Genetic Engineering: Theory and Applications
Professor Vishal Trivedi
Department of Bioscience and Bioengineering
Indian Institute of Technology Guwahati
Module 9 Characterization of Isolated Product part 1
Lecture 31
Electophoresis (part 3)

Hello everyone this is Dr. Vishal Trivedi from Department of bioscience and my engineering, IIT Guwahati and what we were discussing in the previous 2 lectures that we have discussed about the electrophoresis, the basic principle as well as we have also discussed about how to perform the electrophoresis and subsequently in the previous lecture we have also discussed about once you got the image then you can do the lot of analysis with the help of different types of software which are available and in a detailed demo what we have shown in the last lecture that how software can be able to help you in terms of analysing that particular image and how that analysis can help you to answer many questions.

So in today's lecture we are going to discuss about how once you are done with the image analysis, what are the different information you will get from that image and how that information can let you to characterise the proteins which we are going to purify using the SDS-PAGE So the first thing what we have to discuss is the image what you have got and how that image can be used to answer many questions.

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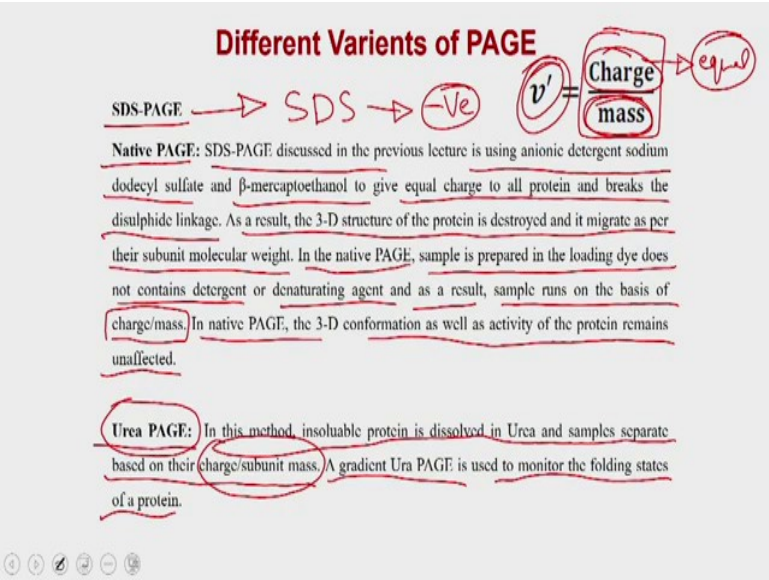

So apart from the SDS-PAGE you have different variance of polyacrylamide gel electrophoresis, so we have the SDS PAGE which we have discussed in the previous 2

lectures where we were using the SDS as degenerating agents and in addition to SDS we were also using the beta mercaptoethanol and both are the degenerating agents who are destroying the three-dimensional structure of the proteins and also the beta mercaptoethanol is breaking the disulphide linkages and on the other hand the SDS is also providing the negative charge to the protein.

So because of that when you are performing the SDS PAGE as we have discussed in the previous lecture that the electrophoretic mobility is directly proportional to the charge by the mass but within the SDS PAGE if you are using the SDS which is actually going to give the negative charge to the protein, the charge portion is going to be equal for all the proteins and as a result what will happen is when you perform the SDS PAGE the proteins, first of all the protein three-dimensionally structure is going to be broken down, the second the protein is going to have…

All the proteins are going to have the equal negative charge and as a result the proteins are going to migrate based on their molecule weight which means the proteins which is of higher molecule weight is going to run slower the routine which is going to be of lower molecule weight is going to run faster because the electrophoretic mobility is inversely proportional to the mass of that particular protein but you do not allow or do not add the SDS as well as beta mercaptoethanol in those cases the protein is going to have its charge, the protein is going to maintain the three-dimensional confirmations and that particular type of polyacrylamide gel electrophoresis is called as the native page, so SDS Page discussed… is using anionic detergent sodium dodecyl sulphate and beta mercaptoethanol to give equal charge to all proteins and breaks the disulphide linkages.

As a result the 3 dimensional structure of the protein is destroyed and it migrate as per their sub unit molecular weight, whereas in the native page the sample is prepared in the loading dye does not contain detergent of the denaturating agent and as a result the sample runs on the basis of charge by mass which means the electrophoretic mobility…since all the proteins are going to have different types of charges either the positive charge or the negative charge and then it also going to have the different types of masses.

So whatever the ratio will come the electomobility will be directly propotional to the charged by mass ratio and that is why in native page the 3 dimensional confirmation as well as the activity of the protein remains unaffected which means the native protein or the native gel or the native page is going to retain the 3 dimensional confirmation of the protein as well as it is

going to retain the activity of that particular protein, what that mean is that if you would like to study the activity of a particular protein.

And if you want to judge or you want to characterize your protein based on that particular activity then you can run the protein in the native page and then you can do the activity staining and that will actually give you the answer whether the protein which you have purified and the purified product what you have got after the recombinant DNA technology whether that particular protein or the factor is catalytically active or not.

On the other hand the 3 dimensional confirmation is also going to be retained, so if you can modulate or if you modulate the conditions you could be able to answer whether the protein is maintaining the native conditions of not. The other variant is the urea page, in this method the insoluble protein is dissolved in urea and the sample is separate based on their charge by mass ratio. A gradient urea is used to monitor the folding state of protein, so urea as you know is a denaturating agent.

Mechanism of urea is very much different from the denaturating mechanism of SDS, so in case of SDS page, the SDS is going to destroy the three-dimensional confirmations and as well as it is going to give the negative charge whereas in the case of urea it is actually going to destroy the three-dimensional conformation but it is not going to give any negative charge, so as a result even in the presence of urea the protein is going to run as per their charge by mass ratio which means the proteins are still going to run as per their charge by mass ratio and that actually have an advantage that in the presence of urea you could be able to monitor, how the three-dimensional conformation of a protein is getting affected if you add different amount of urea and that is how actually if you run the urea page you can be able to use that particular page to monitor the protein folding or the unfolding kinetics.

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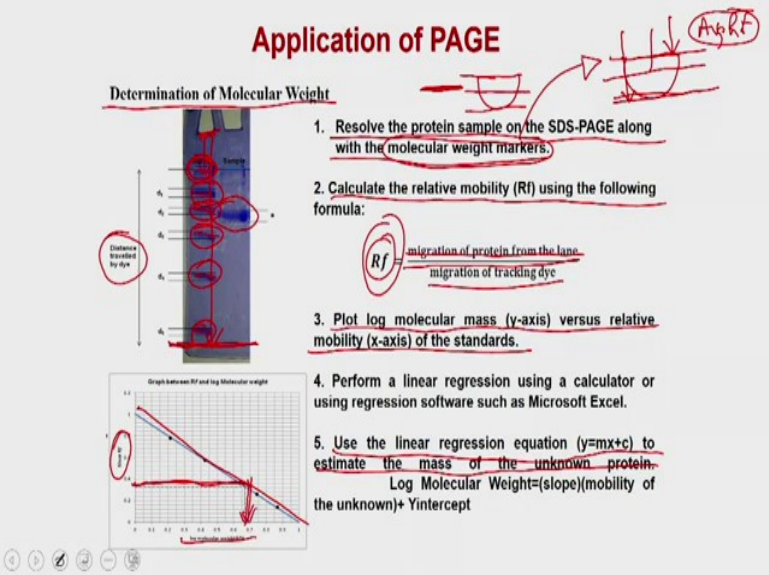

Now, let us move on to the application, so in the previous 2 lectures we have discussed how to run the page and how to analyse the image, now we are going to discuss what is the application of polyacrylamide gel electrophoresis, so one and the foremost application of the SDS pages that it will allow you to calculate the subunit molecule weight of a protein, how to do that? So if you want to use the SDS Page to determining the molecule weight of protein what you are supposed to do is you resolve the protein sample on SDS Page along with the molecule weight markers, so molecule weight marker is a kind of recipe which you are going to get from many biochemical or many biochemistry companies.

What did molecule weight marker is that it is a mixture of the different proteins which they mix and that is how it is actually the decided that which protein band is going to be of which molecule weight, which means it is actually the standard protein which they mix and that is how you know what is the molecule weight corresponding to which brand for example in this case we have different types of bands which we are going to use and all these molecular weight markers the proteins which are present in the molecular weight marker we already know what is the mass of this particular band and what is the mass of the other band.

So, because you know already you can use that to drive a calibration curve, so once you have done with the resolution of resolving the samples on the SDS Page along with the molecule weight marker, what you are going to do is, you are going to calculate the relatives mobility Rf using the formula RF is equivalent to the migration of protein from the lane which means from here versus the migration of the tracking dye.

So you can see that the tracking dye we have taken a marker, so this is the place until which the tracking dye is running suppose for this particular band we would like to calculate the Rf value, so what we are going to do is we are going to run a line through the middle of that particular band and then we are going to measure the distance, so that is the top value and then we are also going to calculate the distance of the dye front and if you divide these two values you are going to get the Rf value for this particular band. Similarly you can do the Rf value for d1, you can do for d2, you can do for d3, you can do for d4 and then you can do for d5.

Similarly, you have to do the Rf value for the sample band as well. Remember when you see a band, normally you see a band like this as you can see here, so many times the students were always having a confusion through what should I take the point for this particular protein, so in those kind of cases you have the approach either you take the Centre of this particular band and you measure the values or the best approach is that you take the value from top and then you take the value from the bottom and then you can use the average Rf value or average distance of these 2.

So you can measure a distance from the point number 1 you can measure a distance from point number 2 and whatever the distance you are getting you can just take the average of that and that actually going to give you the Rf value with the minimum error possible, although it is always advisable when you would like to use the SDS page for the calculation of molecule weight you should load very little amount of proteins.

So that it should form a band which is of thin layer and which should not have any thickness because if you load a very large quantity of the protein then it is going to form a band like this and that actually is going to create trouble because then you will be confused whether I should take this as a point to measure the Rf value or whether I should take a point here to calculate Rf value or whether I should take a middle line or middle point in this particular band and either of these situation are going to give you some amount of error that is why it is recommended that you load very little amount of protein, so that it should just form a very very thin land or with thin band actually on to the SDS page.

So once you have calculated the Rf value for the marker proteins you have calculated the Rf value for your sample then what you do is you plot the Loc molecule weight versus relative mobility of the standards, so what you are going to get, you are going to get Rf versus the log molecule weight and you are going to get a negative calibration curve, then you use the linear

regression equations to estimate the mass of the unknown protein, so you know the Rf value of your own protein and that is actually you can use to calculate the molecule weight of the protein which you would like to calculate the molecule weight. So then you can use the Rf value of your unknown sample and you can be able to calculate the molecule weight.

