Immunology
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Lecture No -28
Tools and Techniques (Contd)

Today's lecture as we discussed or we told I told you that I am going to discuss about immunoblotting another very important and very popular technique that we use many things can be done with this mostly the Eliza what we discussed in last , Eliza we can detect and estimate the antigen. But in Eliza what we cannot do is we cannot tell the molecular weight of the antigen right because there is no scope of knowing the molecular rate.

In this technique we can estimate not as good as Eliza but we can estimate basically relative estimation and we can detect definitely at the same time we can also tell the molecular weight of the antigen that we are interested or looking for. Also we can go for whether antibody is there or not how good the antibody is that we can do. But normally, normally this technique is mostly we see for characterization or do for characterization of the antigen or the protein but same definitely antibody also we can do.

So let me discuss very quickly that what is this immunoblotting? Immunoblotting to what we can do and what is the basic thing what actually how we can know the molecular weight of the protein.

Let us start so this particular technique immunoblotting is also known as Western blot. You know northern blot you heard about southern blot. So this is Western blot.

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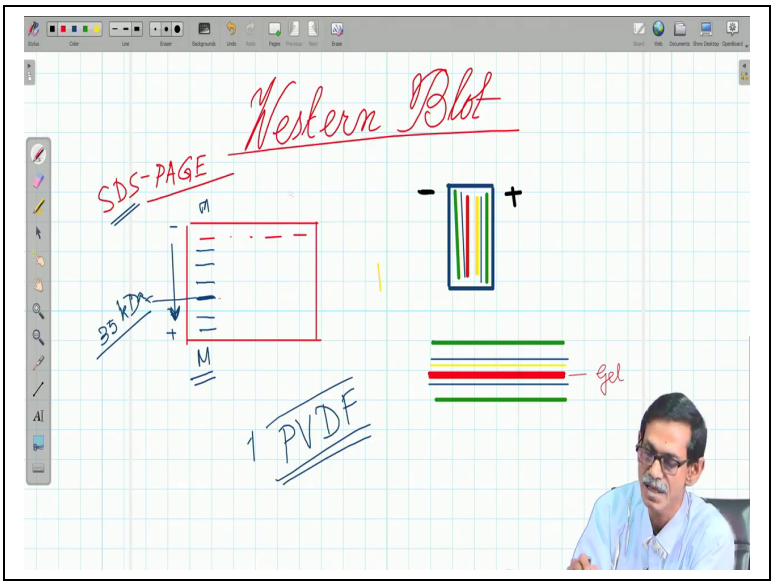

Western blot this is also you know blot. Western blot the first thing is it is no in antibody indigenous acquired. First thing we run a protein gel polyacrylamide gel electrophoresis is also known as PAGE. Many time we run SDS page is the sodium dodecyl sulfate page this is the denaturing job. You can run native gel anyway you have to run a protein gel. So in protein gel what we can do is in protein gel you know that you have to make a gel first and this gel you run you make a gel up you make a gel polyacrylamide there are certain well.

So what we do is you load protein and run and this is the negative and this is positive if it is SDS SDS makes all the protein negatively charged. So when you run or give the electric current for the negative the potential is like this then protein will migrate from negative to positive because all the protein molecule in the solution sorry so all the protein molecules in the solution is negatively charged. So if you pass an electric current or we looked must start electrophoresis protein you will migrate this oil and that is also not visible you can stain it by Coomassie staining and see the protein.

But suppose if the Coomassie system if it is a Coomassie staining what we will see is after staining and distilling suppose there are I am just drawing one lane so that we will get multiple band right in a given solution in a extracts a Leicester there are so many proteins. They will distribute according to their molecular weight because lowest molecular weight will be here highest molecular weight will be here.

Because heavy cannot run fast and in one of the length I this will be known protein that is normally we call it mark what kind of known putting that way I know that the bottom one is 14 kilo Dalton second one is 21 kilo Dalton then 35 then 45 then 66. So that what is this, this is called known marker. This is marker it is called ladder also sometimes. But normally in case of protein molecular weight marker that you can buy commercially.

When you buy a marker solution which band is what molecular ate that also will be given with the seat that when you buy something. So, exactly you can tell who each band is what size. So now if this is the band suppose this is 14, 21 this is 35 kilo Dalton this is known. So any protein in this region any protein in this region this protein is very similar look you can tell that it is very close to 35. So it is most likely 35.

You cannot tell exactly, to tell the exact molecular order of the protein you have to do the mass spectroscopy but normally we do not do it. So here so this one but here up to here so many proteins so not necessarily that suppose you know your molecular weight of the protein is close to 35 assume that you know your protein. But in cell there are 4 to 5 thousand different proteins are there and there are many may be 35000. So when you are seeing one single man here suppose thick band that does not mean that there is only one protein.

