Experimental Biochemistry
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Lecture – 53
Western Blotting Technique

Welcome back everyone, so, today we will learn how to do western blot to detect a particular protein. So, in this week we are going through this, different blotting techniques. So, there are mainly three types of blotting techniques southern blotting, northern blotting and western blotting. So, if you want to detect a specific DNA in a sample then you have to go for southern blotting. If you have to detect a particular RNA then you have to go for northern blotting and in case of protein you have to do western blotting.

So, all the three blotting techniques have this basic theory; that means, that you have to either you have to separate either the DNA, RNA or the protein by doing gel electrophoreses. And when you separate all the types of DNA, RNA protein gel electrophoreses they are separated by the size. So, then you have to transfer the DNA, RNA or the protein to a particular membrane. So, that membrane basically absorbs the DNA to RNA or the protein. And then using that membrane, you can detect your whatever DNA or RNA or the protein you want to detect. So, this three kinds of blotting technique has a same protocol and if you have go through the theory classes then you will understand what is the main theory behind those blotting techniques. .

So, in this lab we will go for this western blot where we will detect the particular proteins. So, western blotting is also known as Immuno blotting, because here we will detect the protein by doing antibody interaction. So, there will be antibody interaction and that is why it is also known as Immuno blot. So, in case of western blotting there are few steps which we need to follow. So, as I mentioned first you need to isolate the first you have to isolate the protein from a sample.

So, basically you have any particular sample cell lysate sample or the tissue and from that you have to isolate all the proteins that are present in case of that particular tissue or any other sample which you have.

From the tissue sample, you have to separate all the proteins based on their size and for that we need to do SDS page, because as in earlier classes also we have mentioned to isolate or the separate the proteins in a sample we have go for this denaturing SDS page that is a gel electrophoresis and that gel is made up of polyacrylamide. So, after you separate the proteins by doing SDS page then the SDS page or the gel is placed under top of the iron membrane or a nitro cellulose membrane nitrocellulose membrane or PVDF. Now all these membrane basically get absorbed, take away the protein sample from the gel and it retains the protein in the membrane.

Now, after that you have blotted the protein in the membrane then you can detect a membrane the protein in the membrane by doing incubation with primary antibody and secondary antibody since this involves this antibodies that is why it is also known as Immuno blotting technique. So, first step is to get the sample ready for doing the, western blot. So, in case of western blot our sample will be proteins. So, suppose we have sterilizer we are want to detect or want to see if a particular protein is present in a particular sample.

So, you have a sample where different types of protein will be present and you want to specifically detect one particular protein that is why western blotting is also known as western blotting as very selective. And it is also semi quantitative, if you can detect a particular proteins presence as well as the what is the amount of protein the relative amount of protein that is present in the particular sample. So, you have the sterilizer where different types of proteins are present, then you go for SDS page. Now for doing SDS page the where we prepare the sample is similar to the normal SDS page.

So, basically have to, you have to boil the sample with the gel loading dye which will contain the SDS and argon of inner blue and if you have sustained then beta Mercaptoethanol and DTT.

So, that will ensure that the protein is denatured basically and it is in the primary form, structured form. So, the protein will separate in the gel based on their size so, not based on the native confirmation, because we are running a denaturing SDS page.

So, after you prepare the sample then you load the sample in the SDS page gel. Now in case of doing a western blot you should always load a particular marker which is already pristine. So, you should be able to see the marker. So, marker means there is a protein

ladder where different sized proteins will be there and you can see those markers if it is pristine while the gel is running. If you do not use the pristine marker then you cannot see where the if the properly the gel has been run or what is the size in the gel. So, till how you have to run the gel, it will be determined by the prestained marker and while doing this blotting.

When you are transferring your protein to a nitrocellulose membrane or PVDF membrane you can also see that the transfer is or not because you can see the ladder proteins ah, because those are pristine and you will be able to see those prestained ladder in the blotting membrane. As well since the gel we have to make a SDS page gel and I,I have already done that and I have already loaded the sample in the SDS page gel and the gel is already running. So, after you run the gel another important thing for western blotting is that you should not keep any length which is empty.

