Experimental Biotechnology
Prof. Dr. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module No # 03
Lecture No # 12
Scientific Questions (Part – 2)

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(Video Ends: 01:00)

Hello everyone this Doctor Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati. And what we were discussing? We were discussing about the electrophoresis.

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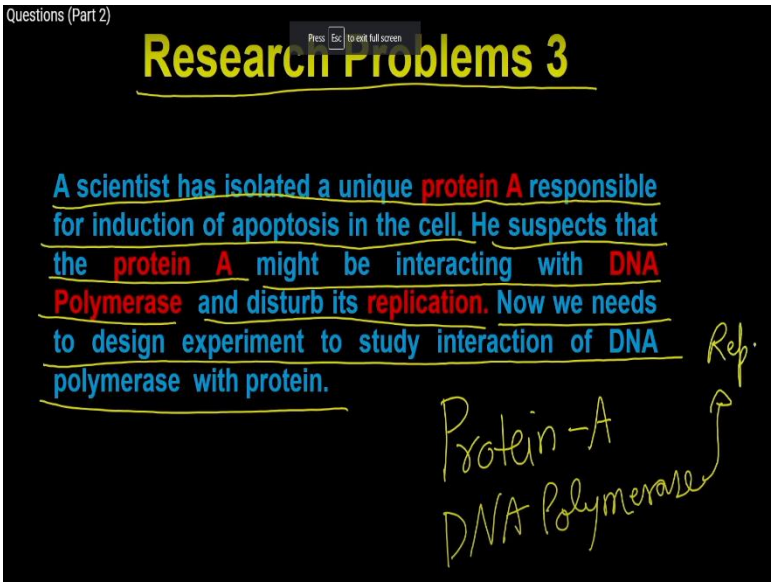

Let us move on to the next research problem. So in the research problem number 3 a scientist has isolated a unique protein A responsible for induction of apoptosis in the cell. He suspects that the protein A might be interacting with DNA polymerase is and disturbs its replication. Now we want to design the experiment to study the interaction of the DNA polymerase with the protein. So here the protein A is interacting or at least it is suspecting that the protein A is interacting with the DNA polymerase.

So DNA polymerase is enzyme which is actually is taking part into the replication or it is it responsible for the replication of the proteins. So that is how by utilizing these you are interested to study the interaction of the 2 different proteins. Although we are discussing the interaction

with the 2 different proteins but with the help of the electrophoresis you can be able to study the interaction of the 2 macro molecules where there will be a change in the mass.

And these is these complexes are fairly stable so that they will not get departed when you are performing the electrophoresis or when you are performing the different steps. So if the complex, are very stable if the complexes are resulting into the increase in mass then you can be able to utilize the electrophoresis.

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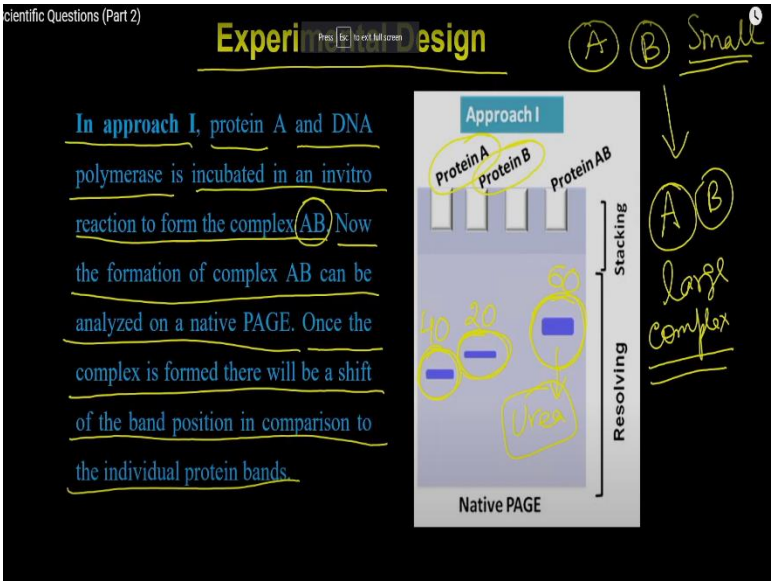

The experimental design we can design the 2 different types of experiment depending on the type of infrastructure you have in your laboratory the type of resources you have in the laboratory. So in the approach 1 or the experimental design 1 what you can do is you can take the protein A and the DNA polymerase clone; them separately and then you incubate them in a invitro reaction to form the complex AB polymers.

