Experimental Biochemistry
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Lecture - 52
Immunoassay Techniques

Hello welcome to week 12, this is the last week of these lecture series in this course Experimental Biochemistry. So, today in this week we are going to cover the final technique and this is most probably one of the most important and complicated techniques that we are going to discuss and it is Immunoassay Techniques. And you will see that everything that we have learnt so, far we will nicely come together in the determination of in the use this technique.

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So, again we are going to talk about different types of immunoassay techniques and we will see that there are basically three types. I will talk about the applications of these and also the practical aspects of these methods.

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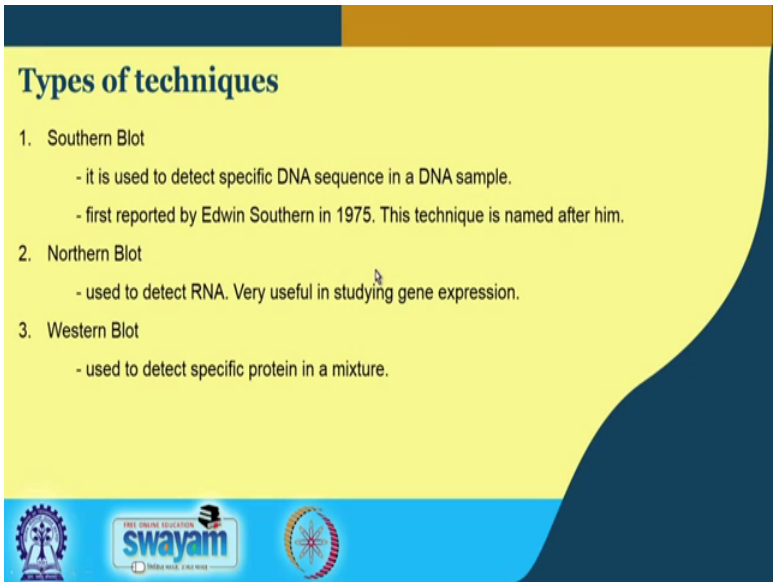

There are three basic types of immunoassay techniques and as you will see their names are southern blot northern blot and western blot. So, southern blot this is a technique that is used to detect specific DNA sequences. So, if you have a sample of DNA, which has all different types of DNA different sequences and you want to know whether a particular DNA sequence is present in it or not then that method of your choice will be southern blotting. And this method was first developed by Edwin Southern and it is named after him.

So, that is why it is called southern blotting later on the other two techniques were developed and people went with sort of a trend to name them as northern blot and western blot. So, what is northern blot? Northern blot is use to detect specific RNA sequences; it is a very useful technique in the study of gene expression. Suppose you have a particular cell type and under a certain condition you want to know whether a particular protein or the mRNA of that protein is expressed under those conditions in that case you will use northern blot to see whether that mRNA is present or not.

The third one is the western blot and this is most probably the most widely used immunoassay technique in current molecular biology labs and this method is used to detect specific protein in a mixture, suppose you have extracted you have a cell I said. So, you it can come from bacterial cell it can come from some memorial cell and you want to see whether a particular protein is present in this cell mixture or not, and in that

case we are going to use western blot. So, you can see that all three major macromolecules are covered by these three different immunoassay techniques. Southern blot is for DNA, northern blot is for RNA and western blot is for proteins and we will look at them in that particular order.

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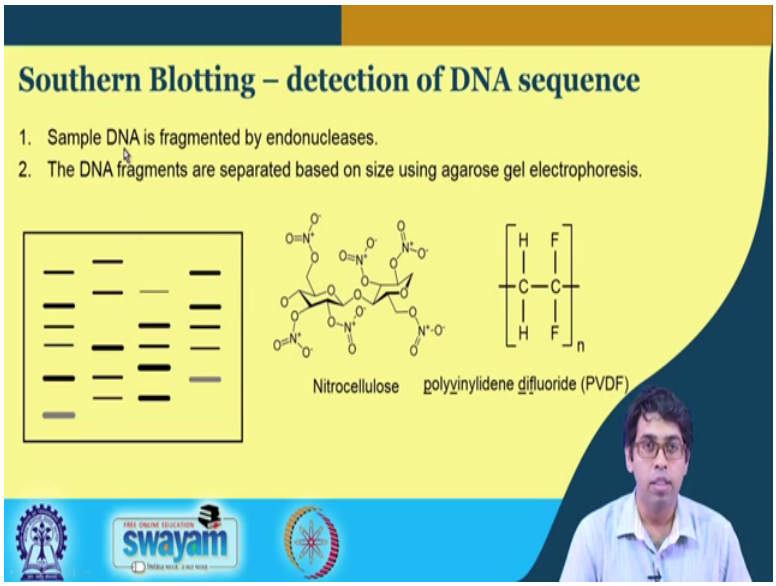