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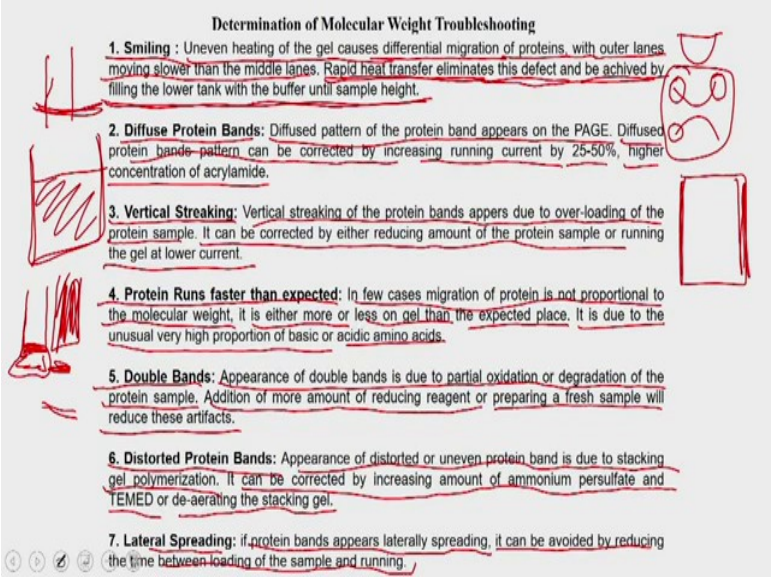

As I said one of the thing is that you can have the band like this but when you can actually encounter many different types of abnormalities in running of these bands or when you are resolving your sample onto the SDS Page and that actually going to give you different types of error when you are calculating the molecule weight or any other kind of analysis, so what are the different types of troubleshooting or what are the different types of bands what you are going to see onto the SDS Page and how you can overcome those problems, so one of the things is called as a smiley bands.

So even uneven heating of the gel causes differential migration of the protein with the outer lanes, the rapid heating transfer eliminates this defect and can be achieved by filling the lower tank the buffer until the sample height, so what is mean by smiley is that you have a band like this or you can have the band like this. What happens is that the corners are actually running either slower or either running the faster. In the case of that the corners are running faster you are going to have different types of smiley or if you are running the slower you are going to get a different type smiley and all these smiley effects are going to create trouble in terms of analysing that particular band also to calculate the RF values, how to correct that and why this happen?

This happened because when you are running the sample on the plates you are actually having uneven heating onto this plate and because of this heating, either the proteins is running faster or the protein is running slower and all the protein molecules are not running uniformly and because of that it is going to create smiley like appearance, so you have to bring coolingness in the system, so what you can do is you can have the rapid heat transfer will eliminate this effect and it will be achieved simply by filling the lowered tank with the buffer until the sample height which means you know that if you remember there was a buffer tank during the SDS Page.

So you can just fill this buffered tanks slightly more, so that the buffer which is present is actually going to maintain the uniform heat and it is going to maintain the SDS Page plates slightly colder and you can actually recycle this buffer as well if you are seeing that the protein is getting heating or your apparatus is getting heat very high then you can actually exchange the buffer or you can put it into ice bags so that you will be… the buffer which you are feeling outside should be very cold.

The 2nd is the diffuse protein bands, so diffuse pattern of the protein band appear on the page, the diffused protein brands pattern can be corrected simply by increasing the running current by 25 to 50 percent higher collection or you can have the higher concentration of the acrylamide, so diffusing protein brands are like you are going to have some kind of streak.

Then you have the vertical streak which means the proteins are going to be present in a streak format which means if they are not going to be present in a very very consized area which means instead of this they are not going to be present as a band they are going to have a streak, so these vertical streak of a protein brand appear due to the overloading of the protein sample.

If you remember in the previous lecture we have discussed that when you load a sample has to be stacked properly but if you load large quantity of the protein what will happen is before the last molecule of the protein is going to be stacked the 1st molecule will start migrating or start resolving and because of that some molecules are going to run slightly ahead of the all other molecules and because of that you are going to have a vertical streak.

How it can be corrected? It can be corrected either by reducing the amount of protein samples or running the gel at a lower current, so if you run a lower amount of protein all the protein molecules will get stacked or if you run the protein at a very lower current it will allow the

protein molecules to get stacked because then the electrophoretic mobility is going to be very low when they are running into the stacking gel and that is how they will get enough time to get stacked. Then protein runs faster than expected, in a few cases migration of protein is not proportional to the molecule weight, it is either more or less on the gel than the expected place, why it happens? It is due to the unusual very high proportion of basic or acidic amino acid.

So if you remember we said that if you are adding SDS to the protein, proteins are going to have equal charge and because of that the proteins are now going to run simply by their molecule weight which means the electrophoretic mobility will be inversely proportional to the molecule weight but what is in observed that in some cases the protein runs either faster or slower and in those cases what people have found that the protein is either having the large quantity of acidic protein, acidic amino acid and because of the acidic amino acid the protein is also having negative charge from the SDS, the protein is also going to have negative charge because of the acidic amino acid and this acidic amino acid actually give them the more electrophoretic mobility what it is supposed to be. Whereas in some cases it is actually contains basic amino acid and in those cases the net negative charge is less compared to the all other protein and because of that it runs slower compared to the molecule weight.

Then you have double band, so the appearance of a double band is due to the partial oxidation or degradation of your protein bands, so sometimes what you see is you see 2 bands which are appearing very close to each other and these are called as the double bands, how to correct that you can add more amount of reducing reagent or repairing a fresh sample with reduce these double band artifacts.

Then you have the distorted protein bands, appearance of distorted or uneven protein band is due to the stacking gel polymerisation. It can be corrected by increasing the amount of ammonium persulphate and temed or deaeration of the stacking gel, so sometimes what happens is the protein is forming a distorted routine band and that is happening because when it is resolving through the stacking gel, the stacking gel polymerisation is not good and because of that the protein bands are distorted.

So correct that you have to add more amount of temed and APS so that the polymerisation should be in uniform and polymerization should be uniform and polymerization should be correct for the staking gel or what you can do is you can also remove the air from the

stacking gel solution so when you do the degassing of the stacking gel the polymerization also going to be better compared to the previous (())(21:01).

Then we have the lateral spreading, lateral spreading is that if the protein brand appear latterly spreading which means suppose this is your lane, the protein is spreading like this, so it is spreading throughout outside the lane actually so it can be avoided by the reducing the time between the loading of the sample and running. So what happens is…why it happens when you load the samples it starts defusing so when you load the sample it goes and sit into the lane but if you take very large amount of time to connect it to the power pack and turning on the power then the bands are started defusing like this.

So suppose you loaded into the well and they are sitting here then they start like defusing like this and because they already defused some amount once you turn on the power these defused bands or defused protein are also started resolving and then they actually does not come closer to each other and that is how they are getting the lateral spreading of this particular protein bands, how to correct that you have to correct by simply loading the protein and then turning on as quickly as possible.

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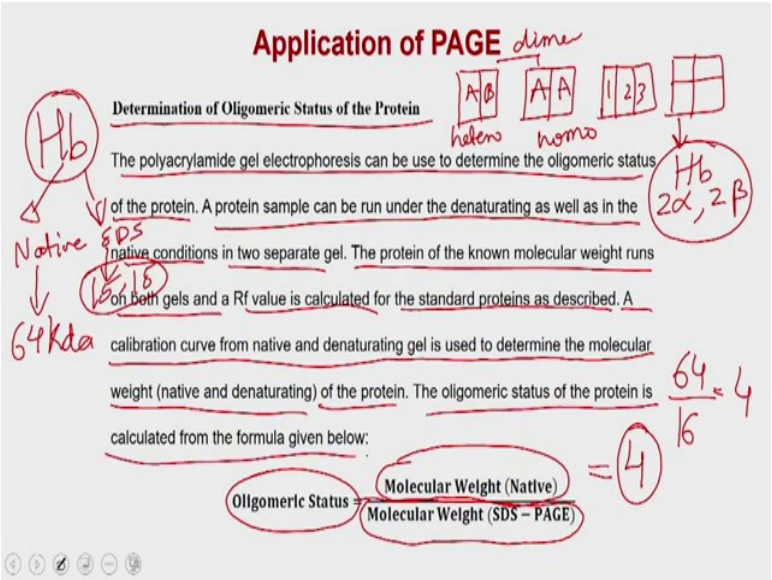

Now, let us move on to the next application so the next application is that suppose you have reduced a protein then you can also determine the oligomeric status of that particular protein, what is mean by the oligomeric status is that it is actually going to tell you that how many number of monomers or how many number of subunits are present in that particular protein so for example if suppose a protein has 2 sub units A and B or it can be AA so this is called

as heterodimer this is called as homodimer, so this is the dimer which means the 2 subunits are present, so these 2 subunits could be 2 different subunits A and B or it could be one subunit but present in 2 copies AA like that.

Similarly you can have the trimers, so you can have 3 units by a 1, 2 and 3 all you can have the tetramers one of the classical example for the tetramers is the haemoglobin where you have the 2 alpha chains and 2 to beta chains. So if you are making the haemoglobin into the E. coli expression system then and suppose you are making the haemoglobin as a substitute for the blood transport or blood transfusion then it is important for you that you should correct and you should verify that the 2 alpha and 2 beta what you have produced into the E. coli and that has given you a tetrameric hemoglobin should be a tetrameric hemoglobin, it should not be dimer, it should not be monomers.

So you have produced alpha subunits, you have produced beta subunits then they get mixed together in the E. coli and that is how you are going to have the tetramer and you can use the SDS Page to determining whether all the 4 subunits are present in your molecule as well or not so what you do is you purify this particular protein, so suppose I have purified the haemoglobin which I have produced in the E. coli expression system and then what I will do is, I will run this haemoglobin into 2 conditions, one I will use the native conditions and the other one I will use in the denaturating SDS Page conditions.

So what will happen is when I run it on the native conditions is going to give me... and then I will calculate the molecule weight in both the conditions I will calculate when it is in the native conditions, it will give me a molecule weight of 64 kilodalton. When it is in the SDS Page I will get 2 bands of 15 or 16 kilodalton because Alpha and beta subunits are 15 kilodalton, so I am to going to get 2 bands and I can calculate the molecule weight of these 2 bands which are corresponding to the alpha and beta subunits and if that happens then what you do is and you want to calculate oligomeric status, what you have to do is oligomeric status is equivalent to the molecule weight what you have got into the native page divided by the molecule weight you bought into the SDS.