Now the information of the complex AB can be analyzed on a native PAGE. Once the complex is formed there will be a shift of the band position in comparison to the individual protein bands. So what you have to do is you take the protein A you take the DNA polymerase and load it on to a native PAGE. Now if these 2 are interacting which means the protein A or the protein B is interacting they are actually going to form the complex of AB.

So these 2 individually are small in number or they are individually are not going to form a complex. When they will come to together they are actually going to form a large complex which means the molecular weight of this are going to be small whereas the molecular weight of the AB complex is going to be on a higher side. So if you load them in native PAGE what will happen is? That the protein A will run at its own molecular weight protein B is going to be run as per its own molecular weight.

But protein AB complex if the complex is formed then it is actually going to be run as per the addition of the protein A and protein B. Which means for example if you have protein A of 40 kilodalton, protein B is 20 kilodalton then this will become the 60 kilodalton and that is how the electrophoretic mobility of the complex is going to be on a slower side. That is why the electrophoretic mobility of the complex is going to be it will run slowly and that is how it is actually going to be away from the A and B.

As a as a verification if you are interested to verify this; what you can do is you can able to add the urea or some de-naturating agent. And that actually should give you the band of the A and B back. So that is the kind of verification experiments that the complex is actually having the A and B together. Other way of verifying the complex is that you can take the complex and then instead of before adding the B.

For example if you take the A and you add the B what you can do is just simply add the antibodies so that it is actually going to bind all the regions available in A to which is available for the B. So as the result of the antibodies the B will not get the suitable sight, for the A to bind and as the result it is not going to form the complex. So there are multiple ways in which you can be able to design the experiment and then you can be able to verify that approach. Now let us go to the next design where you can address the same portion.

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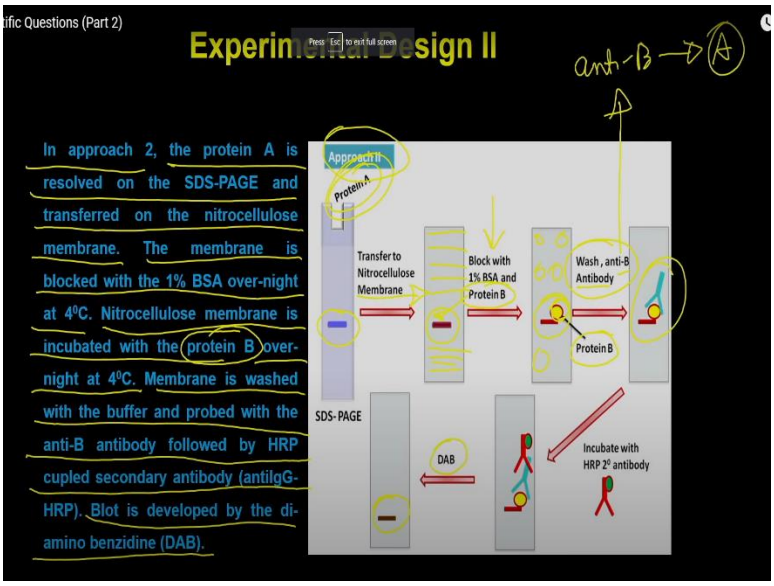

So in the approach 2 the protein A is resolved on to the SDS PAGE and transferred on to the nitrocellulose membrane. The membrane is then blocked with the 1% BSA overnight at 4 degree. Nitrocellulose membrane is incubated with the DNA polymerase or the protein B overnight at the 4 degree. The membrane is washed with the buffer and probed with the anti B antibodies followed by the HRP coupled anti secondary bodies.