Suppose you have mixture of DNA molecules and or you have a big genomic DNA and you want to know whether a particular sequence is present in this DNA or not. So, the first thing that we do is we fragment the DNA into smaller sizes, and that can be done using different types of endonucleases. So, endonucleases are enzymes that we know which cut at specific sides we talked about that in the recombinant DNA technology lectures and so, endonucleases are used to cleave the DNA into smaller relatively smaller fragments.

But still these are very big fragments. So, we are going to use agarose gel electrophoresis to separate out these fragments. So, this is what we are shown sort of schematically showing here that let us we have DNA from four different sources; and we have used a particular type of endonucleases to cleave them into different fragments and now we separate this fragments in the agarose gel electrophoresis. So, the smaller fragments. So, if this is the positive terminal and this is the negative terminal the smaller fragment will be here and the larger fragments will be up here. And we can see that all these different fragments have separated based on their size.

The next thing, that is done in case of all these blotting techniques. So, this is common for all the blotting techniques that use some sort of electrophoresis to separate your sample in case of DNA we are using agarose gel electrophoresis. Now what we are going to do is, we are going to transfer these molecules into a different membrane so, that we can do further ach um steps on that particular membrane. So, we have to transfer these DNA molecules which have separated out nicely into a different membrane.

Two types of membranes are very popularly used; one is nitrocellulose and the other one is PVDF polyvinyl Polyvinylidene Difluoride. In case of nitrocellulose if you look at the structure you will see that it is a very positively charged membrane and we know that DNA molecules are highly negatively charged. So, you can imagine that it will be easy to transfer DNA molecules from the agarose gel to the nitrocellulose membrane. Similarly in case of PVDF the fluoride ions the fluoride atoms are highly electro negative. So, these carbons are electro positive. So, again you can have a negative charge and delta positive charge interaction between these two and you can transfer the DNA molecules on to the PVDF membrane.

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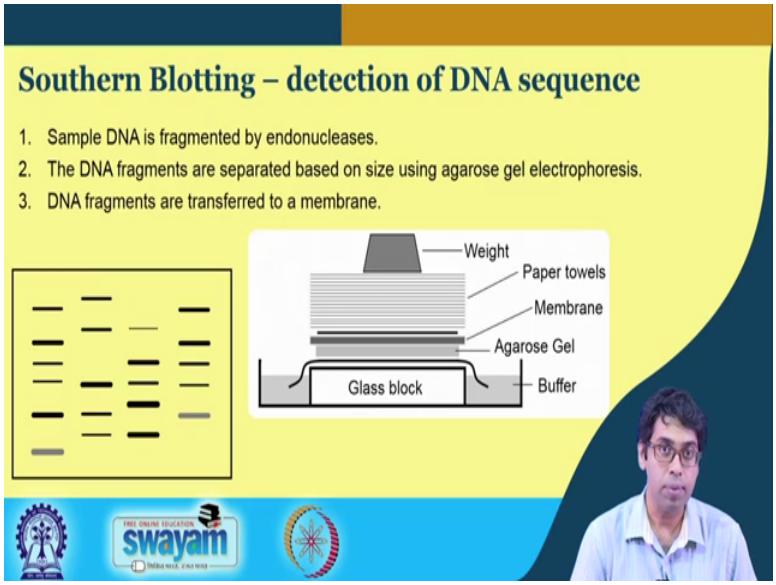

So, the way it is done is just by using this membrane. So, this is a very cruet set up that you can use, where this is a glass block it is just a support on which the agarose gel you can see that the agarose gel is here. So, this agarose gel is kept and this agarose gel is the same gel that we have run.

So, all our DNA samples are separate out into according to their size in this agarose gel and on top of that agarose gel this black one is the membrane. So, let us see it is the nitrocellulose membrane. And then on top of that we will put a stack of paper towels and then put some heavy weight at the top. So, that this whole set up is nicely compressed and with time the DNA molecules will move out from the agarose gel into the membrane.

