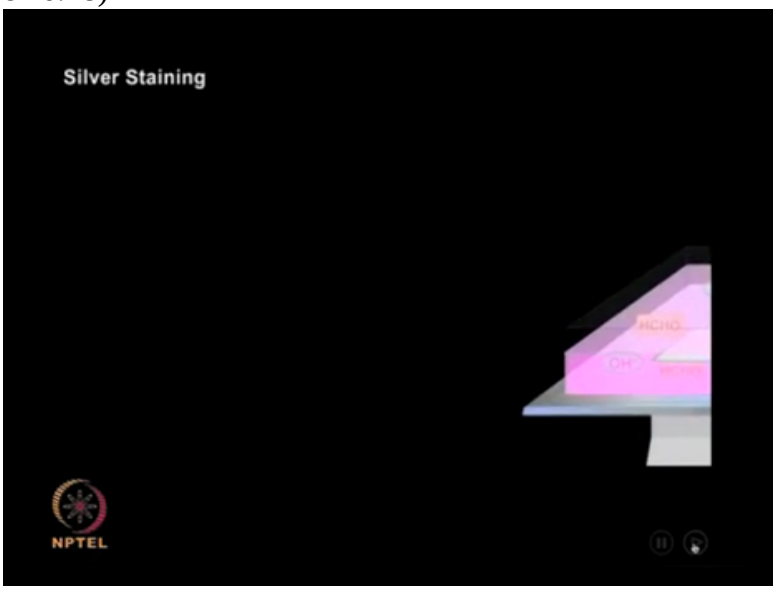

Again a gentle rocking will allow

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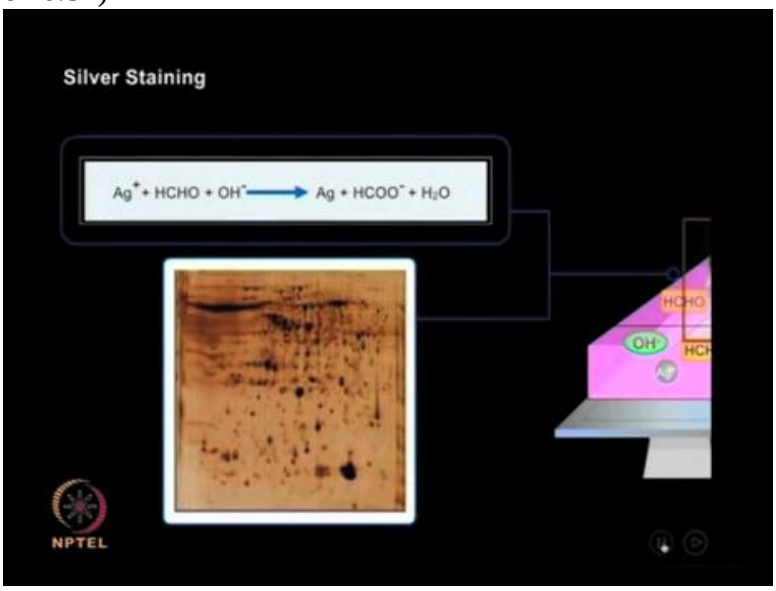

for uniform staining pattern. Finally the gel can be viewed as dark bands or dark spots against light background.

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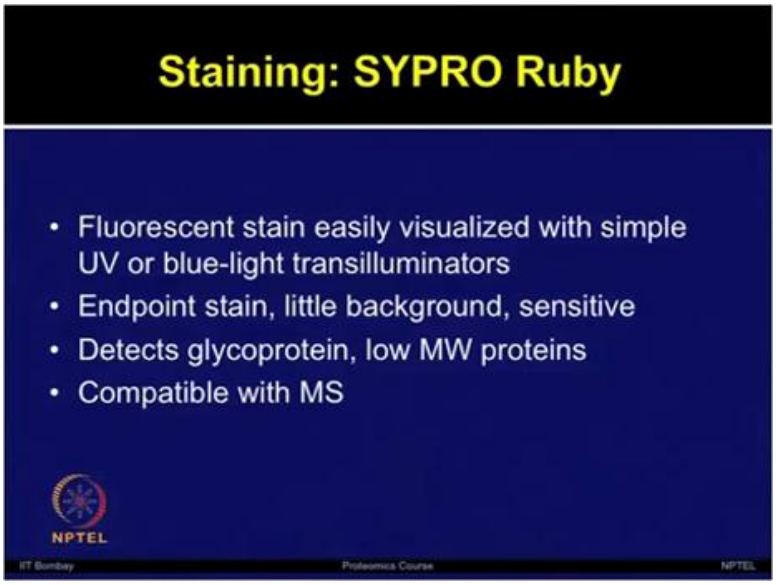

So let's start with SYPRO Ruby staining. This is one of the fluorescent stains which is usually visualized on the gel in the UV or blue light transluminescence.

This stain is very sensitive and it is endpoint stain. So very little background is used unlike the silver stain where there are lot of issues with the very dark background, SYPRO Ruby has overcome those limitations.

This is also one of the very sensitive stains and it can also be used for studying different types of post-translational modifications such as glycoprotein.

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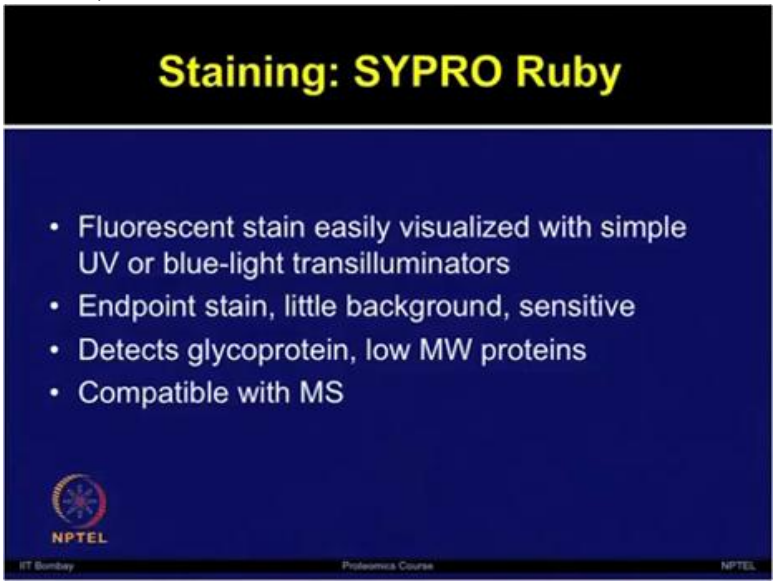

Often, even low molecular weight proteins which are low abundance proteins, those can also be visualized by using this type of stain. So SYPRO Ruby is one of the very useful stains which is very compatible for doing further processing with the mass spectrometry.

If you want to separate the proteins you have to visualize them. After that, your end aim is to characterize those proteins, what those proteins are and further you want to do experiments on those. In that light, SYPRO Ruby is very compatible for mass spectrometry and you can further characterize your proteins.

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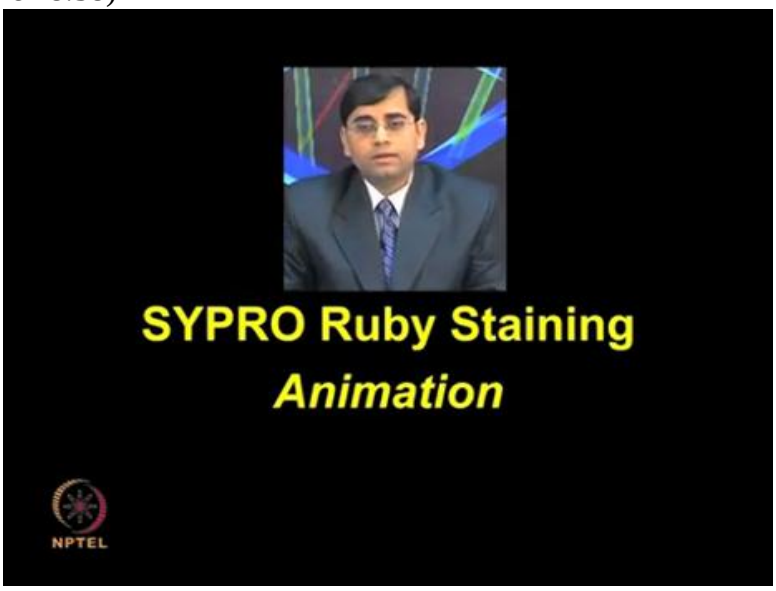

So now I will show you the steps involved in performing SYPRO Ruby stain. So I will show you one animation.