The blot is developed by the diamino benzidine DAB. So in the approach to what you are going to do is first you resolve the protein A and approach to is to only going to work when the protein A and protein B are actually forming a very stable complex. Where the three dimensional structure of the A is not playing a role informing the complex. Whereas the three dimensional structure is forming and it is playing a role and it is providing as scaffold which is actually being attached as which is actually being utilized by the B to bind.

Then this approach is not going to work then you have to go with the approach number 1. So if the 3 dimensional structures, is not important but the surface chemistry or the smaller regions of the T dimensional structure is important then this approach could work. So what you are going to do is just first run the protein A onto as the SDS PAGE so it will going to form a band then what you will do is transferring onto a nitrocellulose membrane and that is actually going to have the protein onto the membrane.

Then you will block and you block it so that all of the surface area of the particular membrane is going to block. So that the protein when you are going to add the protein B it should not bind to other places and then you add the protein B. So if the protein B will have 2 options either it will go to bind to the protein A or it will not bind. If it will not binding then it is not going to give you the binding of the antibody as well. So then in that case second antibody will not bind.

So suppose the protein B binds to the protein A then you wash so that washing will remove the non-specific protein B which is bound to the other region of this membrane and the you add the anti B antibodies. So anti B antibodies are only going to detect the protein B. The only this you have to worry about it is that the anti B antibody should do not detect the A because if it detects the A then it is actually going to give you the false results.

Which means it is actually going to give you the result the band at where the A is present whereas the B is not present. So if the anti B is whatever the anti B are going to use should not have any cross reactivity to the A protein. Now what you do is you do the simple western blotting and you are going to develop the blot with the help of di-amino benzidine and that actually is going to give you a stain at a same place where you have the protein A.

And as I said because you are utilizing antibody you can also run the B separately to know where the B is appearing because the position of the B is going to be different from the position of A. Number 3 you can also run the protein A directly probe that with the anti B so that you know that the anti B does not react with the anti-protein A. So these are the verification and the control experiment which you are going to do to see that the result what you are getting are authentic and they are reliable.

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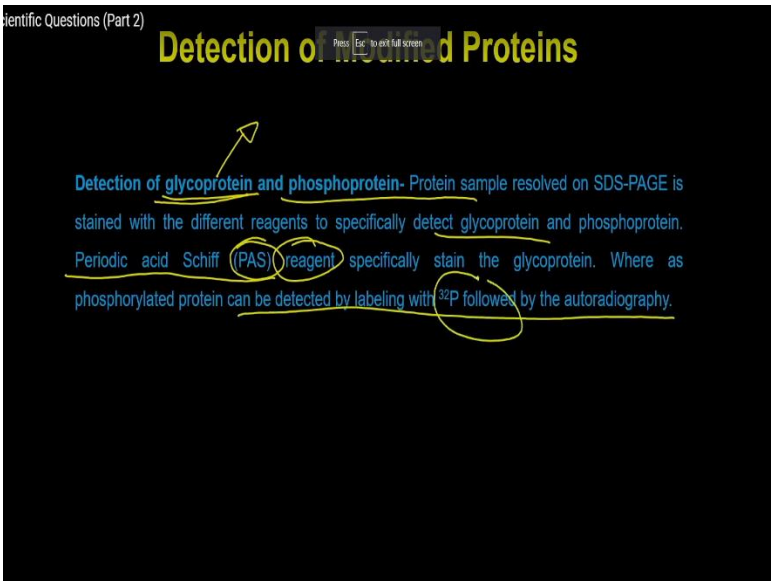

Now apart from this you can also use the SDS PAGE to detect the different types of protein modification. For example you can use the; detect the glycoprotein the proteins where the glucose molecules are attached or you can use it for the phosphoprotein detection. So in these case what you are going to do you are going to run the proteins on to you SDS PAGE. And then you can use the specifically in reagents to detect the glycoprotein for example in the case of glycoprotein you can use you can just stain the gel with the periodic acid Schiff based or the PAS reagent.