So, if you do not have any sample then just no loading buffer or the loading dye, because if you keep any lane in the SDS page gel empty then what will happen that the lane side at the side of the lane if there is any protein, it will try to squeeze and try to the gel will be very weird and you do not want that size and the shape of the band should be proper that is why you should not leave any empty lane in the SDS page. So, it is a good habit you should not leave any empty lane.

So, always load if you do not have enough sample always load. Some amount of loading dye in the gel. Now after you run the gel then we will go for this transfer. Now in case of transfer you can go for this capillary action.

So, basically you put the filter paper then the membrane and then the gel and all the filter papers are soaked in buffer and by capillary action the gel that the proteins will transfer from the gel to the membrane, but this will take a long time and may be from few days even. So, to reduce the time we will go for electro transfer. So, in case of electro transfer what will happen is the way we separate the protein in SDS page, we put in, in a electric field where the protein will move from.

So, during electro transfer what will happen is that the protein will come out from the gel and it will go to the membrane and get stuck to the membrane. Since, the membrane stores the positive side the negatively charged protein will go and stick to the membrane and then we will take up the membrane and then we will go for blocking.

So, it is important to always block the membrane. So, what is the meaning of blocking? So, basically here what we will do is. So, the proteins that are present in the gel it will go and get stuck to the membrane, but that there are lot of space in the membrane which will be empty, because there is not everywhere in the gel the protein will be present. So, there are empty sides in the blame membrane and if you do not block and go for directly for this anti primary antibody incubation. Then what will happen? The primary antibody should be able to bind to the protein of your interest it should be specific, but if there is any kind of empty space.

Since, it is a membrane and it has a affinity towards protein then the primary antibody will also get attached to this empty spaces and there will be a huge background noise. So, the primary antibody will detect your protein as well as there will be other places where you can see your the antibody, because it was empty in the membrane and it will get stuck to the membrane. So, to reduce a noise in the membrane or gel you have to go for blocking.

So, in case of blocking we go for this non-specific protein is incubated with the membrane. So, what will happen in any place where the membrane does not have protein or any protein then that will get non-specific protein will get absorbed to that particular membrane. And then when you go for primary antibody incubation the antibody will specifically bind to the protein of your interest, and other places since there is already blocked by different types of protein, it will not get attached to it.

And when you detect primary antibody with the help of secondary antibody you will only able to see your particular protein of interest and not any other places. So, blocking is a important step in case of western blotting. Then you have to go for different kinds of washing. So, you have to wash the gel and go for this primary antibody incubation. During primary antibody incubation the primary antibody is raised against your protein. So, the primary antibody will selectively detect your protein and it will get attached to the primary antibody your protein of interest, but you cannot detect the primary antibody that is why we need the secondary antibody. So, after the incubation with the primary antibody and several wash steps then we will incubate the membrane with the secondary antibody.

Now, secondary antibody is raised against the primary antibody. So, the secondary antibody will bind to the primary antibody. So, in this way the your protein will bind to say primary antibody and the primary antibody will get bind to the secondary antibody. And in case of secondary antibody there is always, detecting molecule that is present in case of secondary antibody either that is a HRP or Horseradish Peroxidase Enzyme or alkaline phosphatase enzyme or different types of enzymes are there attached to the secondary antibody.

So, after binding to the secondary antibody you give a particular substrate for the particular enzyme that is present in your secondary antibody and depending on the substrate you can detect the position in where your protein should be there. So, wherever you see the detection band that is where your specific protein is there in the membrane and depending on the amount the if there is a; if you are using a peroxidase enzyme or a phosphatase enzyme, there will be a light production. So, Chemiluminescence will be there. So, depending on the amount of light that is produced by the secondary antibody or basically the enzyme that is attached to the secondary antibody you can also semi quantitatively measure how much protein you have in the sample.

So, basically if you have different types of sample you run different samples in the same gel. And in the same gel you try to detect the amount of protein that is present and quantitatively you can see how much protein is there in a different samples.