You can see that this is again a paper which is dipped into this buffer. So, there is capillary action and this capillary cation takes the DNA molecules from the gel to the membrane that is the transfer step. So, in this step what we have done is we have transfer the DNA fragments on to a membrane.

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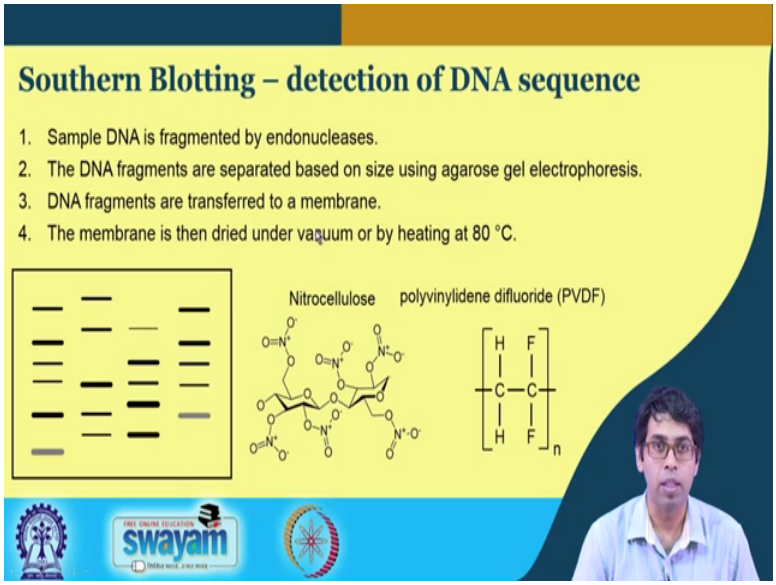

And again just the membranes are negatively charge. So, they can bind to the DNA molecules and once you have this transfer complete you try the membrane under vacuum or by heating it at 80 degree centigrade and we have to do that for several hours so, that we get a really nice dry membrane.

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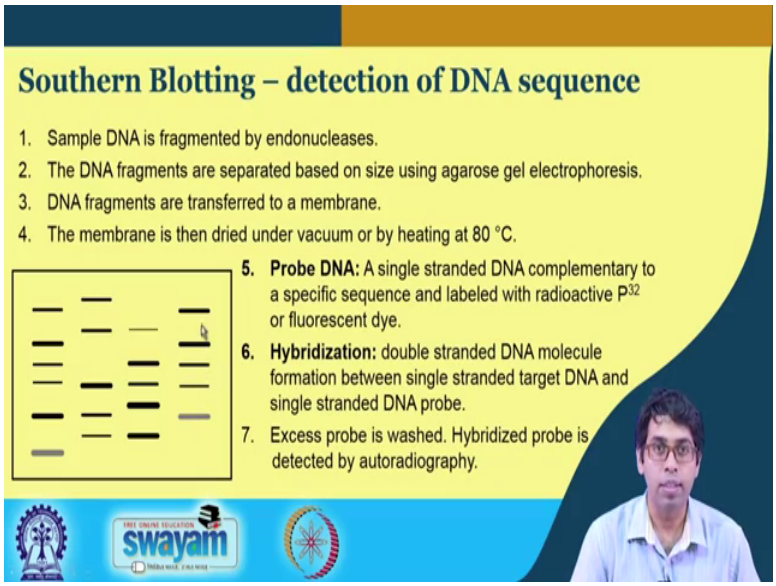

Once you have the your DNA molecule thus sample transfer to the membrane, then there are several things that we do the next step is use of a probe DNA. So, this probe DNA is a single stranded DNA which is complementary to the specific sequence which we are trying to probe.

So, you want to know whether a particular sequence is present in our sample or not. So, what we need to do is we have to synthesize the complimentary strand to that sequence and it is a single stranded DNA and this DNA is also labeled with either a radioactive phosphorous or some fluorescent dye and these are basically for detection. So, that we can see now and this probe DNA is also not very small. So, they are typically in the range of 500 base pair nuclear tides long or they can be even longer than that.