And that PAS reagent is only going to react with the carbohydrates and that actually is going to give you the reactivity and it will going to give you the reactivity next to the protein where the protein is present next to SDS PAGE. Similarly we have the specific reagents for the detecting the phosphor protein as well. Apart from that if you want to detect the phosphor protein you can also be detect simply by incubating the protein with the 32 pill abled and then you can do the autoradiography with utilizing the x ray films. And that actually is going to give you the signal wherever you have your protein present in the SDS.

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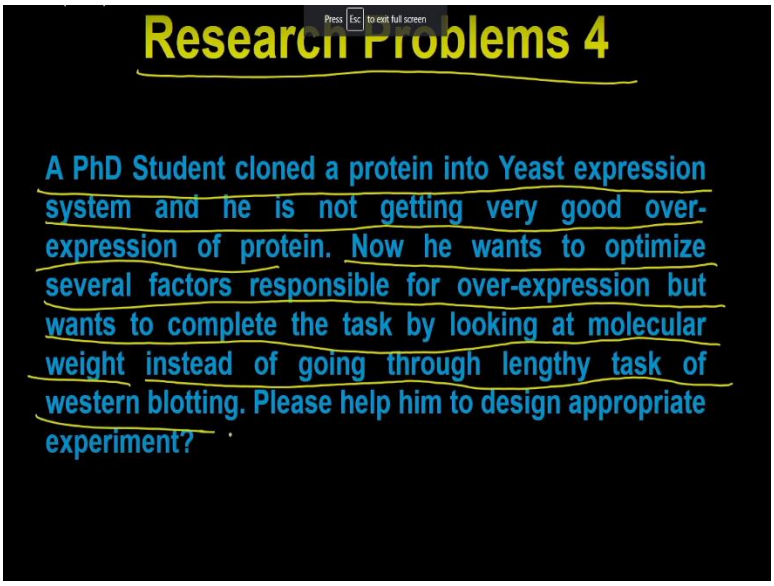

Apart from these experiments the; so let us take up another example of research problem. So in the next research problem what you have is that a PhD student cloned a protein in yeast expression system and he is not getting very good over expression of the protein. Now he wants to optimize several factors responsible for over expression but wants to complete the task by looking at the molecular weight instead of going through lengthy task of western blotting.

Please help him to design the appropriate experiment. So what is the problem is that a PhD student, have cloned the particular protein and it is over expressing in the yeast expression system and he does not have the very good resources. So that he can be able to perform the western plotting and you know any all the other hand he hold he just simply want to optimize the expression. So that so he also wants to do the things in quicker mode because when you do the western blotting, is the lengthy procedure where you have multiple steps. So that is how you want he wants to do the things very quickly mode.

So he is looking for to see the staining procedure so which is actually going to be much sensitive compared to Coomassie brilliant blue. And that is how he can use that the sensitive method to know whether he is getting a protein of that particular molecular weight in the gel or not. And whether if it optimizes the different factor for example if we optimizes the induction time or induction concentrations or consideration of the induced cells and all that whether that is changing the appearances of that particular molecular weight or not.

So one of the appropriate technique; for this is that he can actually stain in the gel with a much sensitive silver staining. So let us discuss about that.

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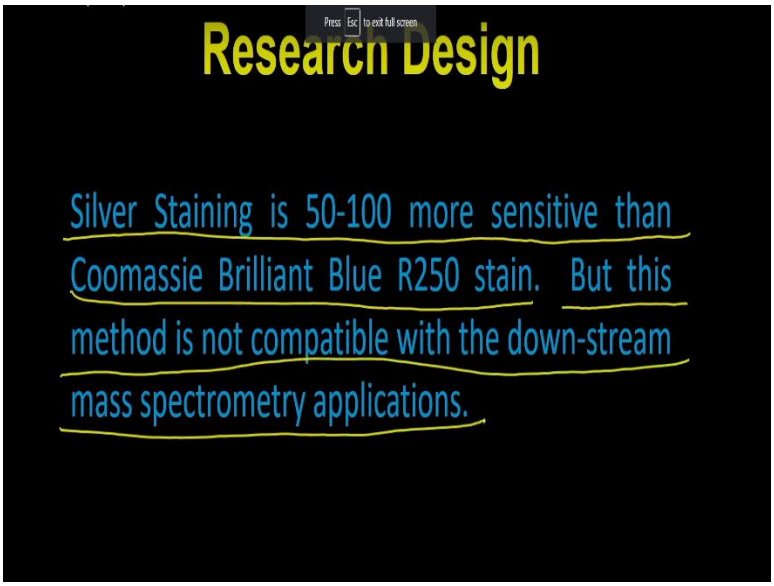

So silver staining is a 50 to 100 times more sensitive than the Coomassie brilliant blue R250 stain. But method is not compatible with the downstream mass spectrometry applications.