And also it will give the qualitative measurement and the size of the particular protein and if at all the protein is present in the sample or not. So, western blotting technique is that is why used in different kinds of detecting tool. So, in case of HIV, if you want to want to get the particular protein present in a patient sample blood sample or not, you can detect that particular protein using western blotting. There are different diseased patient where western blotting helps to detect any particular diseased protein that is present or not in the sample.

So, thereby western blotting is a useful technique for selectively detecting and semi quantitatively measuring the amount of protein is present in the sample. So, the first to begin with here I have already started SDS page gel.

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So, here I have loaded my sample, it is a normal 15 percent SDS page, I have loaded my sample and along with the prestained marker. So, now, I can see the marker and how long it has gone where it is there and I can depending on the prestained marker I can stop when I want to stop the gel.

So, before I stop that as I mentioned that there is a membrane to blot. So, mainly two types of membranes are used nitrocellulose and PVDF; in case of nitrocellulose, it has a very high affinity towards the protein, but the problem with nitrocellulose membrane is very fragile.

So, you cannot it is basically it will break soon or you cannot handle it properly. So, a PVDF membrane is a great choice for this kind of method, because it is very sturdy and it also have higher affinity. Another thing is for nitrocellulose membrane the noise the background noise is very less, but in case of PVDF membrane, if you are not careful enough and if you skip the washes or not properly do the washing steps then there will huge noise.

So, in case of PVDF membrane, you need to do the washing steps thoroughly. So, so, reduce the background noise while detecting your protein. The another difference between nitrocellulose membrane and PVDF membrane is that in case of nitrocellulose membrane, you do not need to activate the membrane.

It is already activated whenever you want to do the blotting, you just soak the membrane in the transfer buffer and place it on the top of the gel, but in case of PVDF membrane, you need to activate the membrane using methanol. So, you have to activate the membrane about 1 to 5 minute in methanol and then you put the membrane in a transfer buffer. So, transfer buffer usually contain trace to maintain the PH glycine and the small amount of SDS, it might contain and also I mean 20 percent methanol it has. So, the methanol will help to retain the shape of the gel.

So, do not skip the methanol part in the transfer buffer. So, mainly the transfer buffer has TRIS glycine and methanol. So, since I have this membrane which I am using this is the PVDF membrane. So, I need to activate the membrane in methanol, but so, there are two types membrane, you can buy in case of PVDF membrane either it is a pre activated membrane or you need to activate in the lab. So, this membrane which we got is a already activated membrane.

So, I do not need to do further activation, but you should always check the level when you are buying the membrane it does not say that it is activated then you go should go for methanol treatment. So, you have in a small box you have a methanol then you put the membrane in methanol for some time and then you get the take out the membrane and put it in the transfer buffer. So, before we go for this blotting, we need to equilibrate the membrane as well as the gel in the transfer buffer for sometime. So, while my gel is running I will put this membrane since, it is already activated I do not need to put it in the methanol.

So, I will put this membrane in transfer buffer. So, I have in this small box I have small amount of transfer buffer here, it is TRIS glycine and methanol another important part of this technique is that you should not touch the membrane or the gel by bare hands. If you do that then there will be some noise and you will get blotty gel. So, using a forcep, I am taking out the membrane and I am putting this membrane in the transfer buffer. So, if membrane you need to activate in the methanol. So, if you do not activate the membrane in the methanol and directly put in the transfer buffer, you will see that the membrane is not getting soaked in the transfer buffer.

So, that time you will know that you need to activate the membrane before putting, it in the transfer buffer because it is a hydrophobic membrane, it will not get soaked into the

transfer buffer. So, here my membrane is already pre-activated and it is already soaking in the transfer buffer. So, I will keep the membrane in transfer buffer in a rocking condition for sometime and in the meanwhile, we will take out the gel and also put the gel in the transfer buffer.

So, I have put the membrane in transfer buffer and to equilibrate the membrane for 10 to 15 minutes. In the meanwhile, we will take out the gel from this plate in a small another box, I have again the transfer buffer and as may be you have noticed that I have very small size I have cut. So, in case of this membrane they come as role also in a precut version, we have it as a role and depending on how much you need. So, basically there will be a two three lanes, I will need only. So, depending on the measurement of the gel where my protein will be there.