Even after that your protein of interest may not be there because that may not express because all pretty not always Express every time that may be your experiment you would like to see whether at a particular condition your protein is expressing or not. In Eliza you can see but whether Eliza is a false positive or giving some nonspecific count you cannot see. So confirmation will be done by this molecular because Eliza positive.

And in Western blot if you can detect and you can tell that to the molecular rate of protein is close to 35 then you are as a scientist or as a doctor or as a pathologist you will be much more confident know what I am telling seeing is fine. Because it is supposed to be 35 it is showing 35 it is positive in both Eliza as well as western blot that means it is confirmatory as well as mental satisfaction and genuinity will be very good there is no nonspecific interaction.

So what we do? So after this after running the gel whatever this band and all these things you would not see. Marker you can buy pre stain marker and you can see but other thing you may or may not see and that for that what we will do is? So that whatever we will do so you take this protein gel we take the protein gel take it out from the gel apparatus I am sorry I cannot show either gel apparatus.

How it open but gel is a very gel like structured thin it is not very thick you have to handle carefully thickness also depends like you can make thicker you can make a thinner that depends on what kind of protein you are isolating. So what we do is? We take this we take this protein gel let me make the color code same so you take this protein gel which is little thick this is the gel of sorry suppose this is the gel this is thick this is gel.

So after running the gel you take it out man this is the resistor sorry for this is your gel you know and we take this gel on top of a filter paper because so that just a support because gel is free. So we take a gel on top of it filter paper. So you take a filter paper we take a filter paper put that gel. On top of this gel we put a membrane same size. So whatever the size you are seeing here same size filter paper same size membrane is what membrane we use either nitrocellulose membrane or the membrane is P P V D F which is very popular now polyvinyl di fluoride membrane poly vinyl die fluoride if anybody is having problem understanding just type in the net PVDF membrane you will get all the details were to do what not to do and how to use those.

Who are interested more and somebody if you already done this experiment for you it is very simple and straightforward. So here on top of this blue line is this quantum and filter paper just to give you support and PVDF membrane that you cut exactly in the same size and you put on top of this gel then you put again then you put again one filled filter paper again a support so what is happening you are taking a filter paper putting your gel then your membrane the PVDF membrane then you putting another filter paper.

So basically you are making a sandwich basically you are making a sandwich of two filter paper in between gel and membrane is there. So two filter paper is there in between gel and membrane

is there so in this orientation so and you have to remember one thing your membrane is in the top I will tell why, I am telling so above it what we use actually there are certain sponge we are just to give another just buffer.

So what is giving we are giving a sponge it is there is no gap between it just for the picture I am giving the gap but it is all in contact and there should be no air gap no air bubbles. So every time what we do is we put one so suppose this is a gel we put the membrane then we put a roller on it to push out all the bubbles there should not be any bubble then this whole thing whole thing so we make this way filter paper filter paper and this then to spawn both side we made this then the full sandwich we make like this.

So we made this way and make like this so this one will fit into the same gel apparatus same gel apparatus. So now what we have is whole thing is you have a sandwich where everything so what we have first we have 1, 2 sponge then we have two filter paper then we have the gel and then we have filter. So the same thing I just put vertically. This mixture is completely packed now we put the filter paper and you remember gel is in the left hand side and the membrane is the right hand side.

Now we start the electrophoresis again what will do it now will give positive charge this side and negative charge this side what will happen? So gel is done like this then now I take the gel put the filter paper and put the electric current positive that side and this is the filter paper say this is the filter paper and this is the gel if the positive is this side what will happen all the protein that was in the gel it will be transferred to this paper, clear.

So, there will be no protein in the; so whatever there it was in the jail in the gel that is transferred to this you know paper I am using everything again or rather let me put this.