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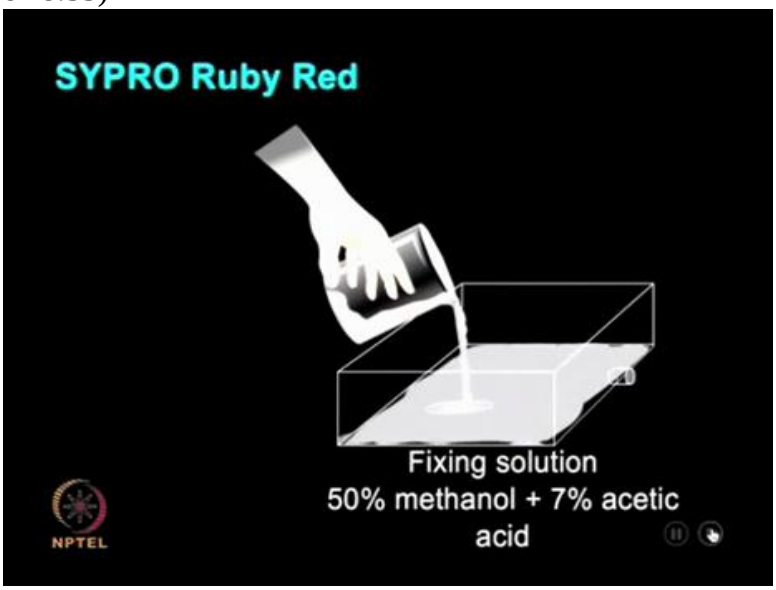

SYPRO Ruby Red, the completed gel is first placed in the fixing solution of methanol and acetic acid

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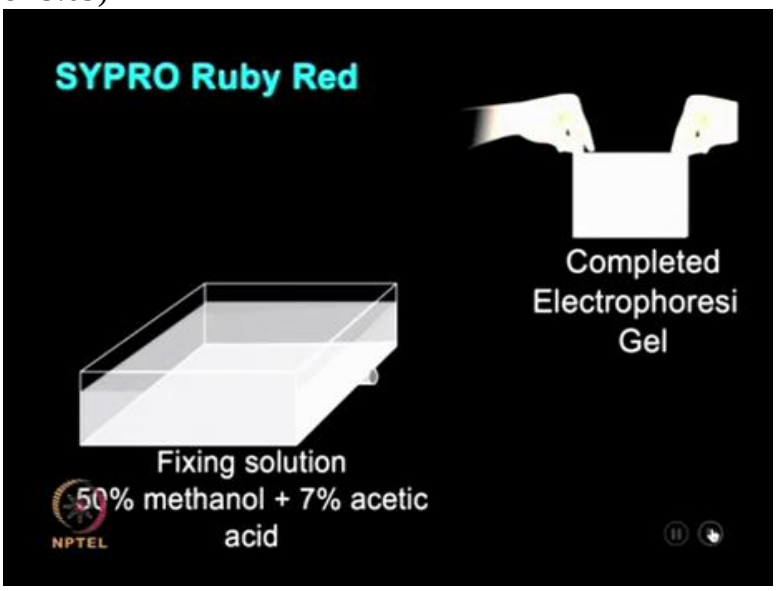

that fixes the protein bands in the gel and minimizes any diffusion.

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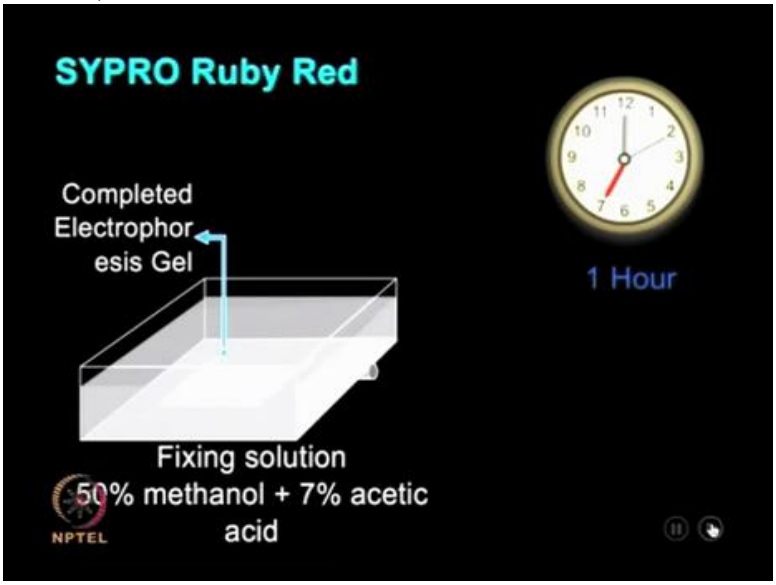

This must be subjected to gentle shaking for around half an hour.

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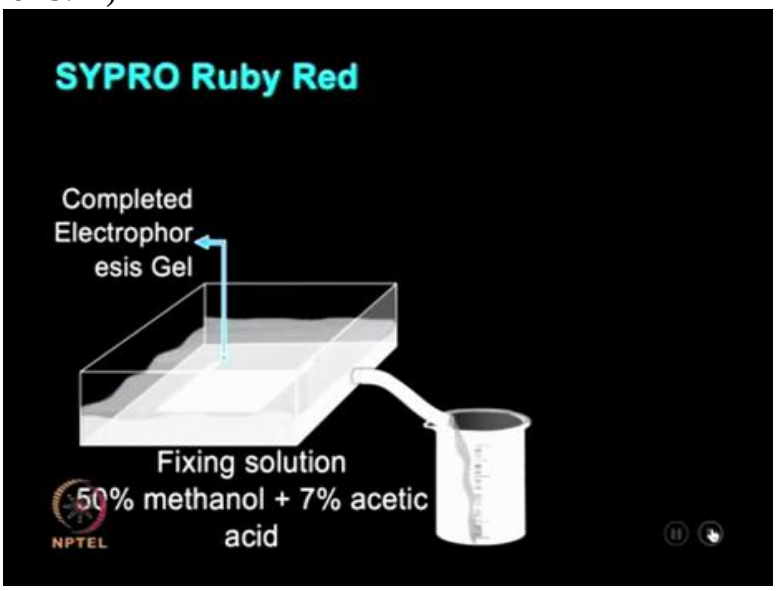

After fixation step

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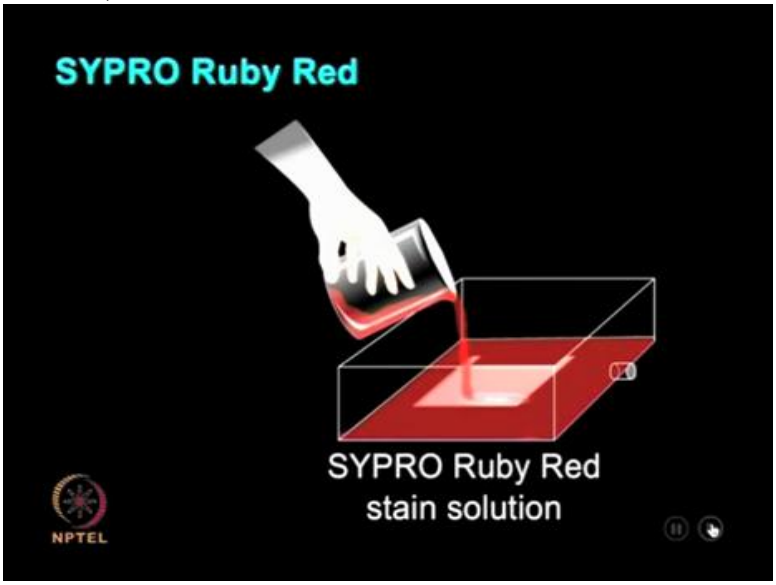

, the gel is soaked in the fluorescent SYPRO Ruby red stain solution.

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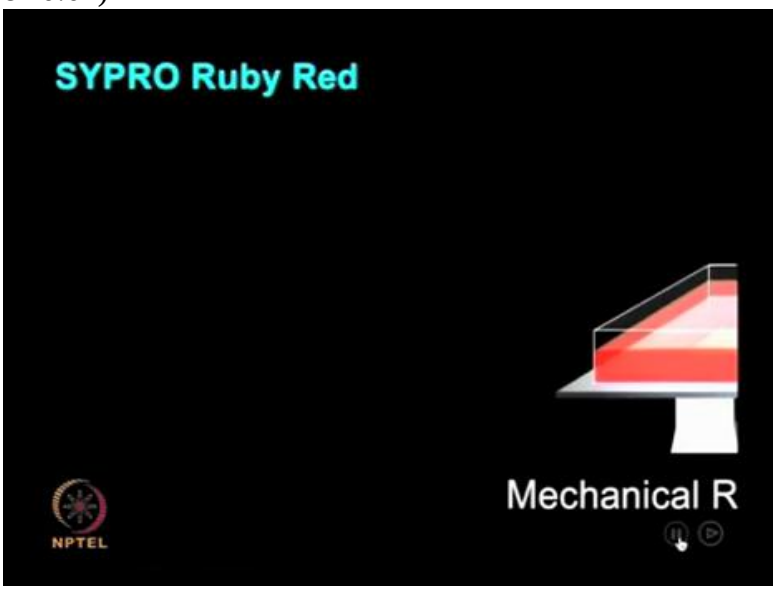

Gel should be kept on the mechanical rocker for uniform shaking.