So if I divided is number by 15 and this number, what I will get is I will get 4 which means 64 divided by 16 which is going to give me 4 that means is that I have produced the hemoglobin which actually contains 2 alpha and 2 beta and that is how it actually showing a oligomeric status of 4 and since we have already verified on the SDS Page that you are getting 2 bands one is of 15 kilodalton another one is 17 kilodalton which is actually the

average of 16, so that is how you are actually confirming that hemoglobin that you have produced in the E. coli expression system is of good quality and it is actually maintaining the similar oligomeric status.

You can go for the head and do some more characterisation experiments to prove that the haemoglobin what you have produced in E. coli is carrying the oxygen and that we can discuss in the later lecture. So a protein, what you are going to do when you are doing the oligomeric status? The polyacrylamide gel electrophoresis can be used to determine oligomeric status of the protein. A protein sample can be run under the denaturating as well as under the native condition in 2 separate gels.

The protein of the known molecule weight runs on both the gel and Rf value is calculated for the standard protein as we discussed in the previous slide. A calibration curve from the native and denaturating gel is used to determining molecule weight in the native as well as denaturating conditions and oligomeric status of the protein is calculated from the formula that is oligomeric status is the molecule weight in the native conditions divided by the molecule weight from the denaturating conditions.

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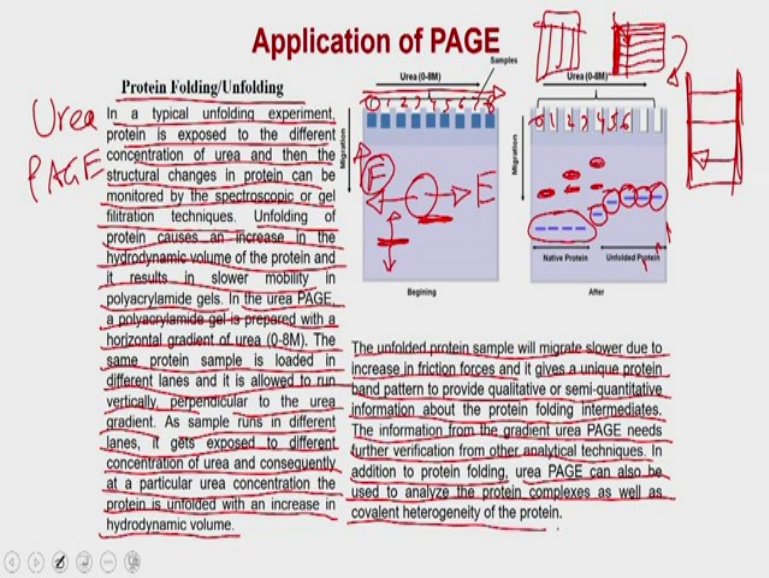

Just now we discussed about the urea page, so urea Page is what you can use to monitor folding or the unfolding kinetics of an unknown protein, so it is important because when you produce a protein you want to know that the stability of this protinest factor is as good as it was reported in the literature, so when you want to see the stability factor you also have to do unfolding kinetics and for doing unfolding kinetics you can do a urea gel, so in the urea gel it

is a typical, in a typical urea gel experiments what you do is protein is exposed to the different concentration of urea and then what will happen is the urea is going to interact with the amino acid residues and that is how it is going to denatured the protein and that can be monitored by the spectroscopic or the gel filtration technique.

Unfolding of a protein causes an increase in the hydrodynamics volume of the protein and it results in the slower mobility in polyacrylamide gel. In the urea page a polyacrylamide gel is prepared with a horizontal gradient of urea which means you are going to prepare a horizontal gradient which means this lane is going to have the 0 urea, this is going to have 1, 2, 3, 4, 5, 6, 7 and 8 molar urea.

Now the question comes how you can be able to cast this particular type of gel, the way we normally cast the gel is like this, we fill the resolving gel like this then we refill the stacking gel, so it will be always in the vertical condition but for the urea page you to have a gradient which is going to be from this direction to this direction which means I have to cast a gel from this direction to this direction which means I have to be in different orientation. For the way the people do normally if you cast these gels is, what they do is they just rotate this particular cassette completely, so what you do is first you rotates this by 19 degree, so in that case it is going to be like this and then what you do is you seal this particular side as well with a glass slide.

So what I have done is I have rotated this cassette like this and then I have sealed this side as well with a glass plate and now what I am doing yes I am putting a urea of different concentration, so I have first put normal acrylamide which is 0 urea then I have put 1 molar urea then I have put 2 molar urea then I have put 4 like that and then we have completed the 8 molar urea and the fold casting is over, so I have not put these stacking gel so far, I have just simply put the resolving gel with containing the different amount of urea.

Once the resolving gel is over then I have moved this and rotated again, then it becomes like this like in a vertical direction and containing the urea like that and then what I have done is I have removed this glass blockage, I have put stacking gel and then I have resolved my samples. As the samples are already containing the different amount of urea, so you incubated your sample in different amount of urea, what urea will going to do is, urea is going to open the structures and as a result the molecular surface area of those molecules or the protein molecules is going to be higher and as you have seen in the previous lecture also

we have discussed that one side if you have molecule on one side this is actually being carried by the electrophoretic mobility on the other side it is going to reflection.

So this side it is electrophoretic mobility, on the other riverside it is the friction, so once be structure is getting open the frictional forces are going to be on a higher side which means, if you do not have the friction the protein may be getting immobilised her because at this point the frictional forces as well as the electrophoretic forces are equal but if you increase the frictional forces what will happen is the bands are start going to be immobilised on a slightly, on a higher side because they are now losing the electrophoretic mobility and because of that you could be able to seek if the protein is getting unfolded, so you will see that the band going up and up like that and that is what is happening.

The same protein sample is rooted in different lanes and is allowed to run vertically opened the color to the urea gradient. As sample runs in different lanes it gets exposed to the different concentration of urea and consequently at a particular urea concentration the protein is unfolded with an increase in hydrodynamics volume. And that point the frictional forces will go up that is what you see the native protein are running more and the unfolded protein will migrate slower due to the increase in frictional forces and it gives a unique protein bands to provide a qualitative or semi-qualitative information about the protein folding intermediate.

So what you see is that initially it got folded, so this is actually that 0, 1, 2, 3, 4 so when you reaches to 4 molar urea some portion of the protein got unfolded and that is how it has created increased hydrodynamics volume and now this will remain intact but when you reaches to the 5 molar, some more portion got opened and once you reaches to the 6 molar the full protein got unfolded and that it still remained like that, so that is how at 6 molar urea this particular protein got completely folded and information from the gradient urea page needs further verification from the other analytical techniques.

So the information what you get from the urea page is actually the semi-qualitative or semi quantitative or Observatory kind of information is means it is going to give you the information that up to the 4 molar urea this protein structure is very stable but when you go to the 5 molar are or 6 molar the protein structure is going to be disrupted and that information is good enough to establish few more concluding experiments using some other analytical techniques such as CD spectroscopy or fluorescence spectroscopy.

So to answer more questions what is opening whether it is a domain which is opening or whether it is some more subunits which are getting detached from this particular protein, so you can imagine that if it is a monomeric protein you will see this particular type of pattern, if it is a dimeric protein what you will see is that the Monomer is getting like this then you will see more bands appearing like 2 bands, 3 bands like that.

If you have a tetramer you will save one all the protein got denatured you will see the for bands because all the 4 bands are going to be separated from each other and all the 4 bands are going to migrate at a different relative mobility, so this urea page in addition to the protein folding urea page can also be used to analyse the protein complexes as well as the covalent heterogeneity of the protein.

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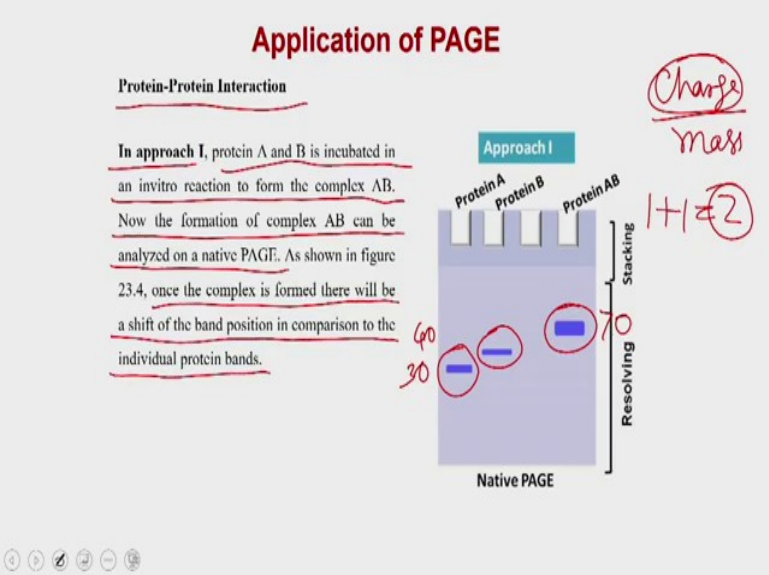

Then the SDS Page can also be used to study the protein-protein interaction, protein protein interactions can be studied in 2 different approaches. In approach 1 protein A and B is incubated in an invitro reaction to from the complex B. Now the formation of the complex AB can be analysed on a native page, as you can see in this particular figure, so protein A is of 30 kilodalton, protein B is of 4 kilodalton and what your C is that it is formed a complex of 70 kilodalton but what you see is that the electrophoretic mobility is not in proportion to the 70 kilodalton because it is actually running based on the charged by mass because we are running a native page.

So it is not like 1 plus 1 is equal to 2 because the masses are being added but subsequent in addition to that the charges are also been mixed with each other and because of that you do

not know or the resulting charge… it will migrate as per the resulting charge by mass ratio, so that it will give you a distinct band but it will not be in proportion to mass because the masses are… it is actually migrating based on the charge by mass ratio because we are running a native page. Once the complex is formed there will be a shift of the band in the position in compare to the individual protein bands.

