Molecular Biology
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Module - 08
Molecular Techniques (Part 1)
Lecture-36 Western Blotting (Part 2)

Hello everyone, this is Dr. Vishal Trivedi from Department of biosciences and bioengineering IIT Guwahati and what we were discussing we were discussing about the different aspects of the western blotting in this particular lecture. And so far what we have discussed we have discussed about how you can be able to separate the molecule based on the molecular weight in the technique which is called as the SDS page. So, we have discussed about the how to prepare the gel, how to cast the gels, how to run the gel, how to perform the stacking and so on. And then at the end we have also discussed about how to stain the gels and how you can be able to visualize the pattern.

If you want to use these gels for western blotting then you are not supposed to stain these gels instead you can actually be put it into the transfer. So, now in the this particular lecture we are going to discuss about how to perform the western blotting. So, now we assume that you are stacking your gel is ready with the protein resolved and then you can be able to use these gels for performing the western blotting. So, in a western blotting what you have is so, is a step 1 you have are going to resolve the sample onto SDS page.

So, that you are going to see a pattern of the gel and now you are going what you are going to do is you are going to transfer these onto a nitrocellulose membrane. And then you are actually going to do a primary treatment, secondary treatment and ultimately you are going to use the suitable substrate to see the bands. Now let us talk about the step number 1. So, step number 1 you have already we have already discussed in our previous lecture that how you can be able to resolve the sample onto the SDS page. Now we will just start discussing about the step number 2.

So, for step number 2 onwards what are the materials required you are actually going to require the following materials you require E. coli cell expressing GFP. So, this is the sample actually this is the sample what you require for doing the western blotting. Then you require the protein standard markers, you require the transfer buffers, you require a transfer membrane such as 0.45 micro meter nitrocellulose membrane or the PVDF membrane, you require a plastic tray, you require spatula, you are require the blotting sheets.

So, 3 mm thick cellulose blotting sheets, then you require the semi dry electro blotting technique units, then you require reagents for forming the electrophoresis, you require the NTGFP antibody that is the primary antibodies and then you require the anti rabbit IgGHRP and then you also require the developing reagents such as the chemi reagents or the western blotting. So, as far as the procedure is concerned in the step 1 you are going to prepare the samples. So, preparation of the sample depends on the sample type for the tissue for solid tissue such as tumor or whole liver or brain it is first mechanically been broken down into the individual cell using a blender, homogenizer or by sonications once the individual cells are obtained it is been processed as described. For cells individual cells are incubated with the lysis buffer containing detergent along with the protease and phosphatase in a butter cocktail to protect the sample from the degradations. And then the step 2 you are going to do the electrophoresis of the sample.

So, this anyway we have discussed in the previous lecture. Then in the step 3 you are going to do the transfer of the protein gel on to the blotting membranes. So, this can be done 2 ways one is in 2 steps first is the preparation of the transfer membrane and the second is the assembly of transfer operators. So, cut the membrane of the same size as gel right. So, suppose you have a gel of this size then you can actually be able to cut the membrane of the same size or slightly bigger size actually because the nitrocellulose membranes are costlier.

So, you should be little careful with the size because you should not take very big so that you are going to waste. Then here you have the variations if you are using the nitrocellulose membrane the place the membrane the transfer buffer and observe that the liquid was wicking the wick the membrane areas appear as white spot need the special intentions right. So, when you are actually going to soak these membrane into the transfer buffer you what you are going to see is the some portion is going to be remain dry. So, it has to be submerged completely so that it should not show you any white patches it should just show you the clean membrane. Then for the PVDF membrane it actually going to be require additional step of charging so that you should be able to convert the PVDF membrane because the PVDF membrane or polyvinyl dihydro fluoride membranes are hydrophobic in nature.

So, they will not going to bind the protein molecules instead you are suppose to convert or impart a charge on to the membrane so that they will be actually going to attract the protein molecules. So, we that you are going to do is by immersing the membrane into a 100 percent ethanol for 15 to 20 minutes. So, when you immerse the ethanol membrane into a 100 percent methanol it is actually going to give you the it is going to react with the membrane and it is going to give a polar environment. Then you decant the membrane methanol and submerge the membrane into a transfer buffer for additional 10

to 30 minutes and that is actually going to do the same is going to make the membrane suitable for transfer activities. Then the you are going to do the assembly of the transferic acids.