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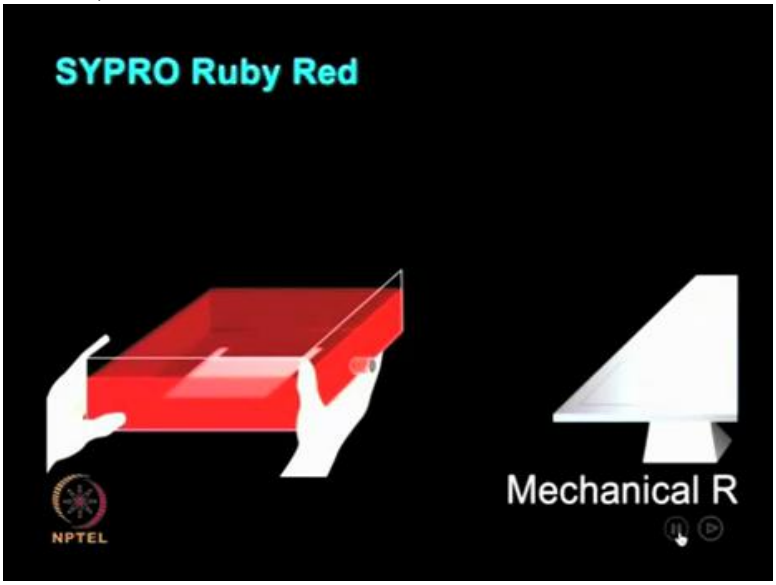

During the process the gel gets uniform staining with ruthenium based red dye.

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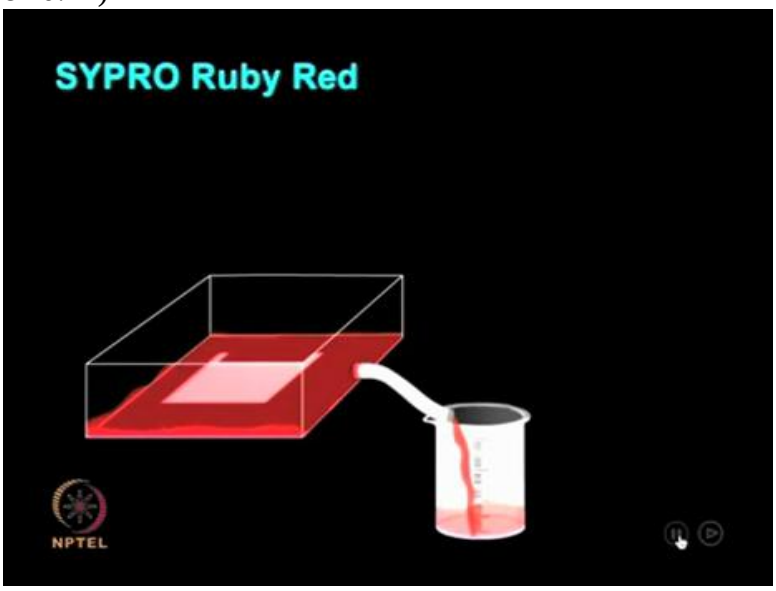

After this overnight step of staining

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… the gel can be washed with methanol

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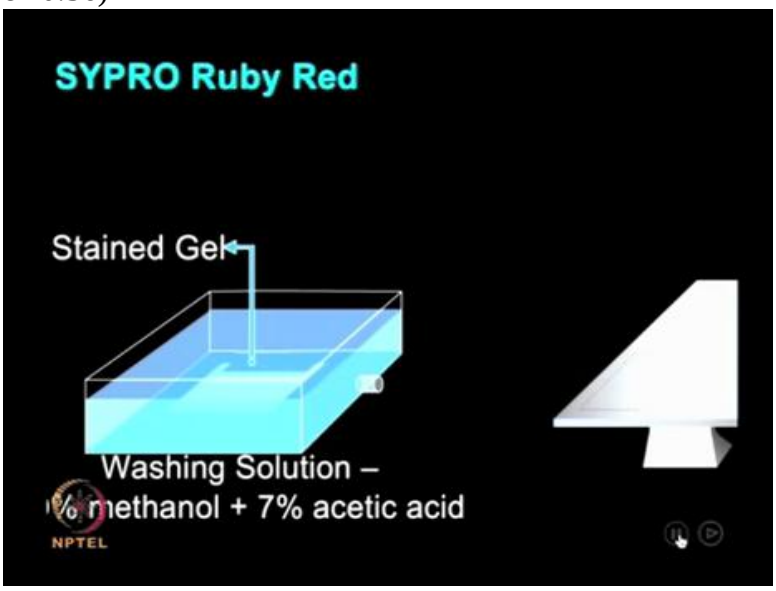

and acetic acid solution.

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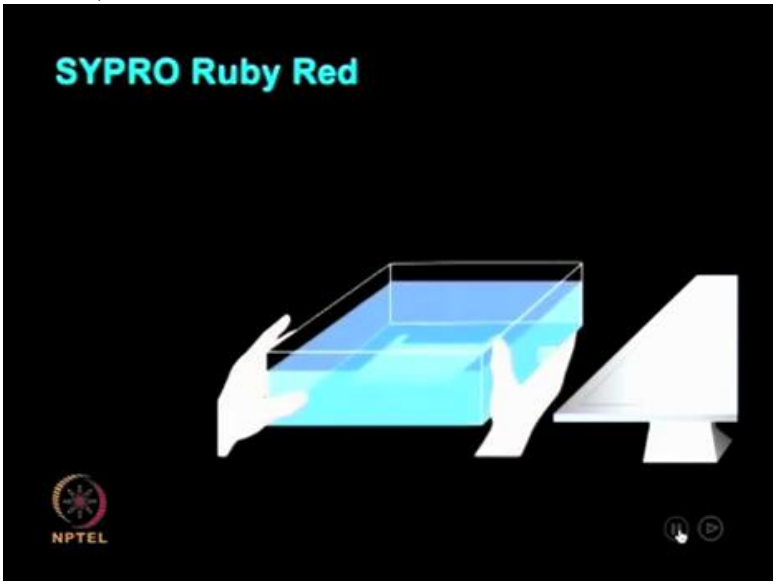

Again you need to keep it on rocker

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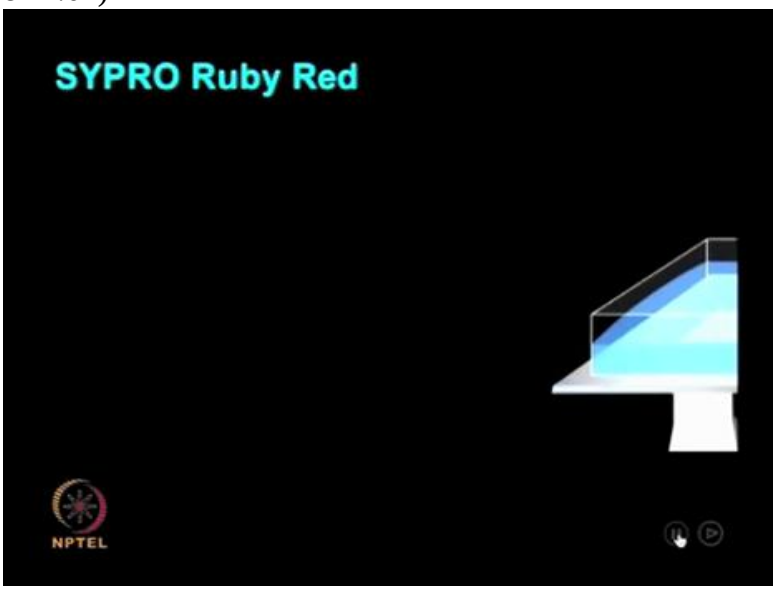

so that uniform destaining can be performed.