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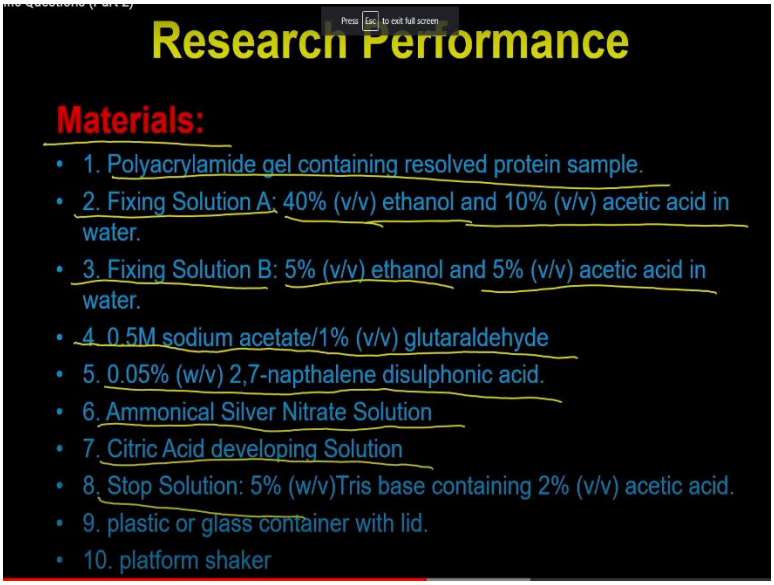

So material what you required for the silver staining is that you required SDS PAGE where you actually resolve the samples. Then you require a fixing solution A. The fixing solution A contains the ethanol as well as the acetic acid. Then you required a fixing solution B which

requires the ethanol and the different consideration of the acetic acid. Then you require the 0.5 molar; sodium acetate in 1% glutaraldehyde.

And then you require 0.05% and naphthalol naphthalene disulphonic acid. And then you require the ammonium silver nitrate solution, citric acid developing solution and then ultimately you required stop solution. So stop solution contains 5 percent risk containing 2% acetic acid and then you require a plastic or the glass container where you are going to perform the silver staining.

And ultimately you also require a platform shaker where you are actually going to mix this reagent. Before we are going to discuss about the protocol of the silver staining you should remember that we are going to deal with the silver solution. And silver is very: very toxic solution. When you perform this experiment or when you perform the silver staining you always have to wear the gloves and you always have to keep changing the gloves.

For example if you are doing some experiment and you doing the silver staining and suppose if you remove the gloves you should not wear the same gloves back because when you are touching these glass when you are touching the gels the silver comes out on your gloves. So when you remove it goes into the inner surface of the gloves. And when you wear it again the old gloves it actually goes into you hand. Silver is the very toxic metal so it goes in to your skin very fast.

And that is how it is going to start showing the toxic affects; may be; not in one procedure or one type one time staining procedures. But when you are doing this on a routine basis you might, accumulating large quantity of the silver in your body and that actually going to be determinantal for your life. So that is why you have to keep all this precaution when you are doing the silver staining.

Apart from that whatever the washing buffers whatever the things will come out from this requirement has to be collected in a bottle so that you remove the silver and throw that into the river or in your sink. You cannot simply throw all these into the sink because then you are contaminating the environment with these silver solutions. And that is very dangerous for the

aquatic animals and as well as for the plants. So while you are doing the experiment you have to be very careful in terms of protecting yourself as well as protecting the environment.