I will cut a small piece of membrane from the role, because membrane is very expensive, do not waste the membrane, do not cut the full membrane as a whole. Now, we will take out the gel from the plate. So, I am stopping the SDS page run.

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As you can see the marker has been separated properly and I know when my protein will be there. So, I have to stop now. And according to the marker I will also cut the gel into small fragment, because not every where the protein is present.

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So, this is a plate which we got, here the protein is present here, I will separate the plate. So, this is the short plate we have and I will keep it here and the gel is stuck to the bigger one. So, now, I will just cut my gel according to the size I want. So, first I will remove this top or the stacking gel basically. So, you do not need the whole gel, do not go for this whole gel blotting, because then you will need long or much more membrane and thus have mentioned the membranes are quite costly, I do not need the whole gel.

So, I will just cut the gel using this cutter. So, this we will just cut I will keep the ladder, because that will ensure when the transfer is done that will tell me. When if the proteins are transferred properly or not lower portion also I will cut, I can also check the size. So, these are the things which we got from the membrane. So, my membrane is of almost this size. So, the gel should be a little bit smaller than the membrane. So, the now that I have separated out my gel I will cut into the smaller fragment, I will put this gel in the transfer buffer to equilibrate.

So, these are SDS page, so, you do not need to be very careful want to break the gel and put it in the transfer buffer. And will keep it in the rocking motion the gel and the membrane in the transfer buffer for 10 to 15 minutes so that our membrane and the gel is equilibrated in the transfer buffer, done. So, 15 minutes is over. So, now, our membrane and the gel is equilibrated enough in the transfer buffer, we also want this tissue paper basically.

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So, these are the blotting papers and this is not normal general tissue paper this is high quality blotting washing paper or Whatman blotting paper and we will soak this blotting paper as well in the transfer buffer. So, after this we will go for this transfer. So, we will make a sandwich. So, just keep the blotting paper or the here in the transfer buffer.

So, due to capillary action it will get soaked in the transfer buffer. So, there are two way, you can do the transfer either of fully wet technique or a semi dry technique. So, in case of wet technique you have to use sponge and so there will be sponge which is soaked with the buffer transfer buffer and in the in between there will be blotting paper the membrane and the gel and again blotting paper and another sponge; in case today for today's demonstration we are going for semi dry transfer.

So, here we do not need the sponge, we just need the blotting paper which is soaked and then we will make the sandwich. So, we will put some amount of buffer as well after the membrane is and a blotting papers are properly soaked. So, as I have mentioned that we are going for this electro transfer. So, you may opt out from the electro transfer, but nowadays everyone go for this electro transfer, because it is quicker you do not want to wait the whole day or the or even more than 1 day.

So, here what will happen is that we will place our gel and the blot membrane and because due to the electricity or the electric field protein will come out from the gel and get stuck to the membrane; so, this is the instrument which we have, at the bottom part

we will take blotting papers. So, at the bottom we usually take 4 to 5 blotting papers, place it here. This should be properly soaked then will put the membrane do not touch the membrane with bare hands, will put the membrane here then on the top of the membrane will put our gel.

So, in the blotting paper on the top of the blotting paper I have kept the membrane here. So, at the bottom there should be 4 to 5 blotting papers which are soaked in the transfer buffer. And now I will put the gel in the top of the blotting paper. So, as you see can see the all the lanes and the marker. So, that is why we prefer to put a prestained ladder in this case of blotting gel. After you put the gel then on top of that again we will put blotting paper. So, this will create make a sandwich. Now in between we do not want any kind of bubble to present between the membrane and the gel.

So, for that what we have is a roller. So, if there is any bubble and that particular position due to this bubble formation the transfer will be hampered. So, with the roller pin, we will just role out if there is any bubble. There should not be any bubble if there is a bubble at that particular side, you will see spot and the transfer will not happen there, make sure that the gel does not slip out from the middle otherwise if the membrane is not in contact with the gel then the protein will not get transferred to the membrane.

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After you are, and there is no air bubbles then will take the top and we will lock it. So, this is the cassette which we have prepared in between our sandwich is there for this transfer.