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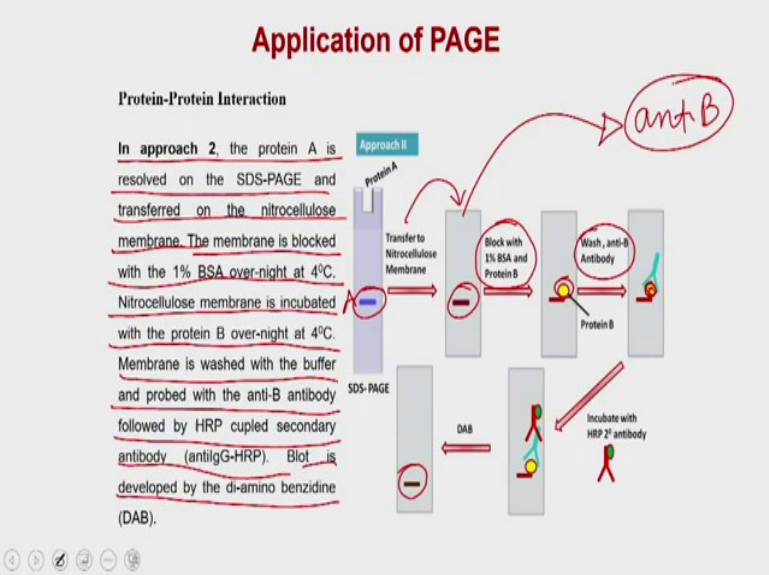

Now in the approach 2 the protein A is going to be resolved on the SDS page and then you transferred it on to the nitrocellulose membrane so you have resolved the protein A which is on to the gel then you transfer this on to a membrane so you have got transferred. Now this protein band is going to be blocked with the 1 percent VSA overnight at 4 degree and then you add the nitrocellulose membrane, you incubate that with the protein B overnight at 4 degree so what you do is you add the BSA for blocking agent and in that blocking agent itself you add the protein B.

So if the protein B has an affinity for protein A, the protein B will go and bind to the protein A then what you do is you wash and now you add the anti B antibodies which means you are going to add the antibodies which is against this protein B. The membrane is washed with the buffer and probed with the anti B antibodies followed by the HRP cupled secondary antibodies, the blot is developed by the DAB, so what will happen is if the B is interacting with A it will be…and then it will be detected by the anti B antibodies and as a result what you will see is you were going to see the band at the position where A is present.

In this particular type of approach what you have to take a precaution is that you have to run a control gel where the A this particular transfer thing is also going to be probed with anti B so that you should ensure that the antibodies what you are using for detecting the protein B is not cross reacting with the protein A because in those cases what will happen is that you will get the signal irrespective of whether the protein B is interacting with A or not.

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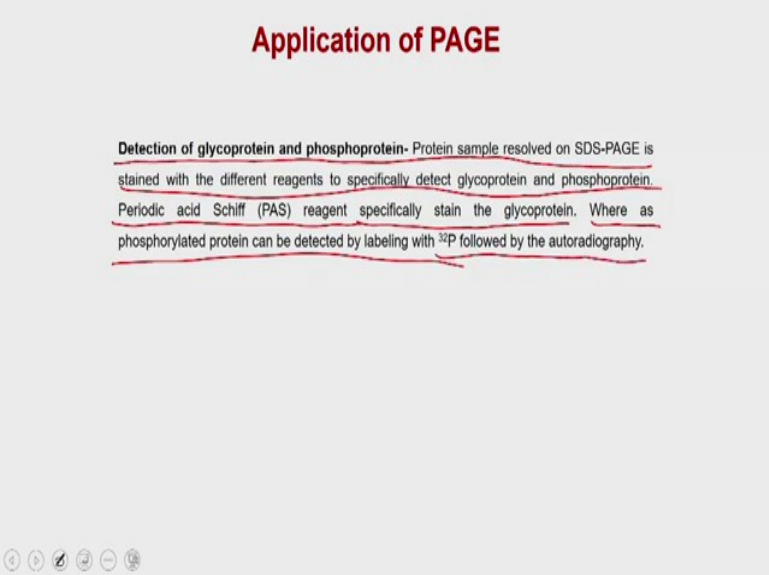

Then you can also use that particular thing is for detecting the glycoprotein and phosphoprotein, so you can run the protein sample and stain with the different reagents to specifically detect glycoprotein and phosphoprotein for example you can use the PAS reagent, the periodic acid shift reagents specifically to stay in with the glycoprotein whereas the phosphorylated protein can be detected by labelling with the P32 followed by the autoradiography.

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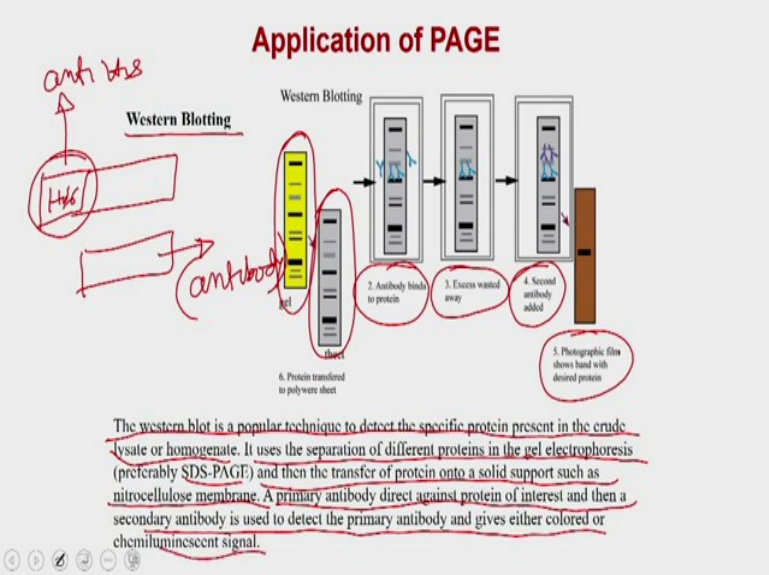

Now we will discuss about the western blotting, so one of the western blotting is a very very robust and popular technique to detect the protein what you have produce is good or bad, so that can be done by 2 methods, 2 ways. In one way what you have is you are producing a protein which is actually containing a tag so if you remember when we were discussing about the affinity chromatography we have put a tag actually, so remember that we have the anti his tag to this protein so what we can do is we can use the antibodies for that tag for example we can use the anti his antibodies, similarly it could be also possible that you do not have the tag but the protein what you are producing should also have the antibodies.

So the prime requirement of this particular approach is that you should have the antibodies to detect the protein which you would like to characterise because that will be used as an analytical tool in this particular technique, so western blotting is a popular technique to detect the specific protein present in the crude lysate or the homogenate. It uses the separation of different protein in the gel Electophoresis like SDS page and then the transfer of protein on to a solid support such as the nitrocellulose membrane.

So what you are going to do is first you run the gel on the gel and then you transferred it on to the nitrocellulose membrane, then you incubate with the primary antibodies, then you wash them to remove the primary antibodies and then you incubate with the secondary antibodies and then you developed with different types of developing agents. A primary antibody direct again the protein of interest, so it could be anti his anti-bodies or the antibody which you have detected for your protein of your interest and then secondary antibody is used to detect the primary antibody and give you either color or the chemiluminesent signals but the western

blotting is very very complicated as well as multistep techniques, so all these steps has to be done in a very very good accordance.

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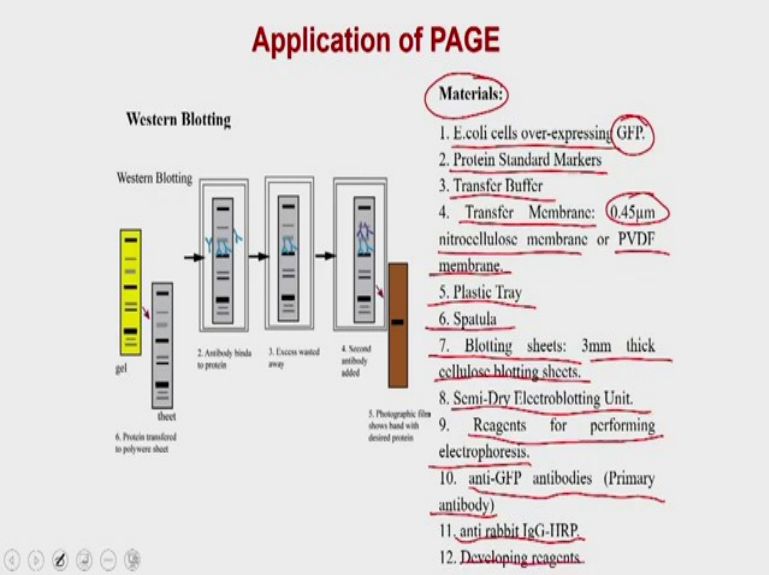

So what is the material you need for doing the western blotting, you have to have E. coli which is overexpressing in this case we have taken an example of GFP then you need a protein standard then you need a transfer buffer, then you need a transfer membrane, transfer membrane could be 0.4 micron meter Nitro cellulose membrane or the PVDF membrane.

Then you need a plastic tray, you need a spatula, you need blotting sheets which are actually 3 mm thick cellulose blotting sheets then you need a semidry electro blotting units and you need reagents for performing the electrophoresis which we have discussed in the previous lecture. Then you need the primarily antibodies, in this case we are using the GFP as a tax. So in this case you need the anti-GFP antibodies then you need anti-rabbit IgG HRP this actually against the primary antibodies and then you need a developing reagent.

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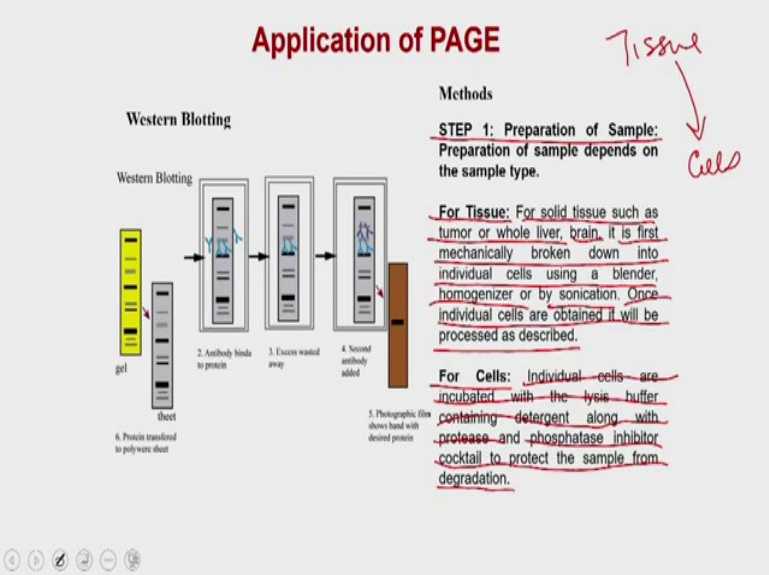

So in the step one you are going to prepare the sample which means you are going to prepare lysate and preparation of lysate also depends upon the sample what you are going to use. If it is a tissue For example if you are using a tissue instead of cell then 1st you have to do is you from the tissue you have to get the cells, so how to do that?