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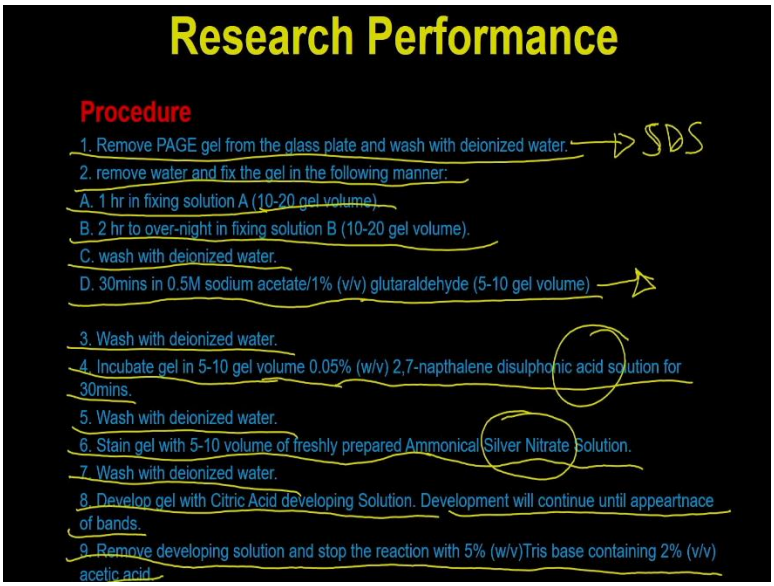

So in the procedure first you remove the PAGE gel from the glass plate and wash it with deionized water. This is required so that you can able to remove the large chunk of the SDS. Then you remove the water and fix the gel in the following manner. You first fix it in the fixing solution 1 for 1 hour then you fix it for 2 hour to overnight in fixing solution B and then you wash it with deionized water.

And then you incubate it in the 0.05% sodium acetate containing glutaraldehyde this is also effective which you are going to use ultimately you wash the gels with the deionized water and then you are going to incubate gel in a 5 to 10 gel volume of 0.05% naphthalene disulphonic acid for 30 minutes. This is actually going to be the activator which is actually going to be activate the protein. So that it actually start accepting the silver.

Then you wash the deionized water and then you stain the gel with the freshly prepared ammoniacal silver nitrate solutions. So this is the silver nitrate solution which actually where from where the; because you have treated the protein with the activating buffer. The proteins are now going to accept the silver atoms. So as a result the silver whatever is present into the ammonium chloride the silver nitrate solution will go and precipitate wherever the protein is present.

And then what you do is you wash it with deionized water. Develop the gel with the citric acid containing the solution and development will continue until the appearance of the band. So at this stage you can keep the gel in a acetic acid solution and the acetic acid solution is going to be keep reacting with the protein bound silver atoms and it will keep giving you some color.

And you can keep observing the color and whenever you think that ok you got the sufficient color in your gel then you can just simply wash the gel and remove the developing solution, and stop the reaction with the stopping solution that is the 5% tris containing 2 percent acetic acid. And ultimately you wash the gel with the deionized water and you can actually do the imaging of this particular gel to analyze the image. And then you will able to know whether the band of your interest is appearing in to that.

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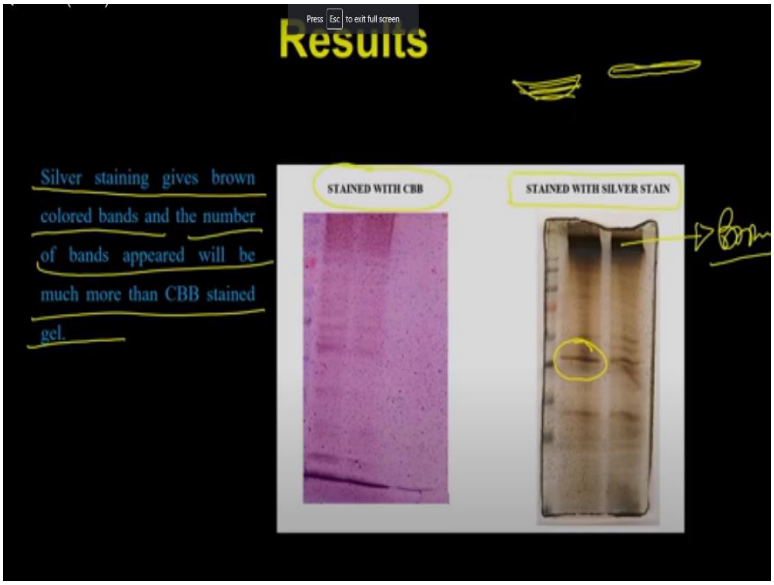