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Now, here we have this trans plot machine basically, it will create this electric current and I will switch it on. So, in this machine I can mention, I can check that voltage and the time till when I have to stop.

So, here I will go for this new and then I will change the voltage to 20 volt and the time to 15 minutes. So, this time and the voltage basically you need to check for depending on your protein. So, I know that I have 15 minutes and I have hold my proteins all will come out from the gel and get to the membrane, but if you see after you see the gel if the membrane, if you see that not all the proteins are getting transferred or few proteins is quite big then you go for time.

So, now I will put this cassette. So, there are two cassette which we can put basically. There are two side A and B, A I am putting. So, simultaneously you can do two blotting transfer. So, now, I have put it in the A and I will go for this run and I will select the A. So, I have met the sandwich and in this blot machine I have started a run for 15 minutes. So, after 15 minutes what we will able to what we will be able to see is that the gel or the ladder basically, because we can see the protein marker as it is prestained we will able to

see that the marker as gone from the gel; the gel will be empty and it has been stuck to the membrane.

So, after we see that all that protein bands that are present in the ladder is gone to the membrane. Then we will stop, if we take out and see that some of the bands are not properly transferred then we will again have to transfer it for some more time. So, it we will wait for 15 minutes and then we will check if it is transferred or to the membrane or not.

So, our transfer of the protein from the gel to membrane has been done and now we will go for blocking the membrane. So, as I mentioned previously that the blocking is a important step in case of western blotting, because if there is any part of the membrane which is not blocked. So, that will be free where the primary antibody can bind. So, apart from binding to the specific protein against which was created the primary antibody will also bind to the free space in the membrane and that will eventually give rise to this noise that are present there after you go for the secondary antibody and the detection of your protein.

So, because if your primary antibody is bound to the particular space where there is no protein, but only the free membrane was there. The secondary antibody will also bind to the primary antibody, because it was raised against the primary antibody and because of secondary antibody is there. You will get the signal from that particular empty space as well and it will eventually cause you the error in the judgment, because the protein is not there you will still see the band and you still get the noise.

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So, now what we will do is we will take out the membrane and check if the transfer is done or not. So, in the gel there is no ladder or no protein is present. So, as I mentioned or shown that it was prestained marker. So, in the gel there is nothing, but in the membrane you can see there is a portion where you can see the ladder present. So, basically the protein has been transferred from the membrane gel to the membrane. Now in a box, we will take blocking solution.

So, mainly the most commonly used blocking solutions are skimmed milk or BSA. So, these are non- specific proteins. So, here I have 5 percent skimmed milk or fat free dry milk which was dissolved in TBST buffer. So, this is a buffer gluten in every washes the secondary antibody the primary antibody all will be in the same buffer. So, the milk is also dissolved in the buffer, it is 5 percent. If you want to use BSA that will also 3 to 5 percent in the TBST buffer you can also use the BSA.

So, here I will put the skimmed milk here and I will put the membrane that has milk protein in the blocking solution. Now I will keep this thing in the rocker in the rocking condition for almost 2 hours.

So, the blocking time is you have to check depending on your protein and the gel, because I am keeping it for 2 hours, because I know after two hours, it will be enough the blocking efficiency will be good, but if you see the later on. If you see there is a much more noise in case of your membrane while detecting your protein you should go for more time in the blocking during the blocking. So, if the blocking is not proper then

there will be noise. So, now, I will keep this in the rocker for 2 hours and in the constant rocking motion. And after 2 hours, we will wash this membrane and then go for this primary antibody incubation. So, it is 2 hours now and the blocking is done.

So, I will remove this skimmed milk from the membrane then I will wash the membrane with one TBST buffer, the same buffer which we use to dissolve our skimmed milk. So, around 3 times we will wash to remove the blocking solution or the protein. You can also keep it for 5 minutes in the rocking motion and wash 2 to 3 times with the solution to sufficiently remove the remove the skimmed membrane.

Now, I will put primary antibody. So, the primary antibody is raised against the protein with which I want to detect and this also is dissolved in TBST buffer, the same buffer which we are using here and the primary antibody.