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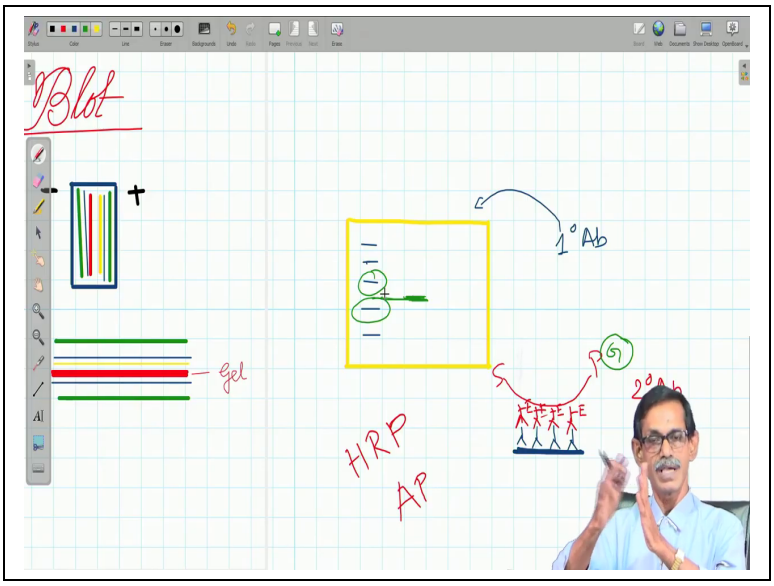

So what will happen is now I have now I have one filter paper which you cannot see anything only thing if you use a priest and marker then you can see the protein band the same protein man whatever you saw before that is visible nothing else your protein band nothing is there. Now what I will do is same as Eliza. Now somewhere your protein is here first I will add first I will add primary antibody into this paper.

So I will take this paper in a tray small tray add the primary antibody solution in give it for one hour what will happen if some protein suppose my protein is here some protein is and which is not visible maybe you cannot see also well it was very faint. So if you have primary antibody out of 1000 proteins my antibody is not going to bind all the protein. If it is specific it will only bind here all the antibody will concentrate where the protein of interest is there.

So now if I draw it thick or separately suppose this thing I am saying so this is the band and your antibody suppose antibody will all come here. So through all the paper proteus protein may be there but antibody is going to bind to the specific band where my protein of interest is that it is like that. So now if I use the secondary antibody against that antibody but now we hope you understand the previous one.

Now if you know secondary antibody it will bind here and the secondary antibody is just like the Eliza which is labeled with enzyme which what enzyme? This is also same enzyme what, if I am

remembering you again HRP horseradish peroxidase or alkaline phosphatase clear. So this enzyme doing the same thing colorless substrate colorful product but there is a nice difference between Eliza and Western blot what is the difference?

Here the substrate is different what why because you have to choose a substrate so that product is colorful but insoluble in water remember that what we will do what we do actually we take a tray we put your paper at primary antibody incubate it will bind then secondary antibody it will in it will into it the red one will go and bind you add the substrate and product will come and the product is soluble it will go away.

But if the product if the product is insoluble what will happen suppose the product color is green another whose colorless and the product color is green what will happen you will see wherever the reaction is happening it is depositing there and gradually you see a green color is appearing. If it is soluble it will go away but if it is insoluble it will deposit on that white paper because the PVD is membrane is white you can see a nice green line.

If green line is coming and if you know that this is 35 this is 45 you can guess this is close to 40 or close to 30 you can calculate very accurately even the relative is because you can plot it and then figure it out from lock scale I am not going in detail. But comparing with the marker you can tell what is the molecular weight of the protein? So if your protein solutions have your protein of interest then only you will get the band.

So even appearance of band is telling you the protein is there. Molecular rate of the band you can understand and that means you can also tell the molecular rate of the antigen this is much more confirmatory than Eliza and that is why HIV test you can go HIV test immediate test is Eliza that some blood sample its HIV positive. But confirmatory test is always done by this Western blot or the immunoblotting technique.

So it is very similar principle same antigen-antibody reaction same enzyme is linked and the similar kind of reaction is happening but device is different here it is first resolved in SDS page or normal page or native page then it is transferred to a membrane then it is like we detect like

Eliza. So Eliza we directly load their protein in well here we resolve the protein mixture. But here what is happening another difference with Eliza says in Elisa protein remain in native form those who are not denaturing it.

Here it is denatured right what is the problem of denaturation? Not much but you few remember my epitope class what I told I am just telling you again there are two types of epitope one is linear epitope and other is conformational epitope. So if the antibody raised again conformational epitopes SDS because protein is denatured conformation is not there. So that antibody will not detect the protein in western blood but they can detect in Eliza because there you are using the protein in native form.

So some protein or some antibody more specifically if it can may be positive in Eliza but showing nothing in western blot that does not mean it is wrong it may be the antibody is against a conformational epitopes that is why it is not picking up for because if you run SDS page it is denatured. But if you are native page and then also you are not seeing it then you have to think something else I am not going to go detail on that I just give an idea of how this native and conformational epitopes works.

So this is going to tell you about the molecular so Eliza and Western both can detect and estimate Western can give you something extra which is which can tell you the molecular rate of the antigen. So this is one another important technique. Next important technique is you know localization.