For a solid tissue such as a tumour or the whole lever brain it is first mechanically being broken down into the individual cells using a blender or homogenizer or by sonication. Once the individual cells are obtained it will be processed we discussed for the cells, if you have individual cells then you are incubated with the lysis buffer containing detergent along with the proteases and phosphatase inhibitor cocktail to protect the sample from the degradation.

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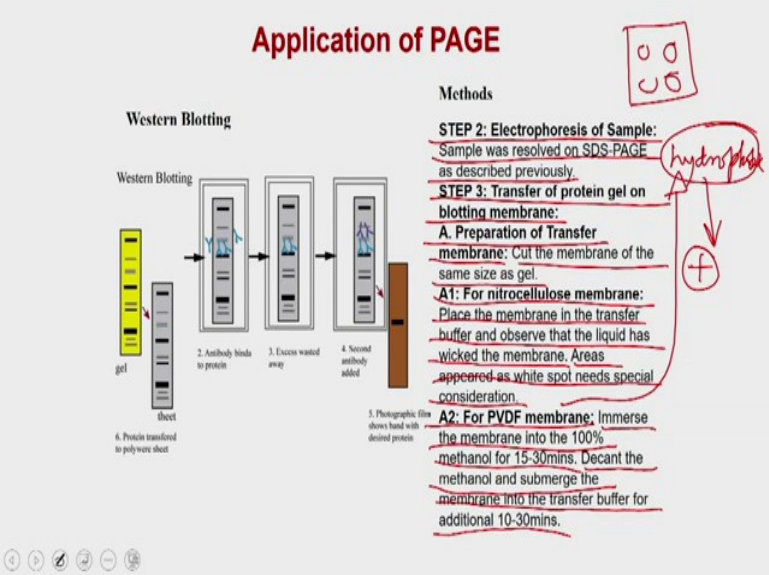

So once your sample is ready in the lysis buffer then you in the 2nd step you are going to resolve the samples onto the SDS page, sample were resolve on to the SDS page as we discussed in the previous lecture, then what you do is in the step 3 you transfer these protein onto a blotting membrane, the transfer is actually a very very crucial as well as important step, so transfer is being done by following multiple steps. In the step 1 what you have to do is before you start the transfer you have to prepare the membrane, so you have took at the membrane of the same size as your gel, so for example if you have gel of 10 by 7 then you cut the membrane of the same size.

Then suppose you are using nitrocellulose membrane then in that case you place the membrane in the transfer buffer and observe that the liquid is going to wet the membrane which means the membrane is going to be wet by using the transfer buffer. The area appear as white spot needs special consideration, so when you do that what happens is sometimes you will see white color spots.

So these white color spots are the places where the membrane has not taken up your transfer buffer, you have to incubate these membrane for longer period of time so that these white spots also going to give you a wet feeling which means the complete membrane should be of uniform color or uniform appearances because all these white color spots are going to give you a non-specific binding of antibodies and they may give you some different types of artefacts.

If you are using a PVDF membrane, so PVDF membranes are actually the hydrophobic membrane so which means they do not like the water, so hydrophobic membrane R need to be converted into hydrophilic membrane, so that it will actually catch up your protein and bind your protein, how to do that? You immerse the membrane to the 100 percent methanol for 15 to 30 minutes then you remove the methanol and submerge the membrane into a transfer buffer for additional half an hour.

That actually is going to make the membrane wet able by the transfer buffer and that actually will allow the transfer of the protein onto this membrane otherwise if you use the PVDF membrane without this particular step, the PVDF membrane is not going to bind a protein or even if it binds a protein it may not be evenly distributed.

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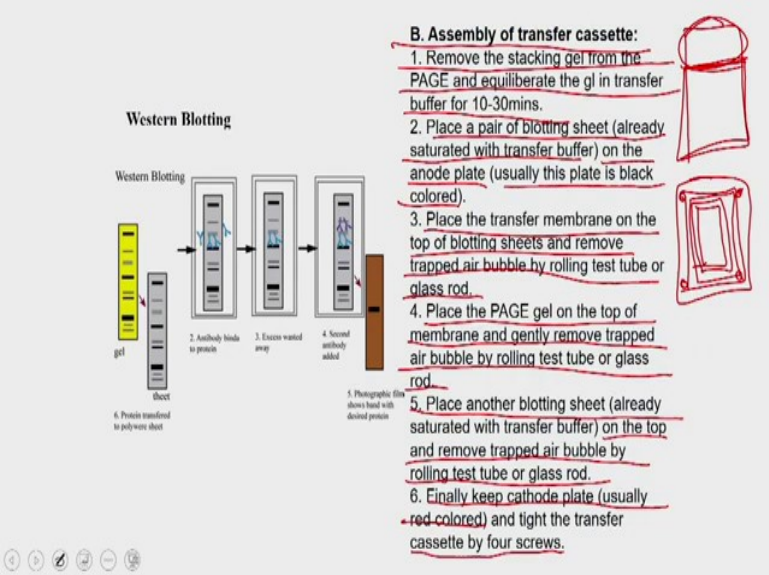

Now once this is done then you have to assemble the transfer cassette, you remove the stacking gel from the pages and equilibrate the gel in the transfer buffer, so your gel is having the stacking gel, you do not need a stacking gel because there is no protein or there is no band present in the stacking gel, so that you have to cut and then you take only the resolving gels. Place a pair of blotting sheets which are already wet with the transfer buffer onto the anode plate, so usually this plate is black coloured.

So your anode plate is black in color upward the 2 sheets of blotting sheets then you place the transfer buffer onto the top of blotting sheets and remove the trapped air bubble by rolling test-tube or glass rod, then you place the SDS page or the gel onto the membrane and gently remove the trapped air bubble by rolling the test-tube or the glass rods, so on the top of these

blotting sheets (())(47:57) the membrane then you remove the bubbles which are present in between and then you put your SDS page.

Then you put another sheet of blotting sheets and if you remove the trapped air or trapped bubbles. Place another blotting sheets on the top of the… and remove the air bubble by rolling test-tube or glass rod finally keep the cathode plates usually it is the red color, so then you put the cathode plate and then you tight the transfer cassette by putting the 4 screws, so every cassette has screws on all the corners and that you have to tie-up so that the whole cassette is going to be one unit and that is how you can just submerged this into the transfer tank.

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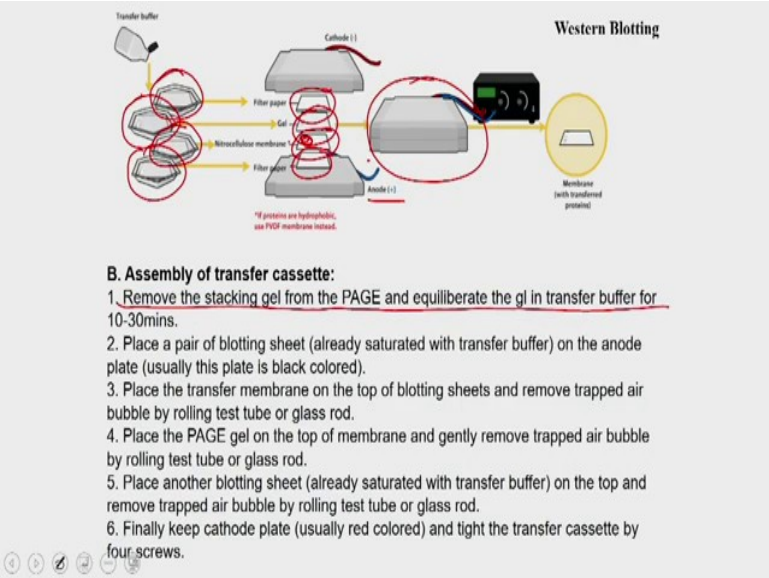

So this is what is you have, 1st you prepare your transfer (())(48:52), so you put the filter paper into the different trays so that they will get wet and then 1st you put the filter papers onto this anode plate then you put the nitrocellulose membrane then you put the gel and then you put the filter paper and in between you have to use a rolling pens so that you can remove the air in between because if the air is present that area the protein is not going to be transferred onto the nitrocellulose membrane, especially the air should not be present between the membrane as well as the gel. Then you remove the stacking gel from the page and equilibrate the gel and once you have done is you put the cathode and then you tie-up with the help of the screw and then you connect this to the power pack and you run the things on 100 volts for…

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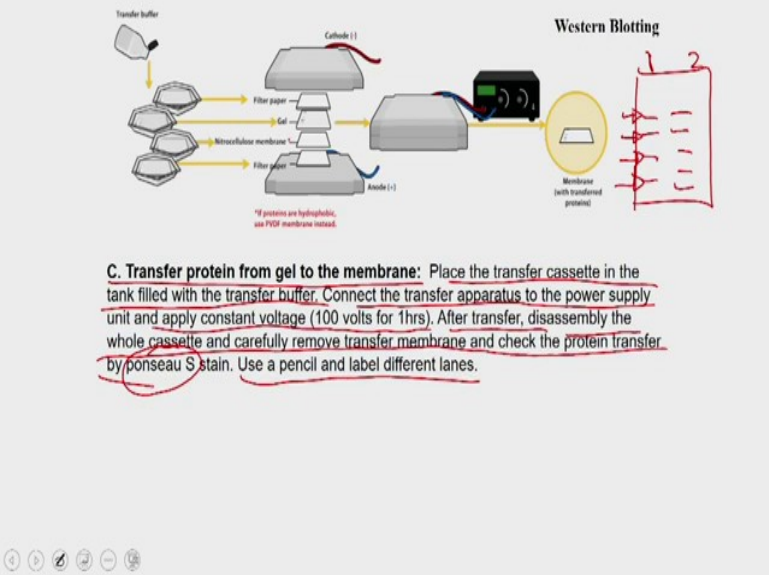

So then you transfer the proteins from the gel to the membrane, place the transfer cassette in the tank filled with the transfer buffer, connected transfer operator to the power supply unit and supply 100 volts for 1 hour. After the transfer you disassemble. So you remove the screws and carefully remove the transfer membrane and check the protein transfer by a dye which is called as ponseau S.