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So, depending on the specificity the amount which you need to ah, provide in the primary antibody have to check. If it is highly specific antibody then you have to go for 1 is 10000 something like that. So, 1 is to 10000 means 10 ML if you give just 1 micro liter of primary antibody or if it is not that specific then you have to go for this 1 is to 5000 like that also.

So, depending on your need you have to select how much amount or antibody you have to provide and it is also trial and error based method you have to optimize the western

blotting techniques. So, if you go for one concentration then you do not see proper bands then you change some parameters like the blocking primary antibody concentration the secondary antibody and like that you optimize a whole protocol.

So, now I will add the primary antibody here. So, it is as I have mentioned, it is a antibody. So, it will specifically bind to the protein of interest and I will keep in the membrane primary antibody in the rocking motion for 1 hour. Now the time is also variable, because sometimes what will happen that the primary antibodies not that specific or you are not getting that much of signal then you have to keep it for longer time.

So, from 1 hour to 1 night, you can keep it in the primary antibody. So, depending on how much time you have and also what is the concentration and what is the efficiency of your primary antibody, you have to reduce or basically optimize the time in which the membrane will be bind with membrane will be in the primary antibody. Now primary antibody will selectively bind to the protein of interest if there is a huge number of proteins, there will be different proteins and they are all will they will separate out based on their size.

Now when I add primary antibody, the primary antibody will selectively bind to the specific protein, because it has this affinity or it has the selection for the particular protein. The other proteins it will not bind and the membrane is also blocked with the non- specific protein with BSA or the skimmed milk. So, the primary antibody will not be able to bind to any of the membrane.

So, only at the membrane where the specific protein is there, it will get bound to the primary antibody and then we will wash it and put the secondary antibody. So, it is almost 2 hours in primary antibody. So, now, I will remove the primary antibody and I will wash it with the TBST buffer. So, I will put few ml of TBST buffer 10 ml almost and keep it 5 minutes in the rocking motion and like this I will wash it for thrice.

So, three times I will wash it with the TBST buffer. So, this is the last time, I am washing. So, I have washed the membrane three times. Now I will remove the buffer and next I will put the secondary antibody. Now secondary antibody is also and dissolved in the TBST buffer and so secondary antibodies are very much specific to the primary antibody, you will need only small amount. So, 1 is 25000, the secondary antibody we

will keep it for suppose 1 to 2 hours again in the rocking condition. Now the secondary, the specificity of the secondary antibody is high and it will bind to the primary antibody also the another important feature of the secondary antibody is that it has some conjugated enzyme.

So, for the detection purpose so, either horse radish peroxidase is there or alkaline phosphatase is there. So, in our case we have HRP that is Horseradish Peroxidase enzyme and what it does is gives a it breaks down the peroxidase and then the it creates the Chemiluminescence. So, basically because of the chemical reaction that it undergoes in substrate and it breaks down the substrate and produces light, and we can measure the light using the chemi doc basically the chemi doc will enable us to see how much light is producing.

So, there will be a band at the specific position and depending on the band we can see we will detect the position of our protein the molecular weight of our protein as well as the intensity depending on the intensity, how much protein is present in different samples. So, I have almost 4 samples here I want to check much protein is there in each of the sample. So, in the secondary antibody, we will keep the membrane for again for almost 1 to 2 hours and then we will wash the secondary antibody away from the membrane and we will put the substrate for the enzyme. So, in our case the substrate basically is a luminal and the peroxide hydrogen peroxide.

So, those two we have already purchased and those two substrates we will make in equi molar concentration and around 100 to 500 micro liter, we will put on the top of the membrane and wait for 5 minute. Then we will just take the membrane and put it in the chemidoc machine and we will see what is the band and if you just plot it in the chemidoc machine, we will able to there is any band is present or not. Now the membrane is almost 1 hour in the secondary antibody; so now, we will remove the secondary antibody and we will wash the membrane again three times with the TBST buffer, we can keep the membrane in TBST buffer itself.

So, we will keep it the TBST buffer 5 minute in each interval and after that we will throw away. And then at the end of it, we will just take out the membrane and put the substrate for this horseradish peroxidase enzyme and then we will then for this development of the band. So, now, after the washing is done, we will take out the membrane and put the

substrate inside on the top of the membrane wait for 5 minutes. So, the enzymatic activity can occur and then we will take out the plot and start for the development of the plot.