The result what you are going to get is very simple. The silver staining gives the brown color band and then number of band appeared will be much more than the Coomassie brilliant blue stained gel. So what you can see is these are same identical gel which we have stained with the help of Coomassie brilliant blue as well as you have stain the same with the silver staining. What you see is the number of bands are very little compared to the number of bands what is appearing into a silver staining all these bands are brown in color.

And they are much more in number compared to Coomassie brilliant blue stain samples. Irrespective of whether you are staining the gel with Coomassie brilliant blue or the silver staining you are always going to see different types of abnormality in the appearances of the bands. For example you can see the appearance of this particular band. So ideally you should see a band which is like this but in these bands does not appear or sometime you see the band which is a thin band.

But these are the ideal bands. You never get these types of bands because you always do the running at a very fast speed or some kind of art effects appeared to the gel. So because of that when you stain the gel you are going to see the different types of the abnormalities in the band appearance. Let see how what these bands.

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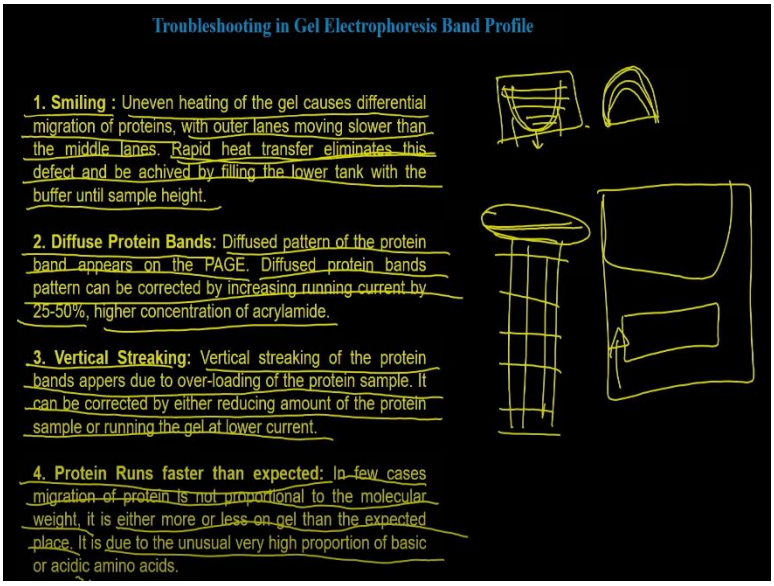

So the first is most common is the smiling of the band. So uneven heating of the gel causes the differential migration of the protein with the outer most lane between slower than the middle lane. So the smiley means this is actually a typical smiley passion of a protein a band. It could be in a upward direction or in a downward directions which means the protein are smiling like this or the proteins are smiling in a reverse orientation.

This happens because there is a few protein molecules are running faster. A few protein molecules are running slower and because of that it actually forms a lump kind of situation. And why it happens? It happens because of the uneven heating because the heating normally

increases the migrations and what you can do is if you want to correct this you can rapid heat transfer eliminates these defects.

And can be achieved by filling the lower tank with the buffer until the sample height. So you know that the lower tank you have a buffer in this you are having a cathode chamber. So if you fill the lower buffer slightly on a higher side that actually is going to provide the enough cooling and that is how you can be able to avoid this smiling affect. Then you have the diffuse protein bands; the diffused protein band appears on the protein PAGE.