We have our sample DNA on the membrane; we add the probe DNA on to that membrane and allow hybridization. So, it will form a double stranded DNA molecule between the single stranded target DNA which is on the membrane and the single stranded probe DNA which we have added and it also carries the label for detection.

now the membrane is washed excessively so, that extensively so, that any unbound probe DNA is removed only the hybridized DNA stales stuck to the membrane and then we can visualize them using auto radiography if the label is radioactive or by the fluorescence of the dye. So, at that point it would look something like this. So, here we have all the DNA molecules.

Let us say this is the membrane. So, we have transferred all the DNA molecules, but only those specific DNA molecules which carry which are complementary to this probe DNA will bind to the probe DNA or will hybridize with the probe DNA and if you look at look at that, then we will see only those two bands and from that we can tell that these are the DNA molecules that are present. So, these are the molecules that we wanted to see these are the sequences that we wanted to see and their present only under these condition, which is the second and the third condition.

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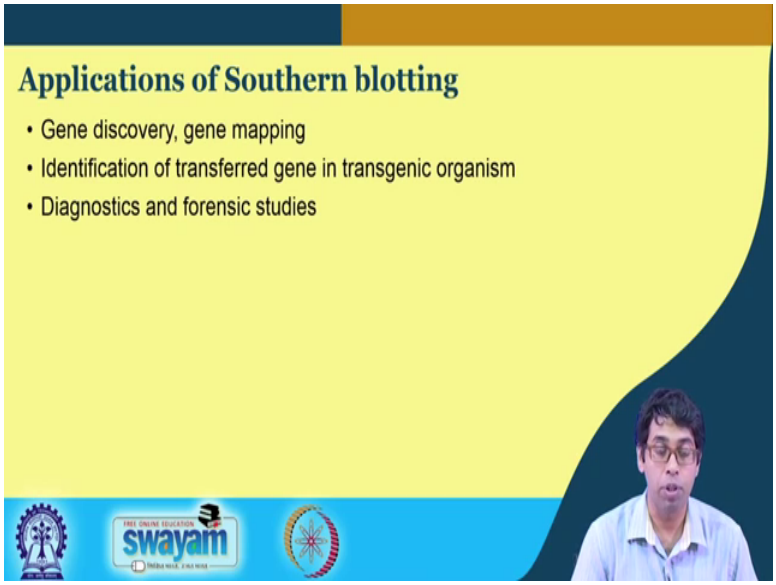

So, southern blotting has several applications. So, it is used for extensively used for gene discovery and gene mapping. So, for example, if we express suspect that a particular gene should be present and we have some idea about the sequence we can try to find whether that gene is present in cell type or in a cell under certain conditions things like that. It is also used to identify transferred gene in transgenic organism.

So, suppose we have created a transgenic organism and where we have added a new gene which is not present in that organism under natural conditions. Now, to test whether our experiment has worked or not; we will use southern blotting techniques and it is also extensively used for diagnostic and forensic studies.

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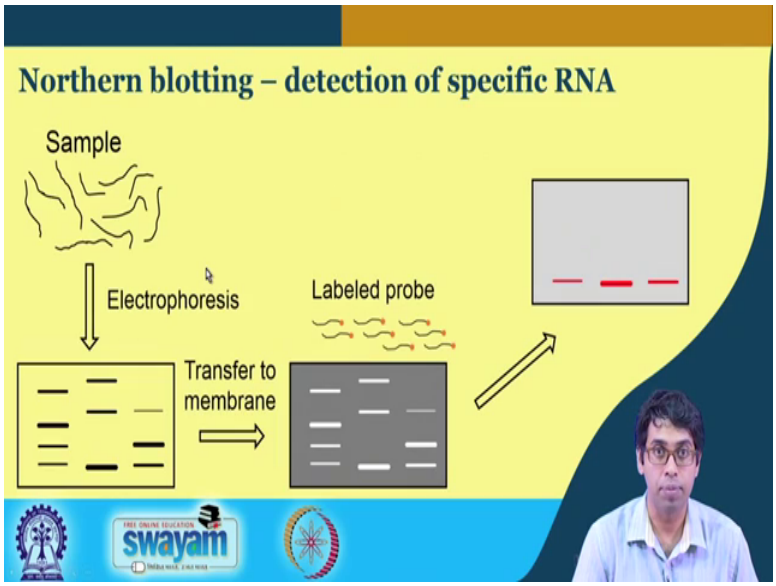