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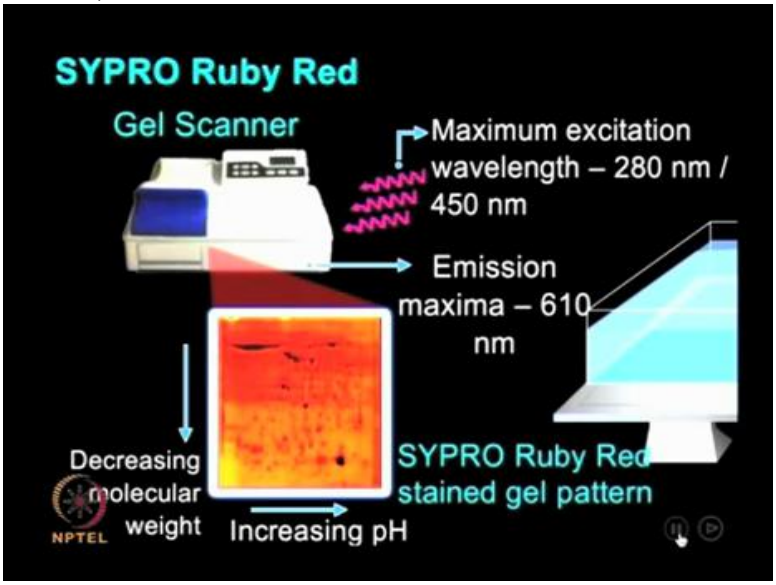

The MS compatible gel is then viewed using UV or laser scanning instrument with an excitation wavelength near 280 or 450 nanometers. The stain has two excitation maxima and an emission maxima of 610 nanometers.

In this animation you can see a gel pattern which is showing you the staining obtained after SYPRO Ruby Red and it is showing separation two dimension based on the molecular weight and isoelectric point. SO now, after looking at this animation, you are familiar that different types of staining methods can be used.

Now let me introduce you to another stain which is Pro-Q Diamond which is used for studying the phosphorylation of the post-translationally modified proteins.

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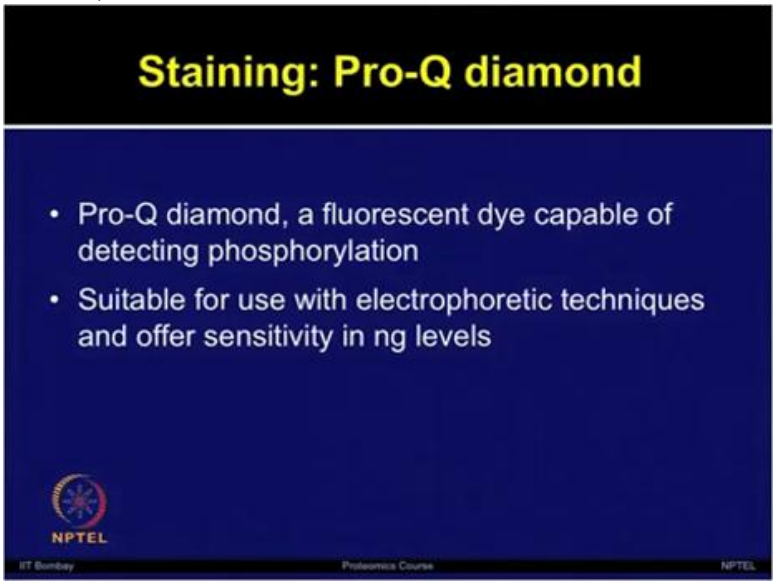

Now Pro-Q Diamond; that is a dye, fluorescent dye which is capable of detecting phosphorylation.

If your protein having some phosphorylation, addition of certain phosphate residues, now this stain is quite sensitive to pick up those patterns of phosphorylation or post translational modification in your gel. This is very suitable for use in electrophoresis and it offers sensitivity in nanogram level.

Now, let's say you want to look at the post translationally modified form of the protein but after that you would also like to know which protein it belongs to. You would like to do the mass spectrometry on these. So often it becomes very difficult if you have just done the Pro-Q Diamond on one gel.

So you would like to do the dual staining.So first you would like to detect only your post translationally modified form of the protein or the phosphorylated form, then followed by; the same gel can be used for further staining with some endpoint stain such as SYPRO Ruby.

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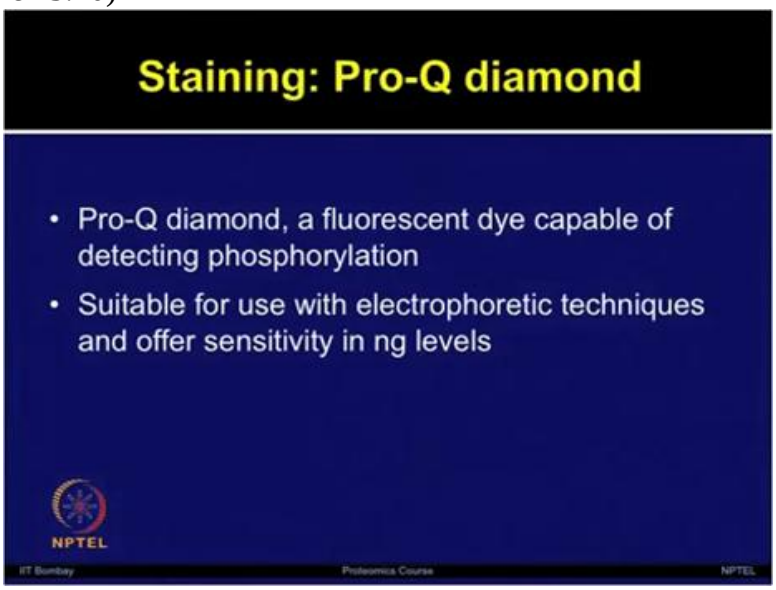

Now you take same gel, further stain with the SYPRO Ruby, so you will obtain two images for the same gel where few spots can show the PTM form and the overall, the global pattern of the proteins can be visualized on different gel.

So, in this regard, by comparing the PTM form or the phosphorylated form along with the complete profile of the gel, one can obtain the very comprehensive picture; these many proteins are separated on the gel, among those, certain numbers are phosphorylated. One can then further excite those spots and do the mass spectrometry for further identification and characterization.

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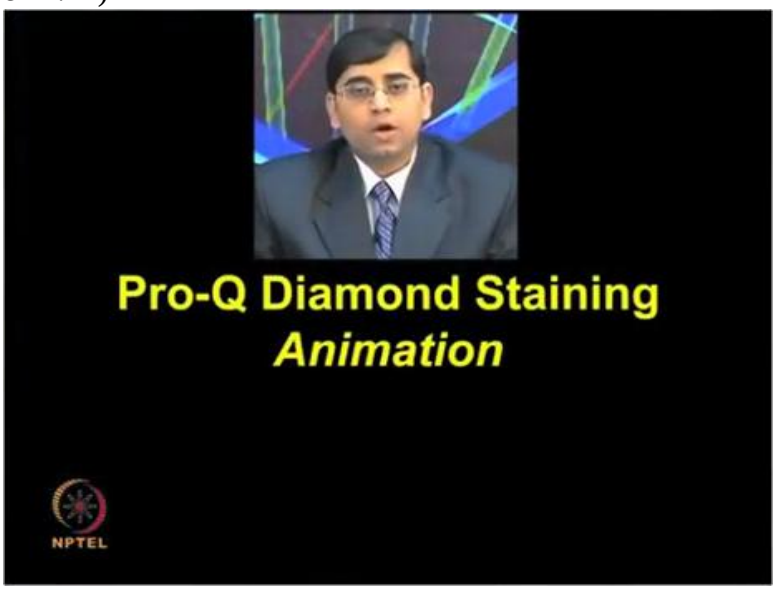

Now let me show you this animation for doing Pro-Q Diamond staining followed by dual staining.