So, remove the stacking gel from the page and equilibrate the gel into a transfer buffer for 10 to 15 minutes. This means you are going to have a stacking gel. So, you are going to have a stacking gel. So, what you are suppose to do because the stacking gel is not required you can just cut this stacking gel and you can only use the resolving gel because the protein bands are only going to be present on to a stacking gel. So, unnecessarily why you should use this portion because otherwise you are suppose to waste the some amount of nitrocellulose membrane.

Then pair place a pair of blotting sheets which are already been soaked into the transfer buffer on to the anodic plate usually this plate is black in color. So, just keep the two one pair of the blotting sheets on to the black plate. Then you place the transfer membrane on top of the blotting sheets and remove the trapped air by rolling the test tube or the glass rod. So, you are going to have a glass rod and that glass rod you should roll it on to the on to this membrane. So, that you all the trapped air between the blotting sheets and the membrane can be removed.

Then you place the gel on top of the membrane and gently remove the trapped air bubble by rolling the test tube or the glass rod. Then you place the another blotting sheets already saturated with the transfer buffer on top of the on the top and remove the trapped air by rolling the test tube or the glass rod. And finally, keep the cathode rate usually the red color and tight the transfer buffer by the four screws. So, you are going to actually having a four screws on top of the four corners. Remember when you are doing the screwing it should be a longitudinally which means if you are doing it 1, 2, 3, 4 your 1 and 3 should be screwed together and 2 and 4 should be screwed together.

So, that you should not have the unbalanced you should not have a any kind of you know the any kind of pressure into this. Because if you do it 1 and 2 then your this portion is actually going to be having a some kind of pressure and as a result it may actually take up some air. Then you are going to transfer the protein from the gel to the membrane. So, place the transfer cassette into the tank filled with the transfer buffer connect the transfer operator to the power supply unit and apply constant voltage for 1 hour after the transfer disassemble the whole cassette and carefully remove the transfer membrane and check the protein transfer by the one should use a pencil and label the different lengths and then you are going to have the step number 4. The step number 4 you are going to do the blocking.

So, wash the membrane with distilled water to remove any remaining poncho stain put the membrane in blocking buffer containing the 5 percent skim milk or 5 percent BSA for the detection of the phospho proteins. So, if you are doing it for the normal protein then you can actually be able to use the 5 percent skim milk as a blocking agent, but if you are doing it for the specific proteins such as the phospho proteins or the glycoproteins and other kinds of proteins then you should do use the BSA because then because the skim milk has lot of alkaline phosphatase. So, that may actually you know destroy the or damage the samples. Then you have the probing. So, in western blotting probing can be done in 2 ways a 2 step probing and the 1 step probing.

So, in a 2 step probing in 2 step probing scheme the membrane is first probed with the primary antibody to recognize the protein of interest. So, and then membrane is probed with the primary antibody with an appropriate dilution for 1 hour at room temperature membrane is washed with buffer containing non-ionic detergent titronics 100 and reprobed with another antibody directed against the primary antibody. The secondary antibody is coupled with an enzyme either HRP or the alkaline phosphatase or a fluorescent dye. Washed membrane is incubated with another secondary antibody with an appropriate dilution for 1 hour at room temperature membrane is washed with buffer containing non-ionic detergent titronics 100 and developed. Use of the 2 different antibody increases the sensitivity as well as giving flexibility to plan the multiple probing.

Then you also have 1 step probing in 1 step probing the primary antibody contains enzyme or fluorescent label for detection 1 step probing is not very common into the western blotting. Once you are done with the probing you can actually be able to develop the probe blot. So, step 6 is the blot development. So, there are multiple way to develop the blot and detect the protein present onto the membrane. So, you are going to have the different types of reagents you can actually have the chromogenic reagents or you can also have the luminescence reagents.

So, in the chromogenic reagents you can have the depending upon the enzymes for example, HRP you can actually be able to use the 4 chloro naphthol or DAB or TMB and the all of these reagents are actually going to be give you the colors. For example, 4 chloro naphthol it is the oxidized product is going to form the purple color precipitate. DAB and nickel chloride is going to form the brown color precipitate and TMB it is actually going to form the dark purple stain. Similarly if it is the enzyme is alkaline phosphatase you but you then you can use the BCIP and ABT and BCIP hydrolysis product in indigo precipitate after oxidation with NBT. If it is luminescent substrates then you can for the HRP based system you can use the luminol and hydrogen peroxide and oxidized luminol gives the blue light whereas, for the alkaline phosphatase you can

use the substituted 1, 2 dioxane phosphate and it is going to dephosphorylate substrate gives the give off the light.