Use a pencil, so once you got the thing you were going to see all these bands the help of ponseau then what you can do is you can use a pencil to mark the markers so you can put a marker you can label the things like 1 and 2 with the help of graphite pencils and that actually will allow you subsequently to know which one is the lane 1 and which one is the lane 2.

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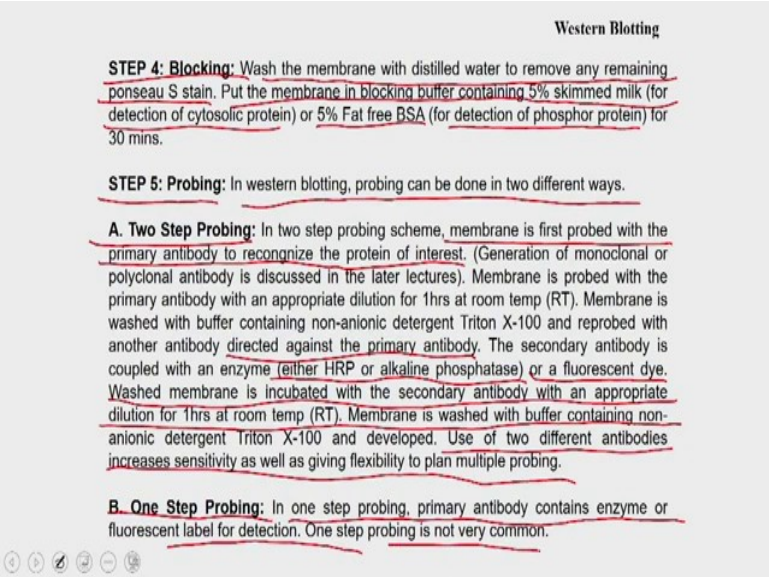

Now in the step 4 once your transfer is over you wash the membrane with distilled water to remove any remaining ponseau S. Put the membrane in blocking buffer containing 5 percent skim milk if you are detecting the cytosolic protein, if you are detecting the phosphorylated protein then you can add the 5 percent fat free BSA because the skimmed milk also contain lot of alkaline phosphate and some other kind of phosphate molecule, so if you use milk as a blocking agent you may get an artefact in the detection of phosphorylated proteins.

Then step 5 you are going to do the probing, so in the Western blotting probing can be done in 2 different ways, a 2 step probing or the single step probing. In the 2 step probing membrane is first probed with the primary antibodies to recognise the protein of interest. These primary antibodies can be generated against the protein of your interest or if this primary antibodies could be against the tag which you are going to use in this case we are using the NTGFP antibodies so you can use the NTGFP antibodies which you can easily purchase from a commercial vendors and then followed by that you also have to put the secondary antibodies which will be again directed against the primary antibodies.

So secondary antibody is not going to detect the antigen or it is not going to detect the protein or the tag, it is only going to detect the wherever the primary antibody is bound and we have discussed in the past that why we use 2 step probing because the two-step probing actually amplifies the signal several folds and the secondary antibodies are normally contains HRP or the alkaline phosphatase as an enzyme which you can use for running the reactions. Sometimes it is also been coupled with fluorescent dye, then the washed membrane is incubated with the secondary antibody with an appropriate delusion for 1 hour at room temperature.

Membrane is washed with buffer containing non-anionic detergent and developed. Use of 2 antibodies increases sensitivity as well as giving flexibility to plan the multiple probing. For example if you are having the 5 membranes what you can do is you can add different different primary antibodies and then you can club them or you can plan the things properly because you have steps whereas in the single step you have to do it very… 1st of all you are going to compromise with the sensitivity secondly you have to compromise with the flexibility that you cannot have the 2 antibodies. In one step probing you add the primary antibody which actually is coupled with the enzyme or the fluorescent level for detection. So one step probing is not very popular or very common.

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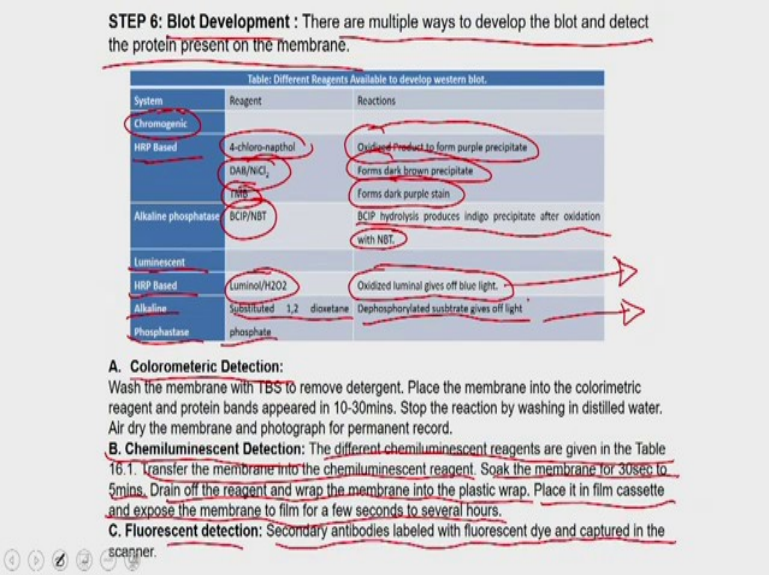

Then what you do is you develop the blot, so there are multiple ways to develop the blot, to detect the protein based on the membrane. You have the chromogenic substrate which are based on the HRP such as the 4 chloro naphthol or DAB or TMB these are the reactions, so if you use a (())(54:06) it will form a purple color precipitate, if you use the DAB it is going to form a dark brown precipitating a user TMB it is going to form the dark purple stain on the membrane.

Similarly you can have the alkaline phosphatase, so you have the substrate which is called the BCIP NBT and the BCIP hydrolysis produce Indigo precipitate after oxidation with NBT, so these are the colorometeric or the coloromerginic substrates, you have the luminescent substrate also for example for HRP you can use luminol or the H2O2 and that actually is going to be oxidised to give you a light and that you can detect in the chemiluminescent system.

Similarly you have the alkaline phosphatase you can use for substituted 1, 2 dioxetane phosphate and that actually also going to give you light which you can detect into the chemiluminescent system, so you have the colorimetric detection is which is actually of these substrates or you have the chemiluminescent detection.

The different chemiluminescent reagents are given in the table you transfer the membrane onto the chemiluminescent reagent soak the membrane for 30 second to 5 minutes. Drain off the reagent and wrap the membrane into the plastic wrap, place it in a film cassettes and expose the membrane for few seconds to several hours. Then you also have the fluorescent

detection, in the secondary antibodies labelled with the fluorescent dye you can capture that into a specific scanner like you can use for different type of scanners to detect the different dyes which are for the si3, si5 or some other color dye and those specialized scanner can be used.

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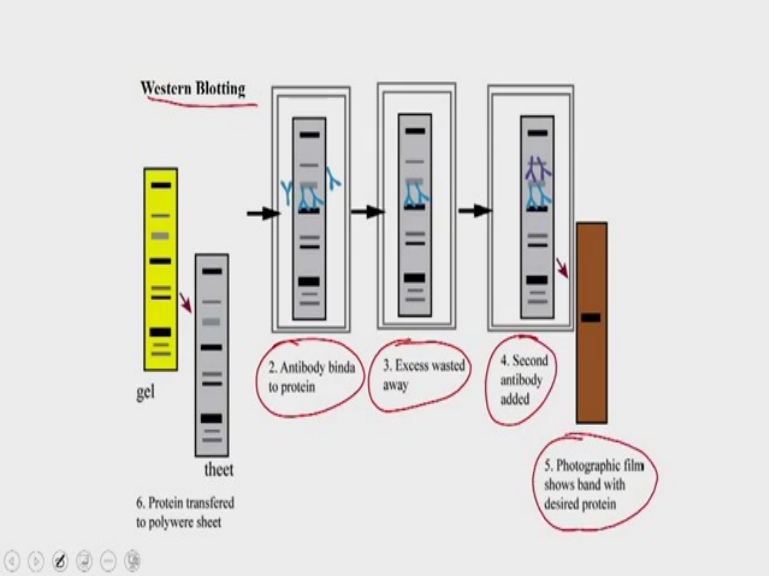

So this what all about the Western blotting where you have the gel then you transfer on to the nitrocellulose membrane then you incubate it with the primary antibodies, then you wash it with the excess wash, then you add the secondary antibodies and then ultimately you develop the blot with the help of different types of substrates either it is colorimetric substrate such as DAB or you have chemiluminescent substrate or the fluorescent substrate, so all these steps are very very simple, they are very easy to perform but they require precautions and they require simple training to do our Western blotting in your laboratory.

So to show you a small demo how to perform the Western blotting we have performed all these steps in our laboratory and Banesh going to show you all these steps so that you will be not only going to get that your theological knowledge, you also going to get the particle knowledge about how to set up these cassettes and how to transfer the gels, how to do primary incubations, secondary incubations and how to develop the blots and once you develop the blots you are going to get the chemiluminescent signal and that actually also can be evaluated using the different types of image analysis software as well.

In this video will demonstrate how to do a Western blot and how to analyse the results using ECR electro chemical (())(57:39) substrate, so here what we will do we have to run gel 1st

then they will transfer, the transfer method how to do the transfer we will show in this video. In previous video we have already shown that how to prepare and SDS page and how to run protein (())(57:58), so in this video particularly we are interested in factors associated with the Western blotting. For doing Western blot we need a membrane and transfer buffer and the transfer medium.

(Refer Slide Time: 58:22)

This one we use to transfer this gel to membrane, so here membrane can be of 2 kinds one is nitrocellulose which has low protein binding efficiency and hydro (())(58:35) nature. Another membrane is PVDF this is hydrophobic membrane and higher protein binding capacity, so the (())(58:47) for Western blot is different for nitrocellulose and PVDF. If you are using PVDF membrane we have to cut the part whether you have ready-made pre-cut blots then no need, if you are taking from a bundle you have to cut precisely how many (())(59:12) you want.

So after that you have to label front on the blot where the front side can be used for transferring the protein and that can be use in further steps also like antibody incubations. So, here if you want to use PVDF membrane you have 2 charge with the methanol, so since the PVDF is hydrophobic membrane you cannot directly transfer the (())(59:47), first you have to keep in methanol for at least 20 minutes, so after this can be called as charging, so after this we will use that for transfer.