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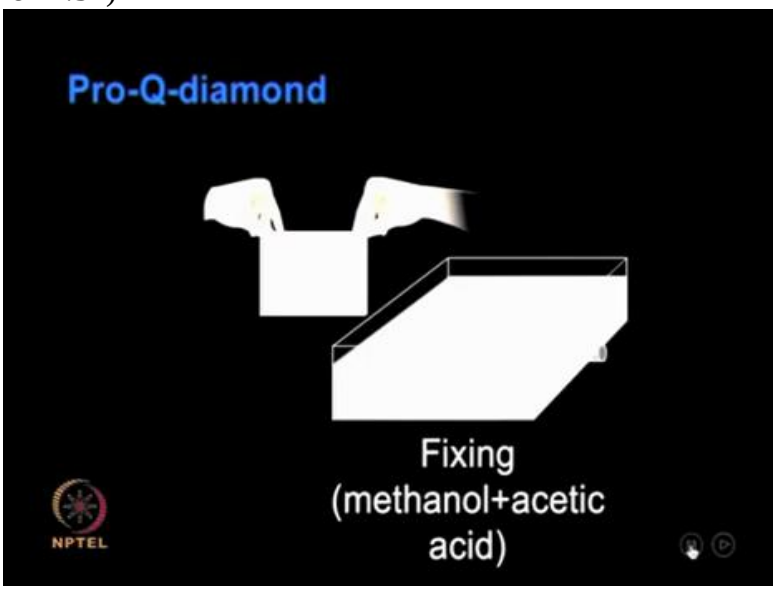

Pro-Q Diamond, this is very useful staining technique for detection of phosphoproteins

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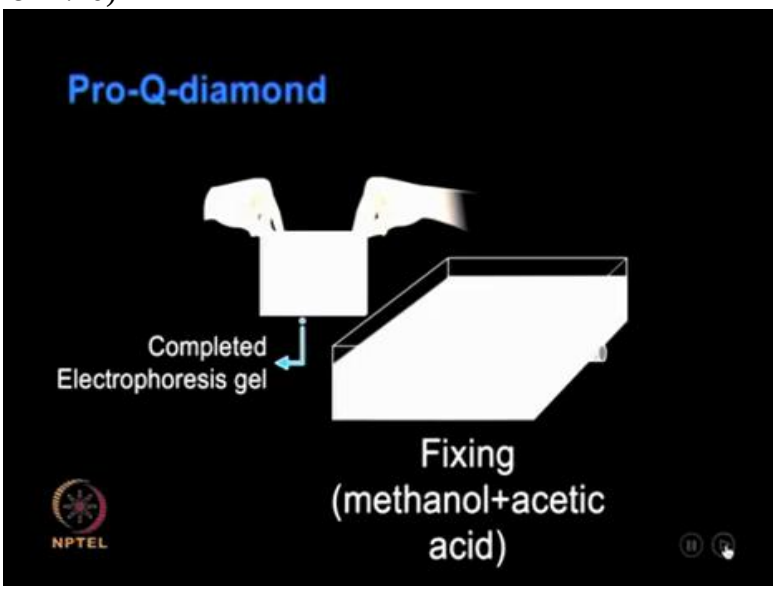

and

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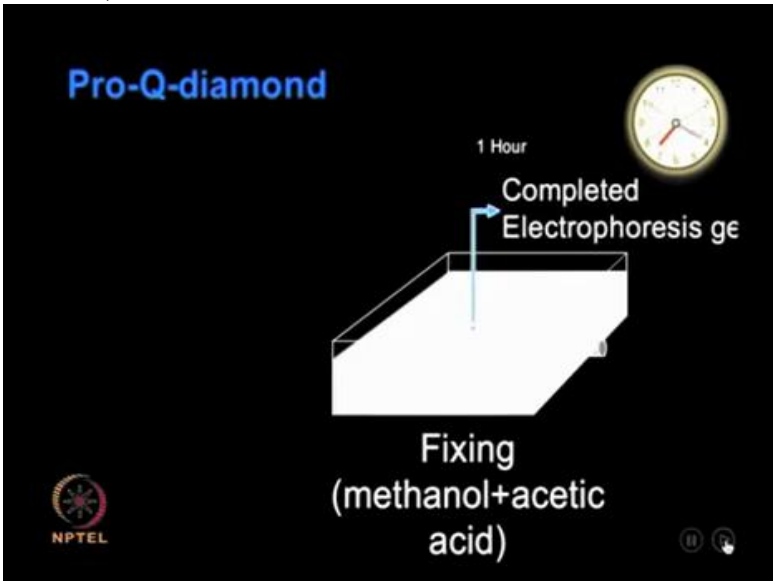

when used in combination with SYPRO Ruby Red,

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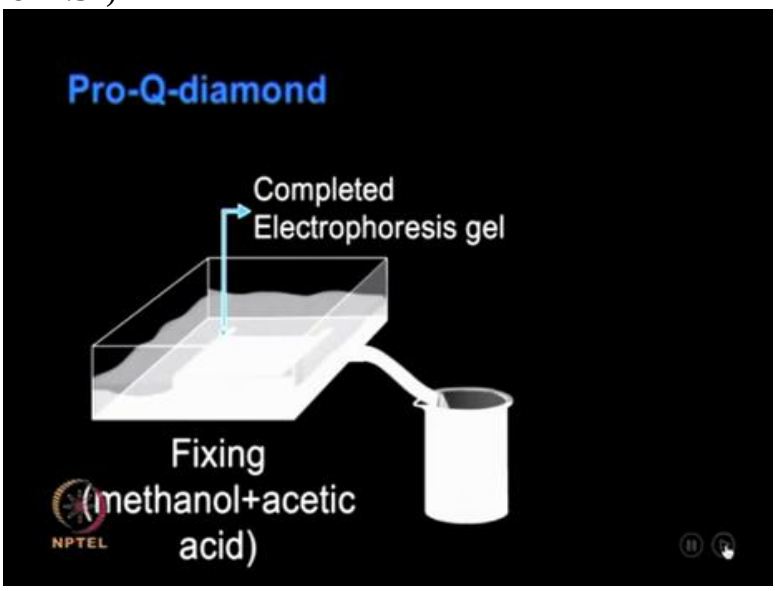

…it also provides a very comparative profile of total protein contents and phosphoprotein contents. The first step is to perform the fixation

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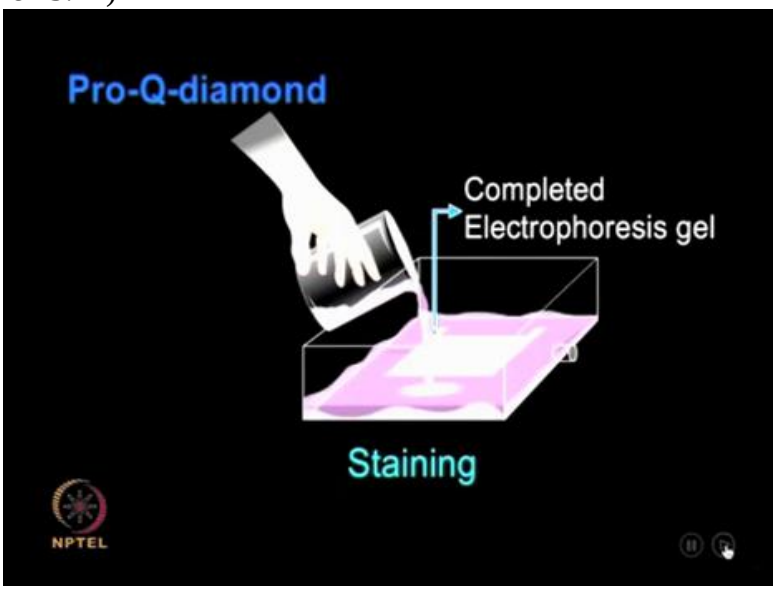

… and followed by addition of Pro-Q Diamond stain. The staining procedure is similar to the SYPRO Ruby Red which was earlier described.

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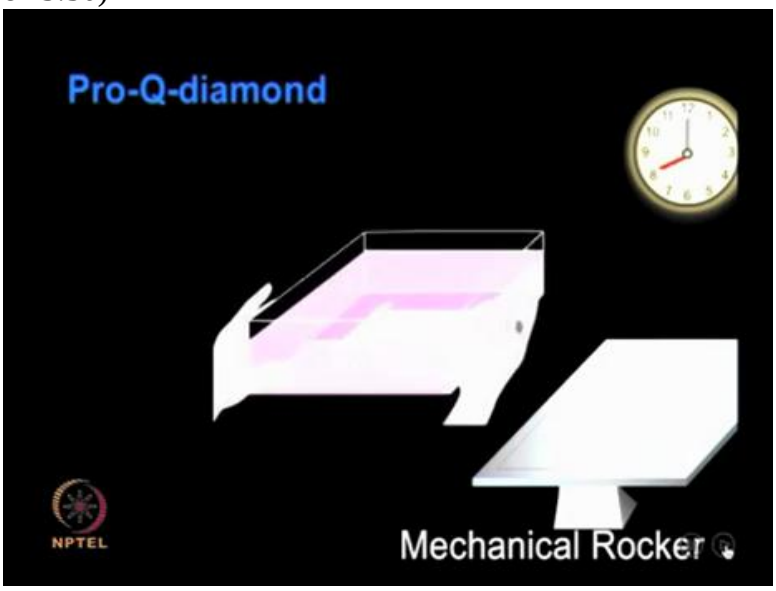