So you can actually have the do 2 different types of detections you can have the colorimetric detections or you can have the chemiluminescent detections. So you wash the membrane with TBS to remove the detergents place the membrane onto the colorimetric reagent and the blot develop into 10 to 30 minutes stop the reaction by washing into the distill buffer air dry the membrane and photographs for permanent records. Then for the chemiluminescent detections the detection the different chemiluminescent reagents are given in the table transfer the membrane onto the chemiluminescent reagents soak the membrane for 30 seconds to 5 minutes strain of the reagent and wrap the membrane into the plastic wrap place it into a film cassette and expose the membrane to film to few seconds to the several hours. Then you can also have the fluorescent detection so secondary antibody labeled with the fluorescent dye and captured into the scanner can be also done. So these are the some of the method the steps what you are supposed to do when you are doing the western blotting.

We are supposed to first run the SDS page followed by the transfer followed by the treatment with primary antibody followed by the treatment with the secondary antibody and then you are going to do a washing step and then you are going to do the development. So these are the some of the very very crucial steps and you also require a lot of precautions while you are performing these steps. So I would like to take you to my laboratory where the students are going to perform all these experiments all these steps and they are also going to discuss about the different types of procedures. So they are actually going to develop the blot with the help of the chemiluminescent reagents so that you will be familiar with the whole process and so on. In this video we will demonstrate you how to do a western blot and how to analyze the result using ACL electro chemiluminescent substrate.

So here what we will do we have to run gel first then we 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 a SDS page and how to run protein samples. In this video particularly we are interested in factors associated with the western blotting. For doing western blot we need membrane and transfer buffer and the transfer medium. This one is we use to transfer this gel to membrane.

So here membrane can be two kind one is nitrocellulose which has low protein binding efficiency and hydro-pulching nature. Another membrane is PVDF this is hydrophobic membrane and higher protein binding capacity. So the processing for western blot is different for nitrocellulose and PVDF. So if you are using PVDF membrane we have to

take we have to cut the part whether if you have readymade pre-cut blots then no need. If you have if you are taking from a bundle you have to cut precisely how many wells 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 used in previous step further steps also like antibody incubation. So here for if you want to use PVDF membrane you have to charge with the methanol. So since the PVDF is hydrophobic membrane you cannot directly transfer the transfer in the aqueous medium. 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. 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.

That is why we need to keep the buffer always in chilled condition and let us start the procedure. So we need a pre-run gel. So we already finished the gel running. In addition to that we also need sparges which will give cushion to the gel so that gel may not be destroyed during the transfer. So this is the cassette we will use for the transfer.

So this is negative side of cassette and this is the positive side. So we are going to keep gel on negative side and positive side the blood membrane. So when we apply voltage from this side to this side the negative protein it will be transferred it will be moved to positive side and it will be captured in the membrane. So first for doing that these sponges we need to keep and also this maybe give 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 inserted the blotting sheet then you have to keep your gel. So here we have to remember that gel after finishing the SDS phase running you have to keep in transfer buffer so that it will give identical condition for equilibration kind of thing during transfer so that protein transfer may be easy. So this is the gel and keeping on the negative side. So after that we have to overlay with the membrane.

Next we have to remove any air bubbles if present. We have to overlay with another blotting sheet 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 like this.

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. First you do not tighten it initially you just keep and after that press the positive side of the cassette then tighten the screws. So all these things should be done in the transfer buffer only unless specified. So this is the chilido transfer buffer.

Now we are going to do transfer. Pour sufficient buffer. Keep this ice pack also if the chilling is not sufficient then there may be heat generation. So in order to prevent that we will use this ice pack. So this will keep the buffer cool till the transfer end of the transfer. So once that is over you directly take out the cassette and keep.

If there is a buffer insufficiency you can add on top of that. Make sure that the cassette completely submerged so that the transfer will be proper and there is no air bubbles. 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 on transfer to transfer it varies.

Generally in our lab we will give at least 2 hours of transfer at 120 volts which is sufficient to transfer even low molecular weight proteins also. But from instrument to instrument also it varies. You needed to optimize before doing transfer. After 2 hours we have to remove the blood and incubate with the blocking buffer.

So I am going to stop here. Remove the cassette. Keep the net ray. Remove the screws properly. Gently remove the sponges. Take out the blood and keep it in blocking buffer. In this condition we have to keep if you are keeping it room temperature it is for 2 hours at least.

If you are keeping in 4 degree Celsius you can keep overnight. The blocking buffer contains skim milk or BSA along with the TUNT. The next one is the Western Blot transfer. It all depends 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 high background during development of the blot. So always use gloves. Apart from that while handling the instrument make sure that there may be possibility of electricity, the shock may happen sometime. So we have to that time also we need to use gloves. And after finishing the transfer you have to clean all the apparatus properly and dry it for the next 10 years.