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So this is pre-soaked in methanol and equilibrated in transfer buffer, so here while doing transfer we need to consider few things, the buffer always should be in chilled condition otherwise during this transfer at high voltage it will generate high temperatures, so that may degrade your protein or decrease the efficiency of the transfer, so that is why we need to keep them buffer always in chilled condition and let us start the procedure. So, we need a gel so we already finished the gel running, in addition to that we also need (())(60:52) which will give cushion to the gel, so that the gel may not (())(61:01) during a transfer.

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Step 2
Transfer

So this is cassettes we will use for the transfer, so this is negative side of cassettes and this is the (())(61:25), so we are going to keep gel on the negative side and positive side the membrane, so when we will apply voltage from this side to this side, the negative protein it will be transferred it will be more to past (())(61:46) and it will be captured in the membrane, so first for doing that these sponges we need to keep and also this maybe gives some non-specific binding to membrane.

So what we will do we will put blotting sheets on top of this, so after this you have to remove air bubbles if any present. So, once you insert the blotting sheets then you have to keep your gel, so here you have to remember that gel after finishing the SDS page (())(62:51) you have to keep in transfer buffer, so that it will give identical condition or equilibration kind of thing during transfer so that protein transfer may be easy.

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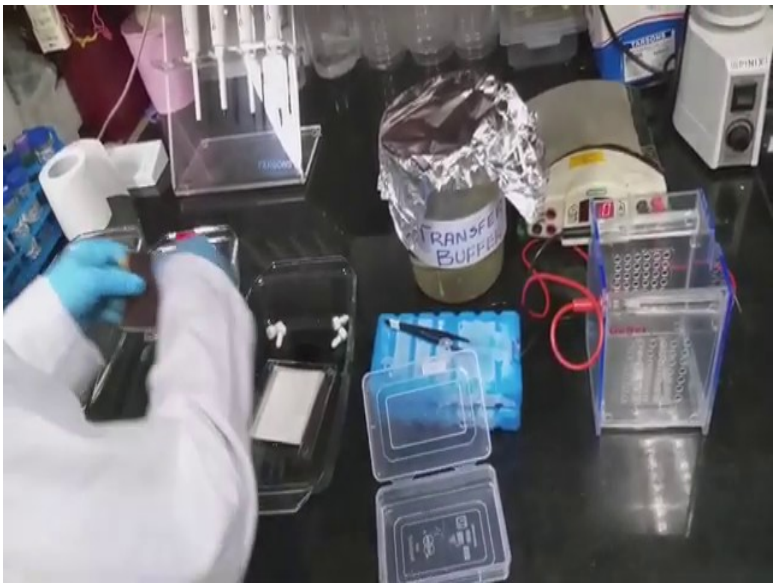
TRANSFER
BUFFER


TRANSFER
BUFFER


TRANSFER
BUFFER

So this is the gel and keeping down the negative side so after that we have to overlap with the membrane. (())(63:39) we have to remove any air bubbles if present, we have to overlap with another blotting sheets and remove the air bubbles each and every time when you introduce something you have to remove air bubbles, so this is the final sheet, so this is the positive side of the cassette, just have to put like this and these are the screws we have to tighten it up then only the contact between the gel and membrane will be sufficient to get transferred. 1st you do not tight initially you just keep and after that press the positive side of the cassette then tight the screws, so all these things should be done in the transfer buffer only unless specified.

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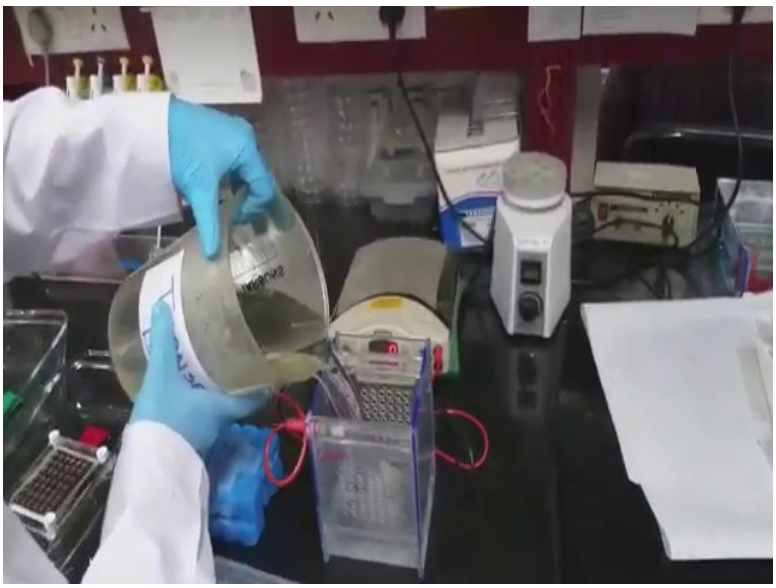
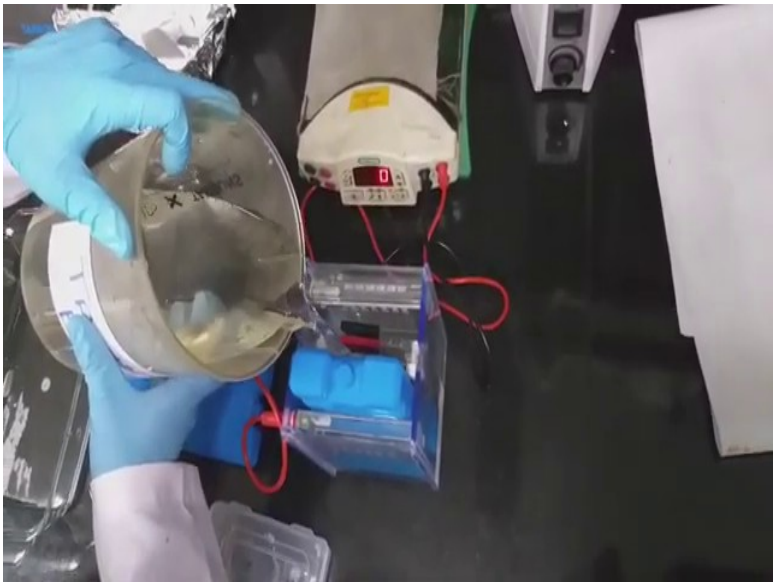



TRANSFER
BUFFER



So this is the chilled transfer buffer. Now, we are going to pour sufficient buffer and a keep this ice pack also, if the chilling is not sufficient then there may be heat generation, so in order to prevent that will use this ice pack, so this will keep the buffer cool till the transfer, so once that is over you directly take out the cassette and keep. If there is a buffered insufficiency you can add on top of that. Make sure that the cassette completely (())(65:52) so that the transfer will be proper and there is no air bubbles.

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So, once the setup is over now you can transfer. Now, transfer is going on, so how much voltage we need to give it depends upon transfer to transfer, it varies generally in our lab we give at least 2 hours sub transfer at 120 volts which is sufficient to transfer even low molecule weight proteins also but from instrument to instrument also it varies. You needed to optimise before doing transfer, after 2 hours we have to remove the blood and incubate with the blotting buffer.

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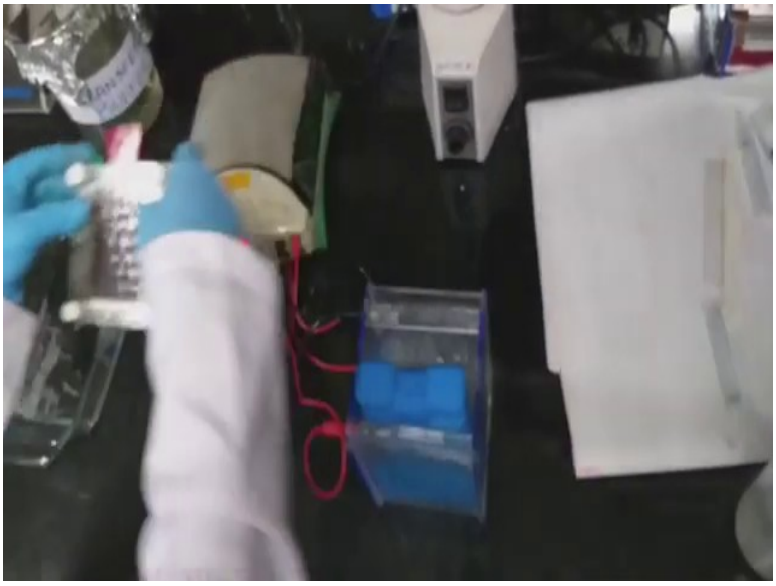

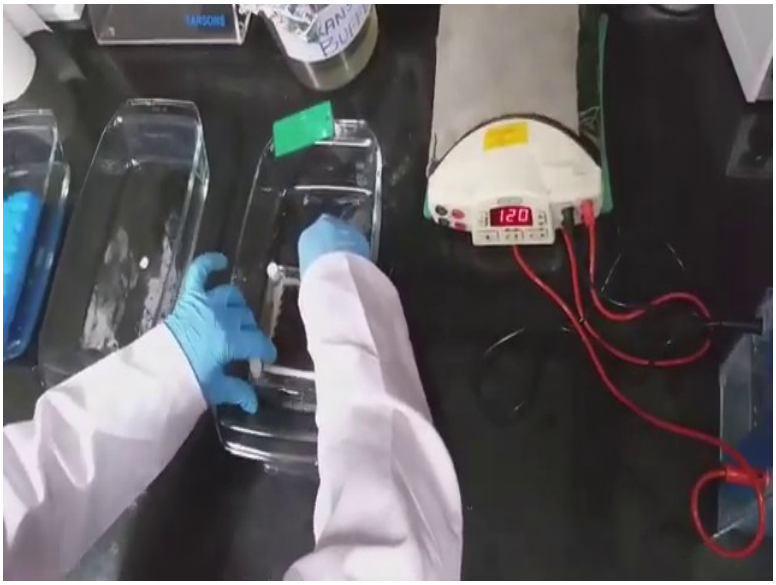

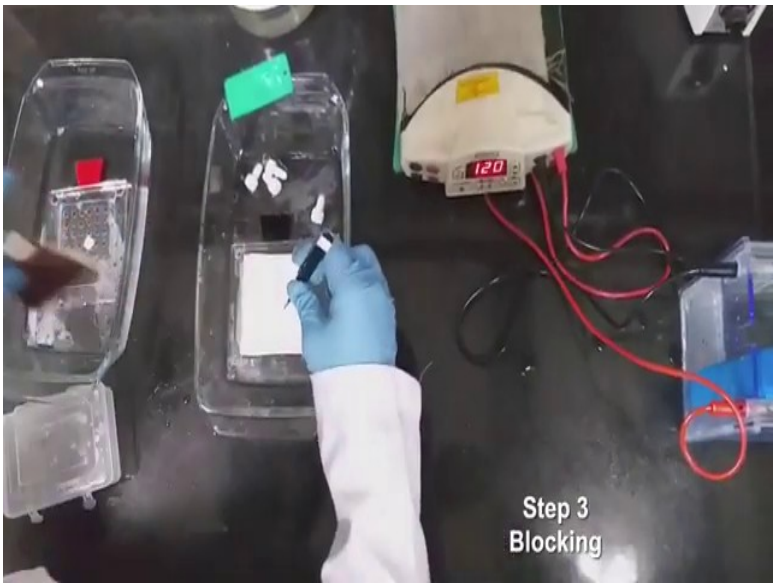

So, I am going to stop here. Remove the cassette, keep it in a tray, remove the screws properly and gently remove the sponges. Take out the blot and keep it in blotting buffer. In this condition we have to keep, if you are keeping it at room temperature it is for 2 hours at least, if you are keeping in 4 degree celsius you can keep overnight. The blotting buffer contents skim milk are BSA along with the (())(67:57).