This fluorescent dye offers more sensitivity down to nanogram level and we get…

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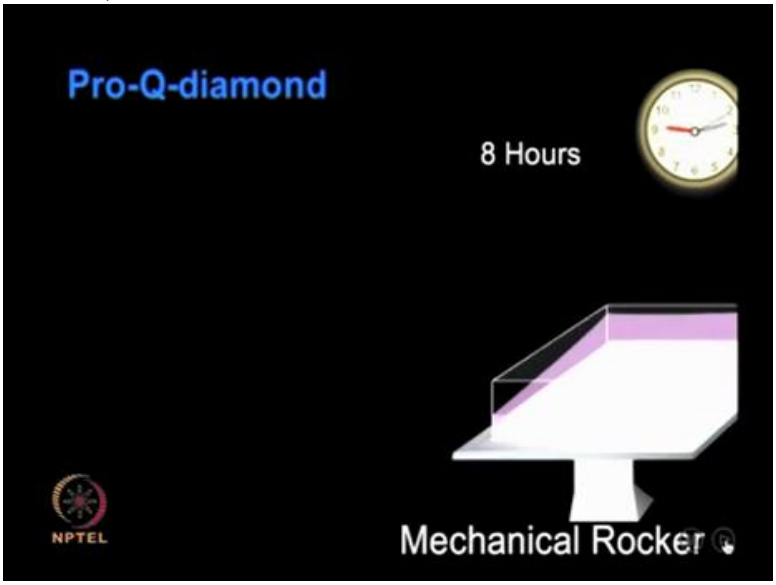

… specifically the phosphorylation taking place at Serine, Threonine or Tyrosine residues. For uniform staining, we need to add staining solution for at least 8 hours or overnight.

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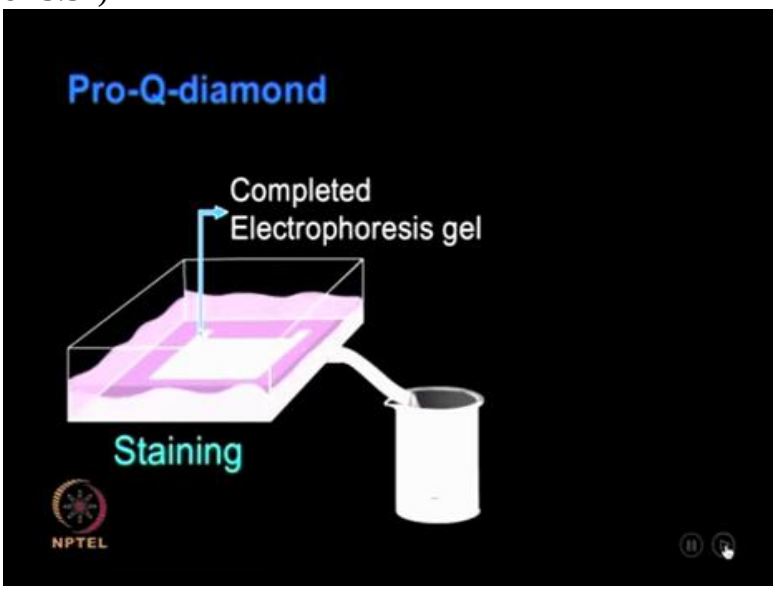

Now staining solution can be removed and …

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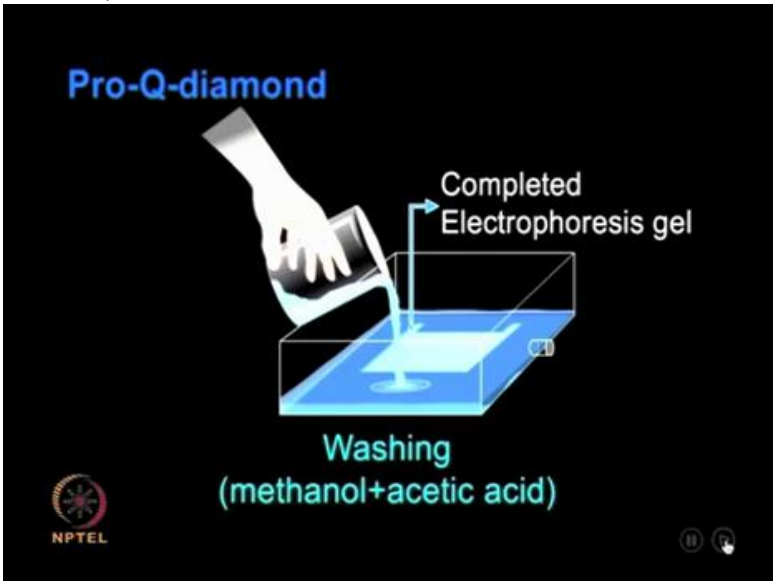

… further washed with methanol and acetic acid.

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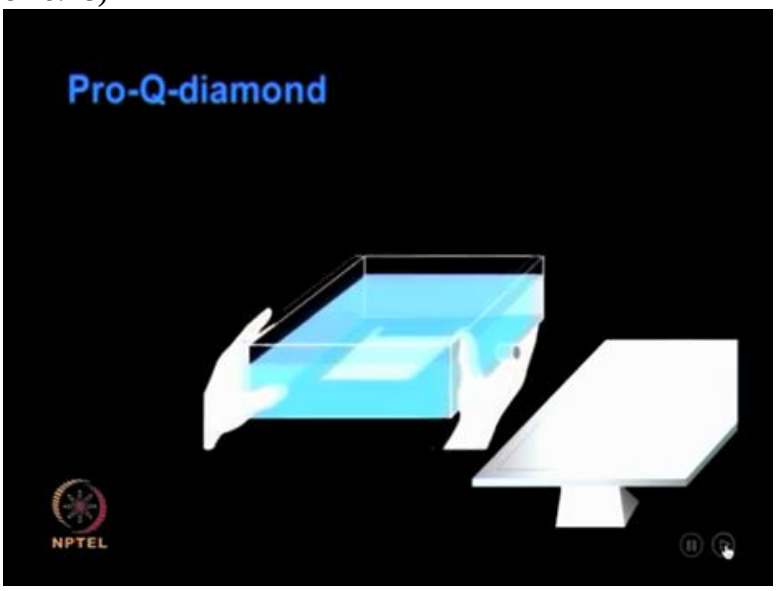

For uniform destaining, it is good idea to keep on gentle shaking…

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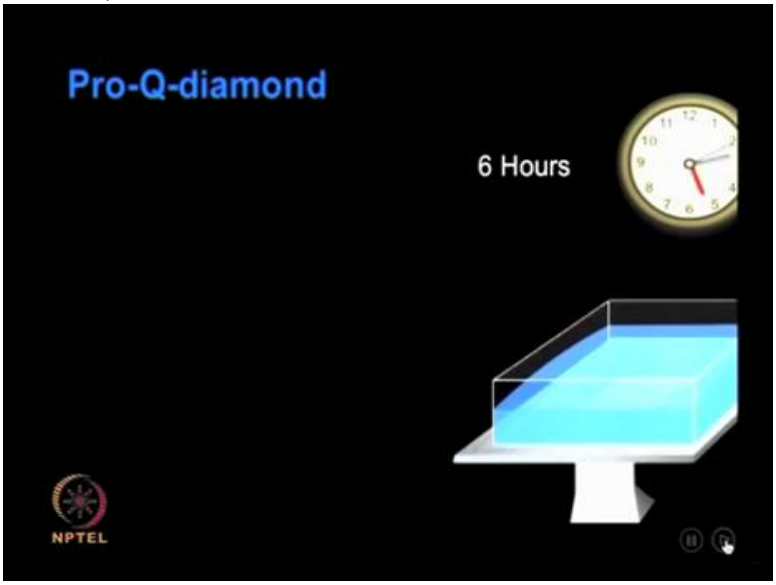

… for at least 6 hours.