After the blocking of the membrane we have to remove the membrane and incubate with

the primary antibody without washing. The main purpose of the blocking is that it will occupy non-specific sites other than the respective protein so that when the antibody comes it will bind to that specific protein and gives no noise. 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 TBST buffer or PBST buffer and again treat with incubate with the suitable secondary antibody. For 5 hours at 4 degree Celsius or 2 hours at 3 hours at room temperature. After that we need to wash properly at least 3 times then we will develop the blot with the electrochemiluminescent substrate.

In earlier western blot how to do western blot we showed how to transfer the proteins to membrane. So we incubated with the primary antibody following secondary antibody and wash with the. Now here we show how to develop a blot. For developing a blot we need chemiluminescent substrate. In most of the commercially available kits Luminal is the one of the substrate we used for this purpose.

So Luminal in presence of hydrogen peroxide and peroxidase agent which present in the secondary antibody. This peroxidase conjugated secondary antibody. This peroxidase converts Luminal to excitated Luminal by deprotonating and oxidizing it. So this excitated product gradually leaves the energy by releasing luminescent products that light will be detected using this instrument. So these are the commercially available chemiluminescent substrate solutions.

So it is available from wide range of companies. We have to mix 1 is to 1 ratio. So we have to take out the blot, drain the buffer whatever present properly. So after that we keep blot in between plastic paper foils. Then we will take chemiluminescent substrate.

So after that we have to slowly press and remove air bubbles. This is the tray we used for developing the blot. So we have to open the system, properly align the tray and then shift blot to the table. Once it is over, we have to just close. Here we have to select application. We want blots that is chemiluminescent and what exposure want? You have two options, manual auto.

In auto two options are there, optimal auto exposure, rapid auto exposure. We will choose optimal auto exposure. So you can enlarge the blot also.

Once it is over, you just say. So this is the developed blot. So as we can see the bands pattern. So this is how we develop western blot through electro chemiluminescent substrate. So in this video we have demonstrated how to transfer 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 laying principle behind the developing the blot. So I

hope this will help you to understand the basic outline mechanism of how western blot works. So what these students have discussed? They have discussed about all these steps.

They have discussed about how to transfer the proteins onto the membrane, how to perform the primary antibodies, how to perform the washing so that and what are the different precautions you should take while you are performing the western blotting and so on. So these are the different steps what you are supposed to perform. Now so what we have discussed so far? We have discussed about the southern blotting. We have discussed about the northern blotting.

We discussed about the western blotting. The purpose of these technique is very clear right for the southern blotting it is actually going to detect the DNA. So what if you see if you try to correlate how you are actually going to answer the different questions with the help of the blotting techniques. So the southern blotting is actually going to tell you that whether a particular gene is present or not right. So it is actually going to tell you about the gene is present or not.

But whether this gene is actually expressing or not right. So whether this gene is performing the transcription or not that information you are going to get with the help of the northern blotting. So northern blotting is actually going to tell you whether this gene is forming the RNA or not right. Because there are genes which are also present but they may not be expressive genes they may be only present into the genome simply by no other reason or they may be expressing but in that condition in which they may not be high skipping genes they may be required for a specific purpose so they may not be expressing. So northern blotting is actually going to tell you whether the RNA is forming or not. So it is actually going to tell you about the transcriptional activity of that particular gene.

So it is actually going to tell you the transcriptional activity of the gene. Then whether the gene is forming the protein or not right. Because there are proteins there are genes which are not expressing there are genes which are expressing but at the only at the RNA level they are not translating into the protein. So that information you will get by the western blotting. So what you see is so western blotting is going to tell you whether there is a translational activity of that translational activity of gene is present or not. So what you see that with the help of these three individual blotting techniques you can be able to get the complete information about a particular gene.

You can actually be able to know how many number time this gene is appeared into the genome right whether the gene is present in genome or not number one number two what

is the location of this particular gene and how many times this gene is present into the genome. Then second question comes that whether this gene is forming the RNA or not whether gene is transcriptionally active or not and that information you will get from the northern blotting. And then whether the RNA what it is forming is expressing and being utilized by the protein synthesis machinery to give you the protein that also can be answered with the help of the western blotting. So all these western all these blotting techniques are complementary to each other they are actually going to give you the in-depth knowledge about that particular gene fragments and so on. So with this I would like to conclude my lecture here in our subsequent lecture we are going to discuss some more molecular biology techniques. So till then we are going to conclude our lecture here. Thank you.