Diffused protein bands pattern can be connected by increasing running current by 25 to 50 percent. Higher concentration of the or with the; higher concentration of the acrylamide. Then you have vertical streaking which means you are going to have single band and then you are going to get a like a streak kind of pattern. Which means the band is going to start from here and then you are going to see 3 kind of thing.

You are going to see the streak like this. The vertical streaking of the protein bands appears due to over loading of the protein sample. It can be corrected by either reducing the amount of the protein sample or running of the gel at a lower current. The third is that the protein runs faster than the expected. So what happen is when the some proteins are very you know charge because you know that the proteins are running from the negative to positive electrodes.

So if they are having the; its own positively negative charges. And you are on the top you are adding the SDS. So it is actually having these slightly higher charges and because of that it runs faster than its molecular weight. So in few cases we migration of the protein is not proportional to the molecular weight. It is either more or less on the gel than the expected place. It is due to very unusual very high proportional of the basic or the acidic amino acid.

So the protein, which are have, a very high concentration of the acidic amino acid those are very fast. The protein which have very high concentration of the basic amino acid there are slower than the expected molecular weight. And all these affects all these defects actually causes the calculation of their molecular weight and as well as the looking at the; you know appearances of these proteins on to the gels.

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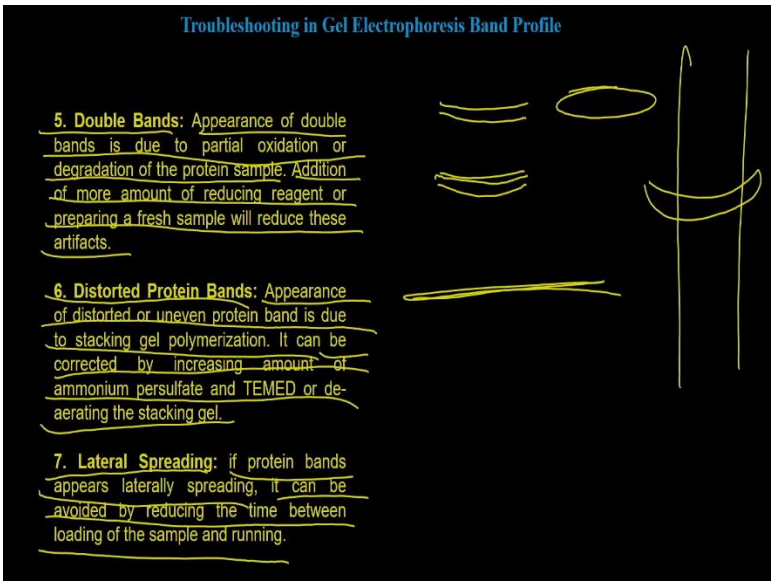

Then you have the double bands means you have a 2 bands instead of 1 band. The appearance of the double band is due to the partial oxidation or the degradation of the protein sample. So happen is that the single protein bands got degraded. So that is why you have the 2 band because this protein got degraded and it is forming a protein of a lower molecular weight. And because of that it actually is not that low that it can separate.

So it will be like forming a band like this. Addition of more amount of reducing agent or preparing a fresh sample will reduce these artifacts. Then you will have the distorted protein bands. Appearance of distorted or uneven protein band is due to the stacking gel polymerization. It can be corrected by increasing the amount of ammonium persulfate or TEMED or de accelerating these stacking gels.

Then you have the lateral spreading. Lateral spreading means the protein is so you have a lane of this and a protein is appearing like this. And this is actually spreading to outside this lane. If the protein bands appear laterally spreading it can be avoided by reducing the time between the loading of the sample as well as the running of the gel. So these are the some of the artifacts and the problem what you are going to see when you are going to run a SDS PAGE.

And when you stain them with the Coomassie brilliant blue or the silver staining these problem can be worked out simply by modulating some of the condition or where you can actually be able to you know change the you know you can increase the buffer or you can make it slower

running and all that kind of things. So these are the thing you have to consider when you are resolving the sample on to the SDS page.

So with this I would like to conclude our lecture here in the subsequent lecture we are going to take up few more exciting experiments where we are going to use the electrophoresis as a tool to answer those questions and to solve those questions. And with these I would like to conclude our lecture here thank you.