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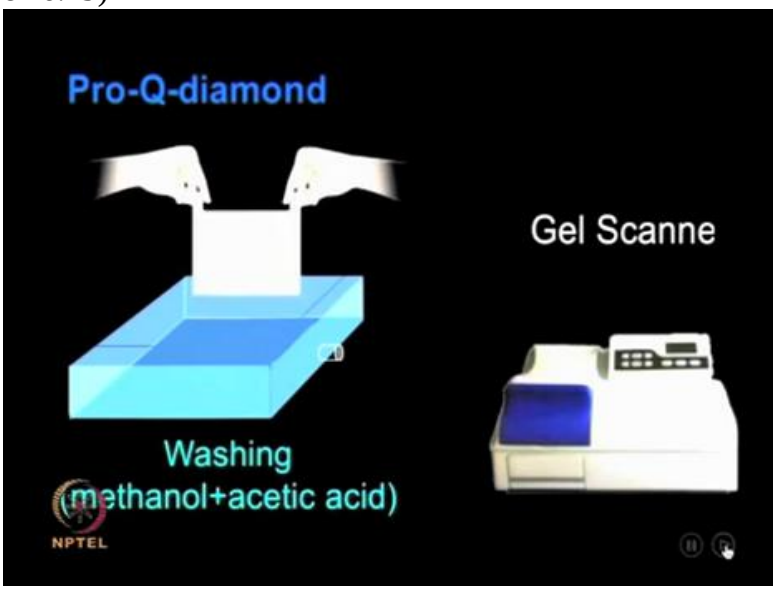

Gel can be further …

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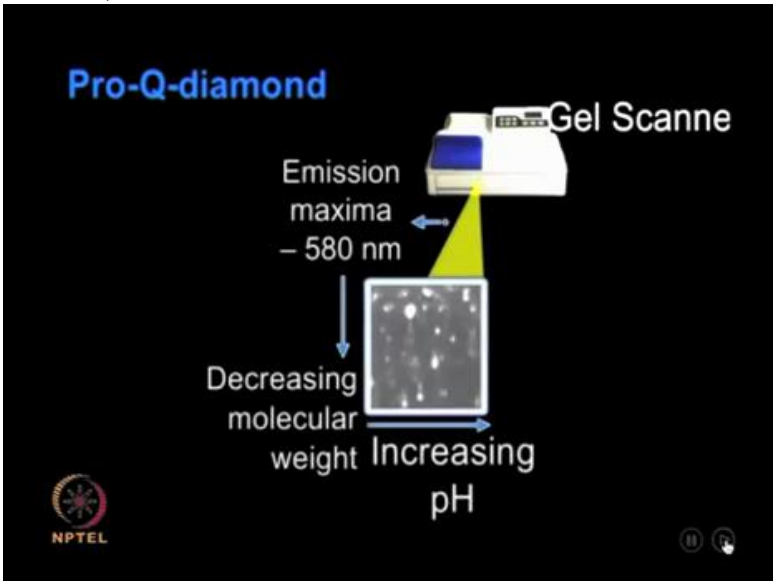

scanned and image can be procured especially at emission maxima of 580 nanometers …

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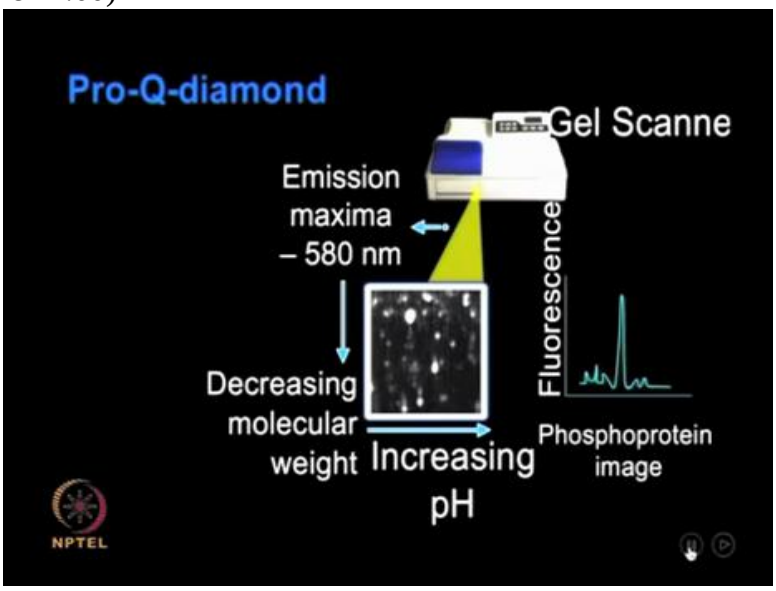

…and it provides an on the 2D, two dimensions, decrease in molecular weight and increase in pH information of all of these protein spots. Often people combine phosphor-stain alongwith…

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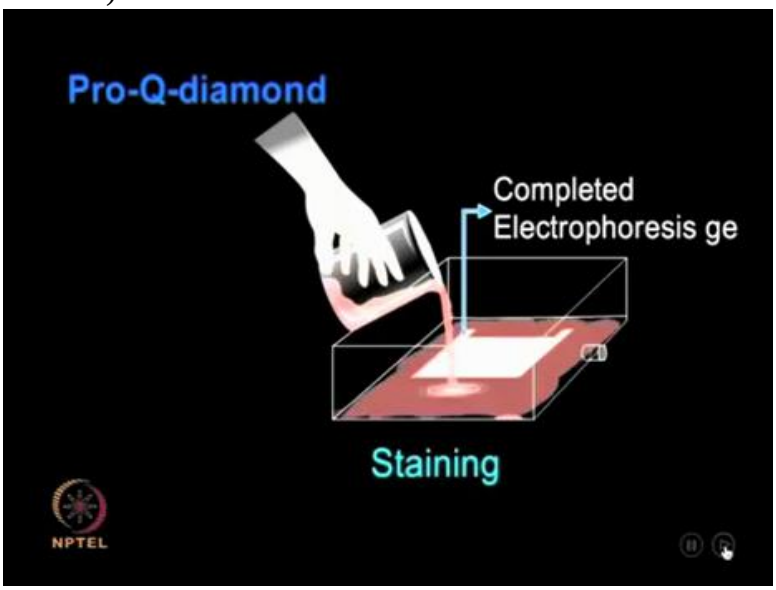

…SYPRO Ruby stain to obtain dual staining information which was described in the lecture.

So in the animation, we have seen that how Pro-Q Diamond can be used to detect post-translation modified form or the phosphorylated form of the proteins and then other staining procedures such as SYPRO Ruby can be used for dual staining.

Now dual staining allows more than one detection protocol on the same gel. So it has lot of merit and I hope after watching this animation, you must be able to appreciate it.

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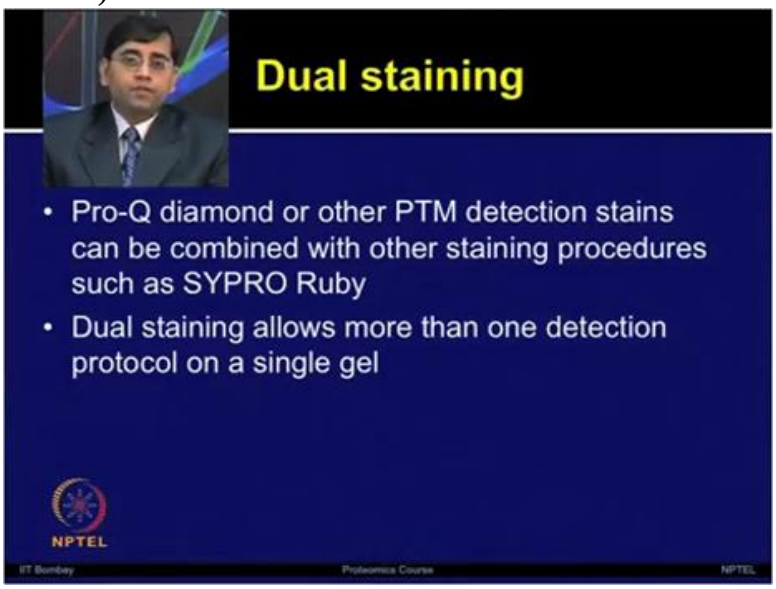

After discussing about different types of staining procedures which are available, now you have separated the protein spots on the gel, now you have visualized that using different types of stains. Again selection of stain depends upon the type of application you are looking for. Often you have limitations of your protein. If you did not have enough protein, you probably used silver or SYPRO Ruby.

If you had enough protein, you went with Coomassie brilliant blue staining. You were interested in looking certain form of PTM; you used Pro-Q Diamond or other specific stains. Now if you want to do some more advanced form to eliminate lot of gel artifacts probably you used Cyanine dyes.