The Western blot transfer is all depend on the efficiency, how precisely you are doing the transfer. For example you should not use your bare hands while handling the blot or gel, so whatever the proteins present on your fingers it will transfer into gel or membrane which will give his background during development of the blot. So always use gloves, apart from that while handing the (())(68:36) make sure that there may be possibilities of electricity shock make happens sometime, so we have that time also we need to use gloves and after finishing

the transfer you will have to treat all the apparatus properly and dry it for the next time use. After the blotting of the membrane we have to remove the membrane and incubate with the primary antibody without washing.

The main purpose of the blotting is that it will occupy non-specific sites other than the expected protein, so that when antibiotic comes it will bind to that specific protein and gives non-ice. So after this we will incubate with the primary antibody for overnight at 4 degree celsius then wash 3 times at least 15 minutes each with the TVST buffer or PVST buffer and again treat with incubate with the suitable second reactive body.

For 5 hours at 4 degree celsius or 2 hours set or 3 hours set in temperature, after that we need to wash properly at least 3 times, then we will develop the blot with the electro chemiluminescent substrate. In earlier, at Western blot, how to do Western blot video, we showed how to transfer the proteins to (())(70:14). So we incubated with the primary antibody following secondary antibody and wash it. Now here we show how to develop a blot.

For developing a blot we need (())(70:31) substrate. In most of the commercially available fix luminal is the one of the substrate we use for this purpose, so luminal in presence of hydrogen peroxide and peroxidise agent which presents in the secondary antibody (())(70:54). This (()) (70:58) converts luminal to (())(71:05) State luminal by deprotonating and oxidising it, so this (())(71:15) product gradually leads to energy by releasing luminescent products. That light will be detected using this instrument.

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Step 5
Development of Blot


LIFT HERE
Step 5
Development of Blot


Step 5
Development of Blot

So this is the commercially available chemiluminesent substrate solutions, so is available from wide range of companies, we have to mix one is to one ratio. So we have to take out the blot, drain the buffer whatever present properly, so after that you keep blot in between a plastic paper foils, then we will take chemiluminesent substrate. So after that slowly press and remove air bubbles.

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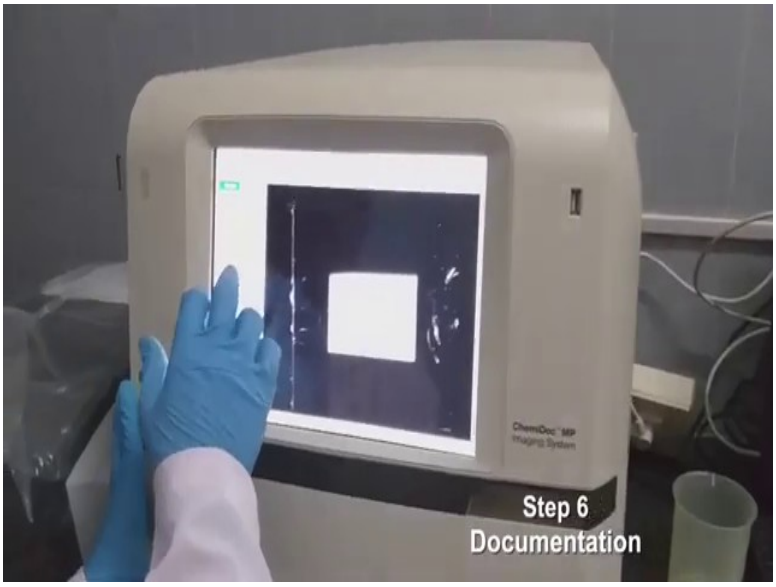
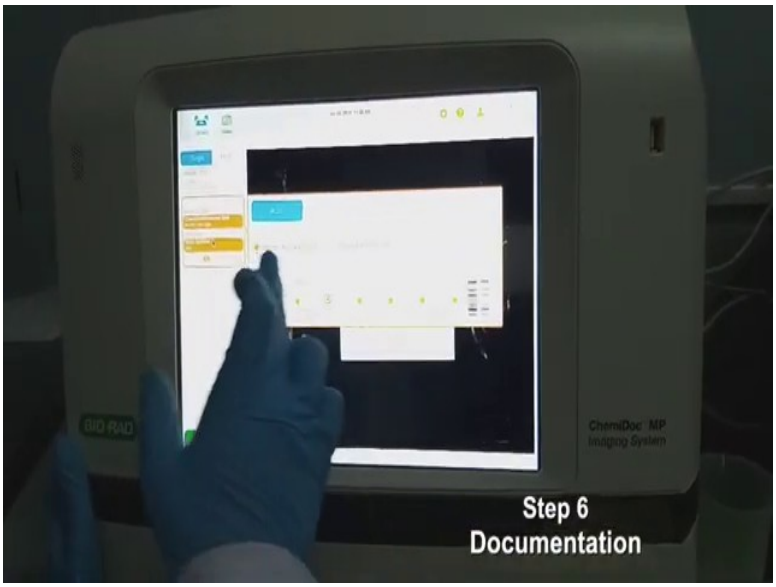
Step 6
Documentation


Step 6
Documentation


Step 6
Documentation


Step 6
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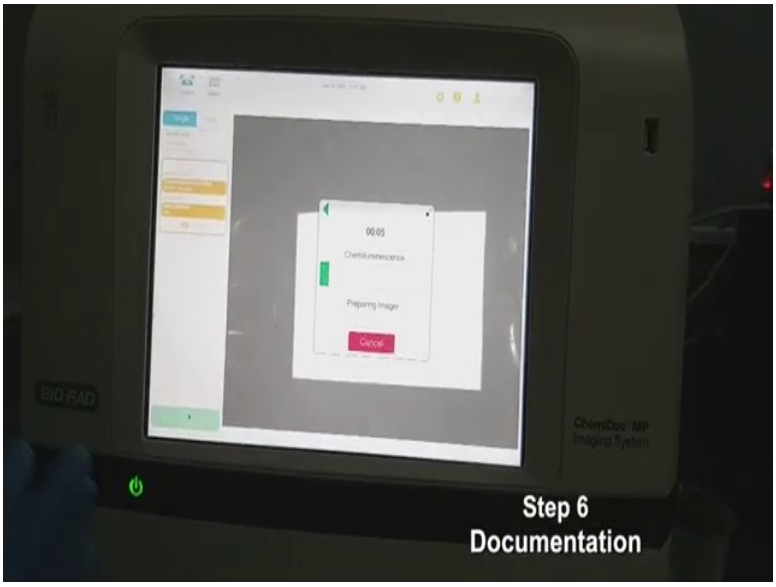
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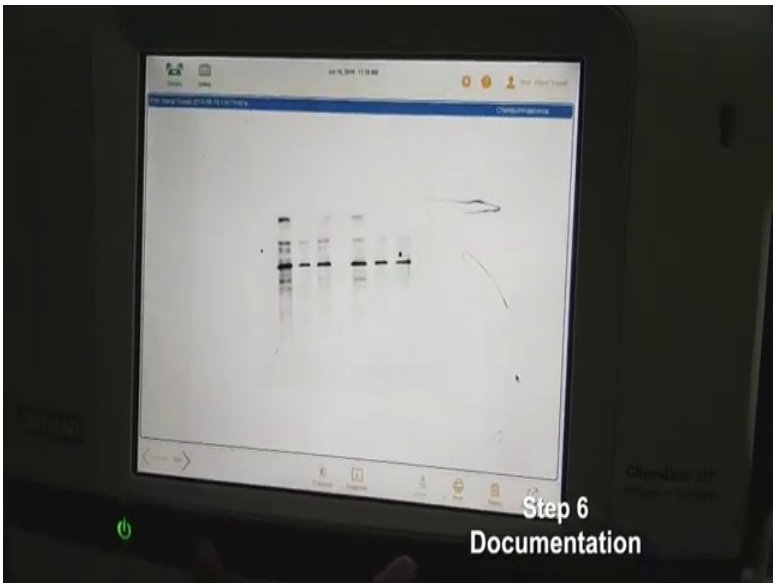
Step 6
Documentation


Step 6
Documentation

This is the train we will use for developing the blot, so we have to open the system properly aligned with (())(73:18) and then shift blot to the plate, once it is over you have you just close. Here we have to select application, we want blots that is chemiluminesent and what exposure want… you have to options manual and auto, in auto 2 options are there, optimal auto exposure, rapid auto exposure.

We will choose optimal auto (())(73:55), so you can enlarge the blot also. Once it is over just say…so this is the developed blot, so as we cab see the bands pattern, so this is how we develop western blot through electro chemiluminesent substrate, so with this we have demonstrated how to transfer the proteins to a blot and what are the precautions need to be taken while doing the western blot and also how to develop the blot and what is the lay in principle behind the development (())(74:43). So I hope this will help you to understand basic outlay in mechanism of how Western blot works.

So in this demo videos, Banesh has discussed different aspects of Western blotting, how to setup the cassettes, how to blot the gel from the… How to transfer proteins from the gel to the nitrocellulose membrane, how to do the blocking and all other steps which are involved in Western blotting which we have discussed in our lecture as well and I hope this demo could have been helpful for you to perform this experiments in your laboratory, so with this we would like to conclude our lecture here and in our subsequent lecture we are going to discuss about how to characterize a product by using different types of spectroscopic techniques. Thank you.