Northern blotting which is the RNA counter part of the same method is used for the detection of specific RNA. So, again here we can see the flow chart and it is exactly the same as the southern blot in the only difference is that the sample here is now single stranded RNA. So, your sample will have a mixture of RNA and again we will separate it out using gel electrophoresis. So, we can fragment the RNA and then we can separate it out using gel electrophoresis, and then we will transfer it to a membrane let say a nitrocellulose membrane just like before. In this case we have single stranded RNA stuck on to the membrane

Now, we will use similar labeled probes, again this probes are have a very specific sequence and we are we want to find whether these sequence or the sequence that is complimentary to this probe sequence is present in this messenger RNA or present in this sample or not. So, then we will get hybridization and finally, we can detect and see that yes we do see our target sequence in this samples. So, now, this is the schematic diagram of northern blotting and it is also the same for southern blotting.

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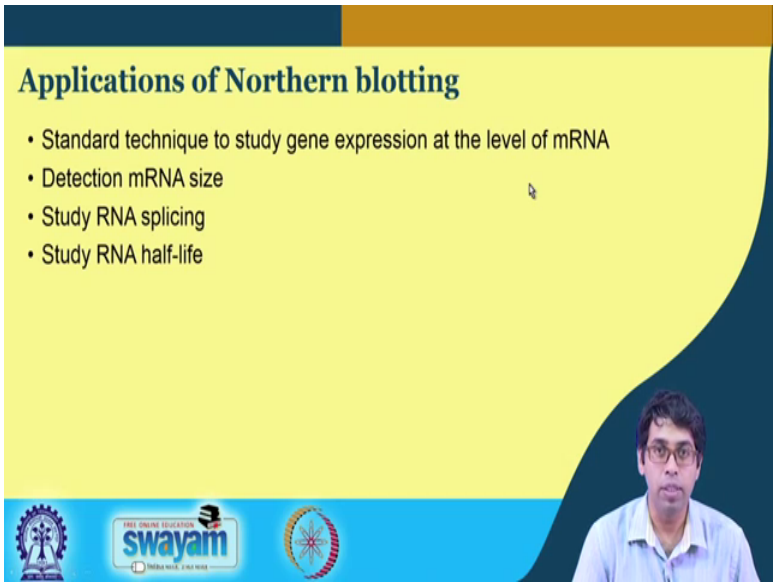

So, northern blotting which is for detection of RNA sequence has again wide spread application. One of the most important application of northern blotting is to study the expression of a particular gene at the mRNA level. So, if you want to see whether this particular gene is expressed under a certain condition or not we can use northern blotting and we will see yes the mRNA of that gene is produced under this condition. You can also detect the size of a particular mRNA, we can study RNA splicing and we can also study half life of a particular RNA it can be mRNA it can be some other types of RNA.

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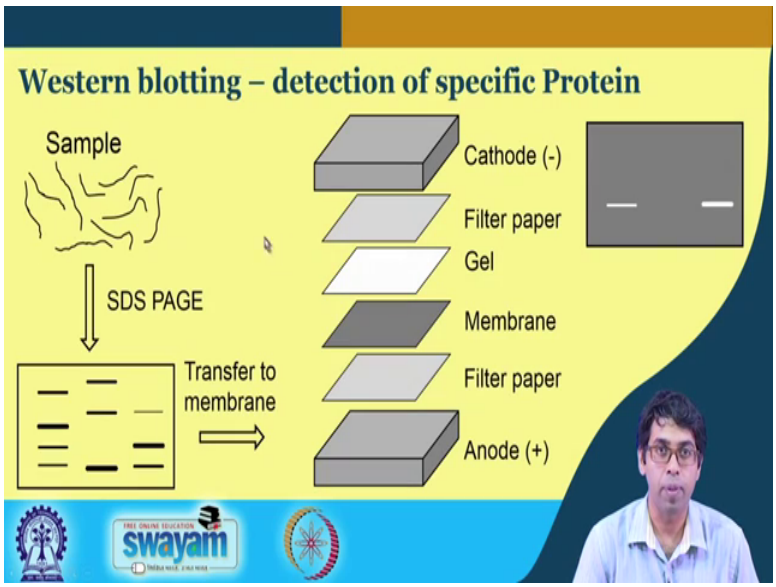

The last one of this three different immunoassay techniques is western blotting. So, just like DNA and RNA western blotting is used to detect a very specific protein. So, again in this case we have a mixture of protein our sample will consist of a mixture of protein and we have to separate out these mixture on to a gel. So, in this case we are not going to use agarose gel we will use polyacrylamide gel electrophoresis. So, we have extensively talked about these polyacrylamide gel electrophoresis in our previous lectures. And since proteins can have different p i so we need to make sure that all proteins migrate in the same manner. So, we are going to use the denaturing conditions. So, this is SDS-PAGE gel electrophoresis.