So after all of these types of staining detection, now you are ready to scan the image because, now you can see all the protein spots available on the gel in different colors; whether it is blue in Coomassie or brown in silver, pinkish in SYPRO or different types of fluorescence patterns in Cyanine dyes and Pro-Q Diamond

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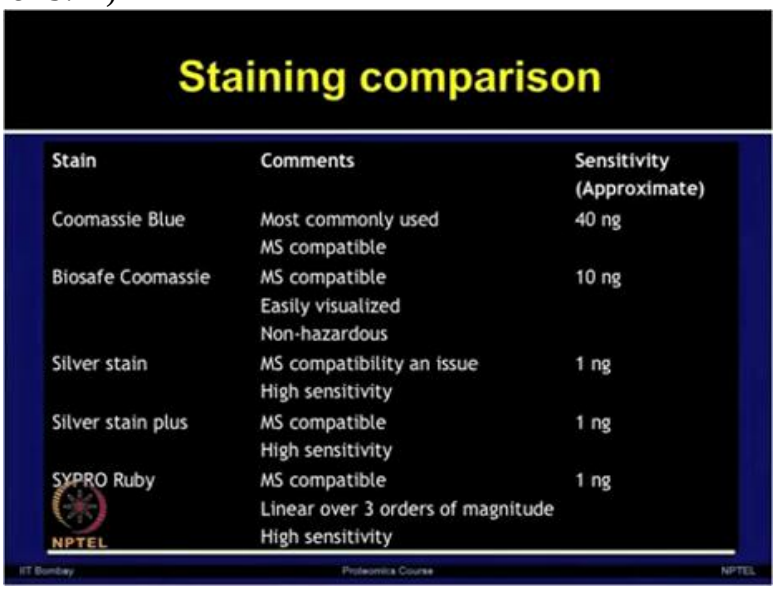

So in a nutshell, here you can obtain information for various stains which are available, their properties and their sensitivity. Please note that the sensitivity is only approximate.

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Gel staining and de-staining

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This process involves removal of the gel from the electrophoresis assembly followed by treatment with the fixing solution, staining solution and finally the destaining solution.

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Gel removal and protein fixation

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Soak the gel present between the glass plates in fresh distilled water to remove any bound SDS.

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Pour the fixing solution containing methanol and acetic acid into another tray.

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Open the glass plates

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and carefully remove the IPG Strip from the gel.

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Then transfer the gel into the fixing solution by gently sliding it off from the glass plate.

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Place the tray on the mechanical shaker for an hour to ensure that the protein spots get fixed to the gel, thereby minimizing any diffusion.

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Gel staining

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Transfer the gel carefully into another tray containing the Coomassie blue staining solution…

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… and place it on the shaker to ensure uniform contact of the gel with the solution.

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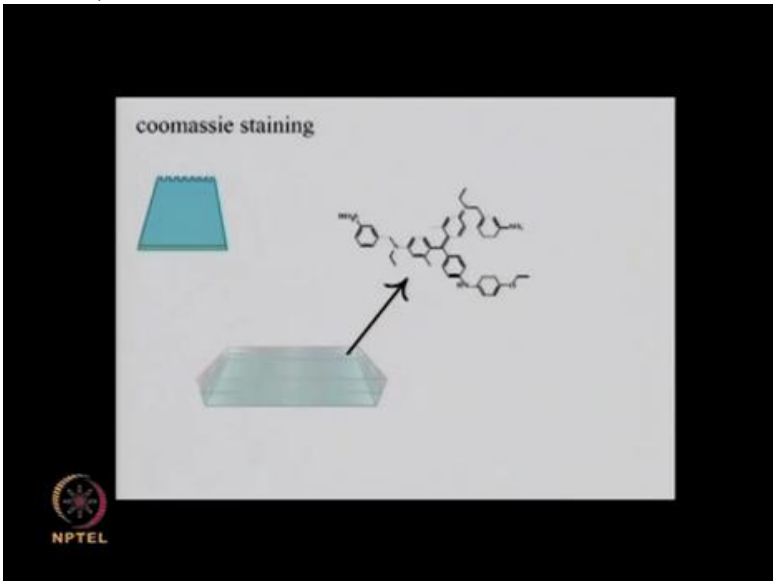

The negatively charged Coomassie dye interacts with protein through ionic and other non-covalent interactions,

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… thereby staining them with a blue color.

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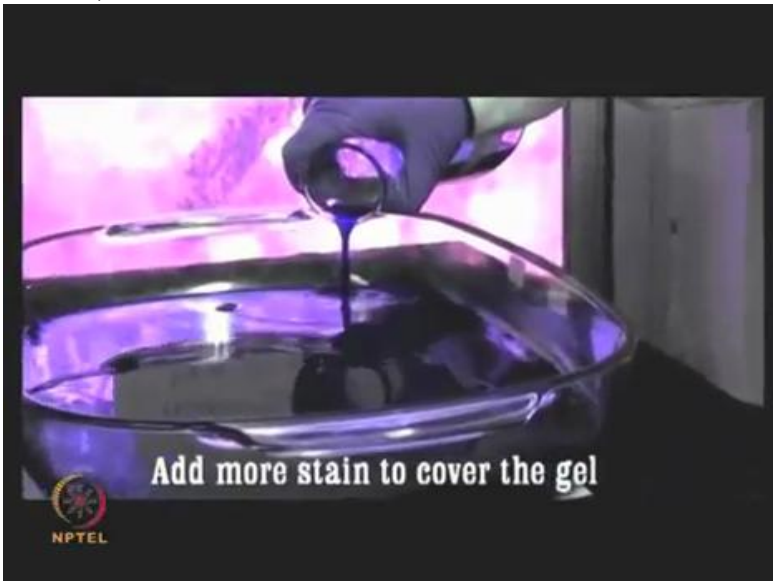

Add some more staining solution such that the gel is completely immersed and leave it overnight for around 8 to 10 hours.

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De-staining

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Drain out the staining solution from the tray and pour the destaining solution consisting of methanol and acetic acid into the tray.

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Place the tray on the shaker for around 6 to 8 hours until the background stain of the gel is completely removed and ….

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… the spots are clearly visible.

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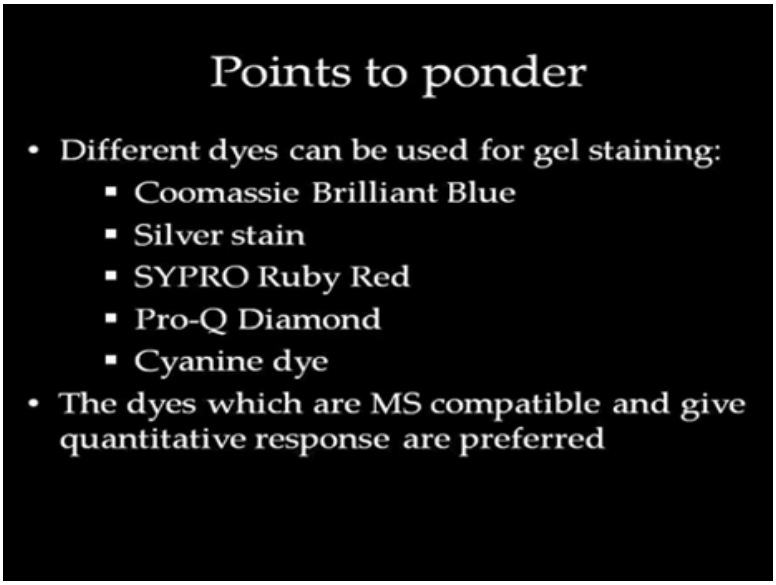

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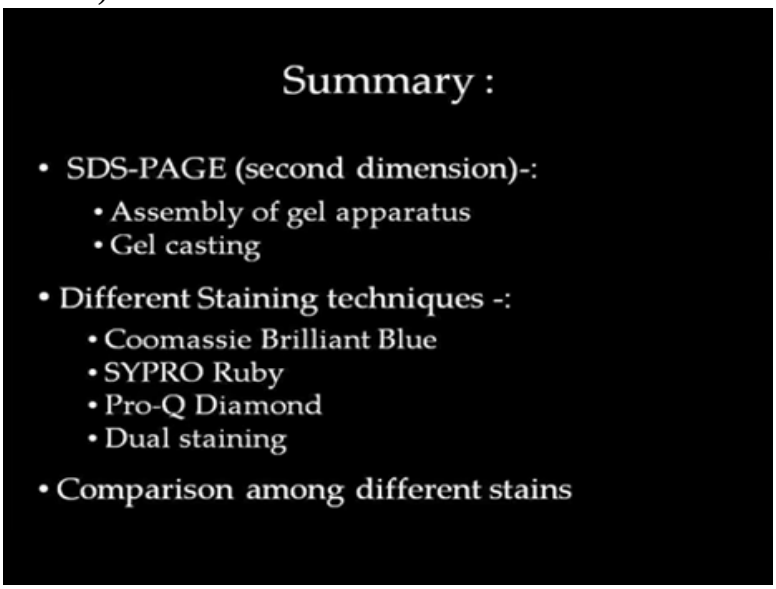

We will continue in the next lecture. I hope you are able to appreciate the Gel-based Proteomics and its potential. Thank you.