Now there are few things during this western blotting technique which we should be able to remember that first you have to run the gel properly. So, that all the proteins are separated based on their size you can either run a native page or the denatured a gel. If you are running native page, you will not able to get the molecular weight of the protein, but if your running denatured or denaturing gel SDS page. And you will get the molecular weight as well and after you run the gel after you cut the small piece where your protein is there for the gel and place it on the top of the membrane and there are two types of membrane nitrocellulose membrane and PVDF membrane.

If you are using a very big proteins then you should go for nitrocellulose membrane. If your protein is small and medium size, you should go for the PVDF membrane. Now PVDF membrane are important and useful in different cases, because it sterdy, it does not break a easily, you reuse it in the membrane for long time. Basically after primary antibody you can restrict the primary antibody and again reprobe the membrane with a different primary antibody.

So, if you are detecting in the same gel. So, if you are detecting different type of protein you can do that with the help of this PVDF membrane, while if you are using nitrocellulose membrane. There will be no re probing and you just can use it once. Now the membrane is important as well as the how much time you go for this gel to transfer that will depend on your protein.

If it is too big protein then it will take longer time to transfer. If it is a small protein, it will easily transfer. Depending on your protein size and the behavior of the membrane, you have to decide how long you have to keep. So, electro transfer is a quick way. So, it is a useful technique the transblot helps to and in 10 to 15 minutes the whole protein will get transferred. After you transfer you should be blocking.

Now, after you develop the gel membrane basically and see there is a too much of noise is present. Then you need to standardize the blocking parameters, but blocking should be done in the BSA or the skimmed milk solution and the timing also will differ. So, if you feel that the blocking is also not proper keep the blocking solution for longer time.

So, that the blocking is proper then only the background noise will be less, if you are using PVDF membrane always you should go for background noise will be high. If you are using nitrocellulose membrane then the background noise is not that much; that means, a nitrocellulose membrane is better, but if your blocking is proper should not be any proper any background noise and also to remove the background noise washing steps should be properly done. So, you should wash the blocking agent away properly the primary antibody away properly, if there is a washing steps are small or it is not properly done then again there will a background noise.

So, do the washing steps properly, if you feel the background noise are much change the blocking parameters change the washing steps or make it longer time. Now after you do the primary antibody incubation then you should go for the secondary incubation, usually secondary antibody incubation takes much lesser time than the primary antibody incubation, because secondary antibody incubation is very much specific to the primary antibody. Now after the secondary antibody incubation, you can go for development. And you can see where the gel or the blot is depending on the size. There might some problem with the blotting.

So, if you see that there is a huge amount of noise again check the washing steps and the blocking solution, if you see that the intensity of the band is very low then probably, you should keep the primary antibody incubation for longer time, because that might be specific to the protein. So, you keep it for a longer time sometimes, you see there is a huge amount of like huge blog of black spots then there might be few reasons for that, that you have loaded too much of protein.

You should not load too much of protein in the gel you should check the amount of protein that you can load and based on that you choose the sample and load that much of protein. If you are loading too much of protein then there will be over saturation and the gel will be very bad also, if you are putting too much of substrate for this HRP enzyme. Then also it will create problem then there will be spots black spots in the membrane and you should also avoid that.

So, a very less amount of substrate is enough to create the make the gel proper. So, during western blot, you should standardize this parameters. So, you should go for this different time and different interval. And based on what you feel is proper for your

protein, you should standardize the parameter and go for this technique. Now it is a very useful technique and it is semi qualitative as well as semi quantitative.

If you have different sample you can get the relative amount of the protein that is present in the sample. If you have a unknown sample and you do not know if a particular protein is present or not. You can detect that particular protein based on this western blot technique and it will also give the molecular weight as well as the amount in a particular tissue sample.

So, western blot technique is used in the molecular biology lab and mostly in case of protein lab in day to day, because it is a very good technique and it is very sensitive as well. So, if you want to detect any particular protein you can detect that.