So, we are going to use SDS-PAGE to separate out our samples. So, this is the very standard simple gel electrophoresis that is routinely used in any molecule biology lab. So, this is our gel SDS-PAGE gel, now we have to transfer these protein molecules on to the membrane. So, in this case to different methods are used you can use simple capillary action to transfer the proteins from the gel to the membrane. So, this is a different set up where what we have used is this is a gel. So, this is our polyacrylamide gel and this is the membrane on two sides we have kept filter paper and then they are compressed using two electrodes.

So, this is the anode which is positively charged and this is the cathode which is negatively charged. Now, if you connect them to a plug the negatively charged. So, this is all the protein molecules are remember they are quoted by SDS. So, they are all negatively charged. So, the negatively charged protein molecules which are on the gel will try to migrate towards the anode which is the positive electrode and hence they will get transfer from the gel to the membrane.

So, we can use this electric potential to transfer these protein molecules from the gel on to the membrane. And once we have done that we are going to again detect these proteins using some way to see whether our specific protein is present in that membrane or not. So, in this case we see that yes the protein that we want to see is present only in the first and the third lane. So, in the first and the third lane and it is missing in the second lane. So, how do we detect a specific protein in this case?

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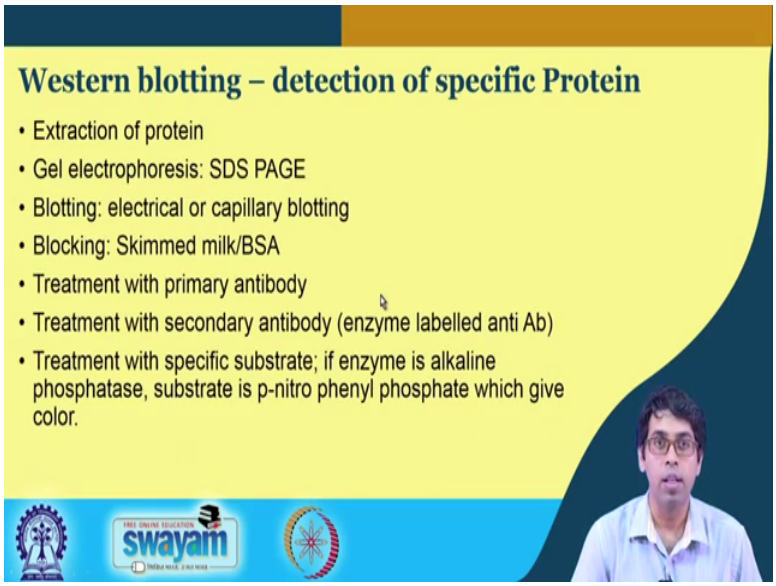

So, this is just an overview of the steps different steps of western blotting and remember that in case of western blotting it is little bit more difficult compare to DNA and RNA because if we want to look at a specific sequence of DNA or RNA we can easily synthesize its complementary sequence, but if you want to detect a specific protein then there is no such complementary protein sequence that can be synthesized. So, here we have to use a different method to detect very specifically whether our protein is present or not.

So, again the first step is extraction of the protein. So, again if it is bacterial cell or if it is some memorial cell, you lies the cells you have all the proteins in the cell I said you remove everything else you have only the protein solution run it on to an SDS-PAGE, separate out the proteins into different lanes we saw that we can use blotting.

So, either capillary blotting or electrical blotting to transfer the proteins from the gel on to the membrane. Now, what we do is we put this membrane into a solution of a standard protein. So, we can use Bovine Serum Albumin BSA or we can use simply skimmed milk. So, skimmed milk does not have fat it is mostly the casein protein that is present in the milk. So, we are basically using another non protein to as a blocking agent.

I will discuss why we need to do a blocking in few minutes and then we treat this membrane with a primary antibody. So, this is the key incase of western blotting. This is just like the probe that we have seen in case of southern blotting and northern blotting.

So, this primary antibody is designed to very specifically bind our protein of interest. So, it will not bind any other protein, it will bind only our protein of interest. So, once the primary antibody is bound we then have to visualize it. So, in case of the southern and northern blotting we can visualize the bound probe using attached be 32 label or attached fluorescence level.

In most cases it is very difficult to generate a primary antibody to a protein and also have it attached with a probe. So, what is done is a secondary antibody is used, which has the probe attach to it in most cases we have an enzyme linked to the secondary antibody and this secondary antibody will go and bind the primary antibody. So, our protein is bound by the primary antibody and the primary antibody is bound by the secondary antibody, and then we can use this enzyme reaction to visualize the particular protein band. So, in this case one example is that the enzyme is alkaline phosphates and we add a substrate so, that when this reaction is carried out we get a color.

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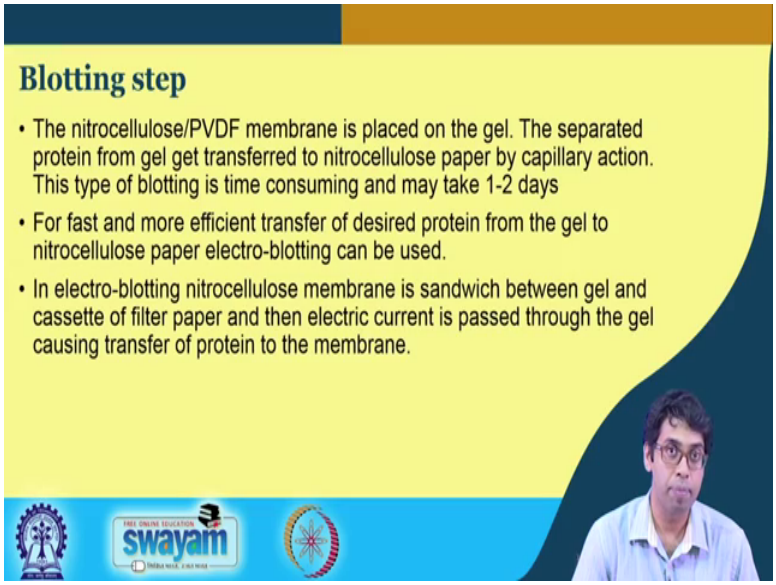

So, I will just cover the important steps which are unique to western blotting. In the blotting step as before either nitrocellulose or PVDF membranes are used. So, they are placed on the gel and the protein is transferred from the gel to the nitrocellulose. So, we can do that either by a capillary action and it will take several days for completion. To speed up the process what we do is we apply a electric potential. So, that the negatively

charge proteins can quickly move on to the gel which is towards move on to the membrane, which is towards the positive electrode.

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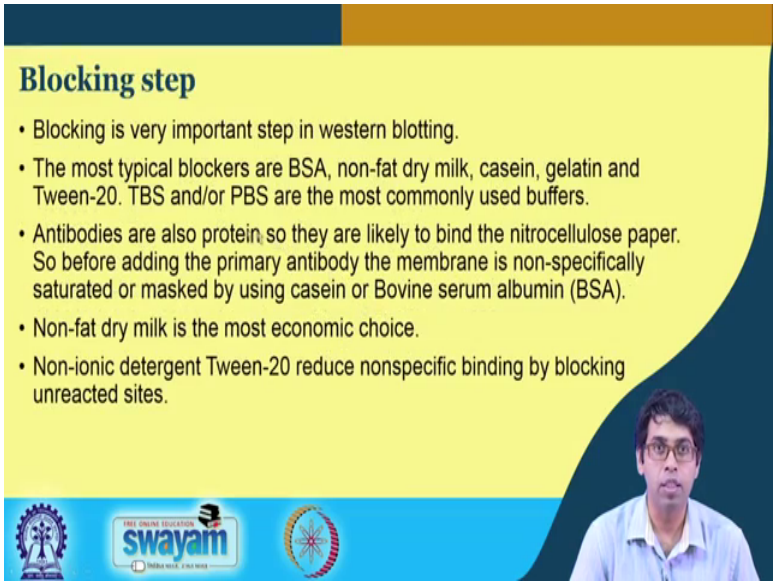

Once we have the transfer step completed then another step is used which is called the blocking. So, blocking is a very important step in western blotting and typically we use BSA or Bovine Serum Albumin or non-fat dye, non-fat dye has casein as the major component and then different buffers are used. So, what is blocking step does is it reduces non specific interaction.

Now it might so, happen that the antibody that we are using may have weak binding for some other protein sequences. This blocking step will reduce all such weak interactions because we have saturated everything using this bovine serum albumin or non fat dry protein dry milk. So, it reduces the non specific interaction and since our antibody has a very tight affinity for our protein, only that interaction will happen and every an all other interactions will be blocked by this blocking agent which is nothing, but another protein.

Non-fat dry milk is the most economic choice and it is widely used by many labs. Some times in order to optimize these steps non-ionic detergents such has Tween -20 are also used to reduce non specific binding by these blocking agents.

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So, now once we have our membrane ready a primary antibody is added. So, primary antibody is specific to the target protein and it will bind only the target protein. The primary antibody binds to the target protein on the membrane, but then we cannot detect. So, to detect another antibody is used which is called the secondary antibody. The membrane is washed thoroughly to remove any unbound primary antibody and is exposed to secondary antibody which is conjugated to a specific enzyme.

The secondary antibody binds to the primary antibody and it carries this specific enzyme. Now the choice of the secondary antibody depends on how the primary antibody was prepared. So, if the primary antibody was raised from a particular animal let say mouse, then the secondary antibody will be a anti mouse antibody ok. If it was raised in rabbit then the secondary antibody will be an anti rabbit antibody because then only this secondary antibody will bind the primary antibody.

And once we have so, we have our protein on the membrane, the primary antibody bound to the protein, the secondary antibody bound to the primary antibody, and then the secondary antibody carries this enzyme alkaline phosphates or horseradish peroxidase these are the two most common enzymes that are used, and then we can simply add our substrate and get a get a color which will tell us where exactly this band is. So, we will know where exactly our specific protein is. So, it will answer two questions one is whether it is present or not and if it is present in which lane it is present.

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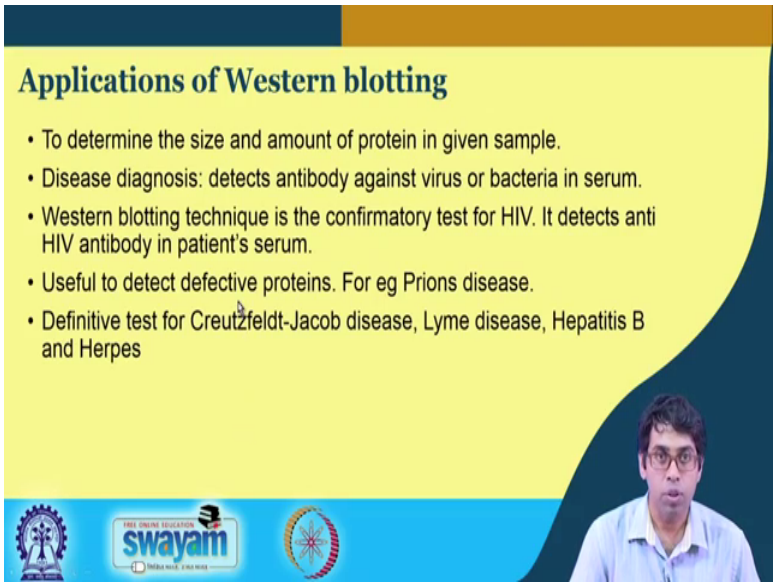

So, there are various applications of a western blotting it can be used to determine the size an amount of protein in a given sample. So, we can answer yes and no type of questions whether a particular protein is present in a given sample or not and if it is present, what is it size because we have run it on to an SDS-PAGE gel. So, we can easily compare the band with respect to the molecular weight marker and we can also estimate the amount of protein that is present in the sample.

It can be used to diagnose several diseases for example, we can use these we can use antibody is to detect whether a particular protein is present or not and that protein might come from a bacteria or a virus. It can be also use to confirm diseases like HIV because anti and HIV antibodies are used to used against patients serum to detect the antigens. So, you can see that all this different applications of western blotting makes it a very unique technique and it is most probably the most widely used immunoassay technique among all three.

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So, again you will find these two references and you will find details about these experiments in these two references.

Thank you.