Experimental Biotechnology
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Lecture – 43
Blotting Techniques (Part-1)

Hello everyone, this is Dr.Vishal Trivedi from Department of Bioscience and Bioengineering IIT, Guwahati and today we are going to discuss about the blotting techniques. So, if you remember when we were discussing about de-electrophoresis we have discussed about the western blotting. So, there are multiple types of blotting techniques, what is been used to detect the different individual molecules. So, let us discuss about those.

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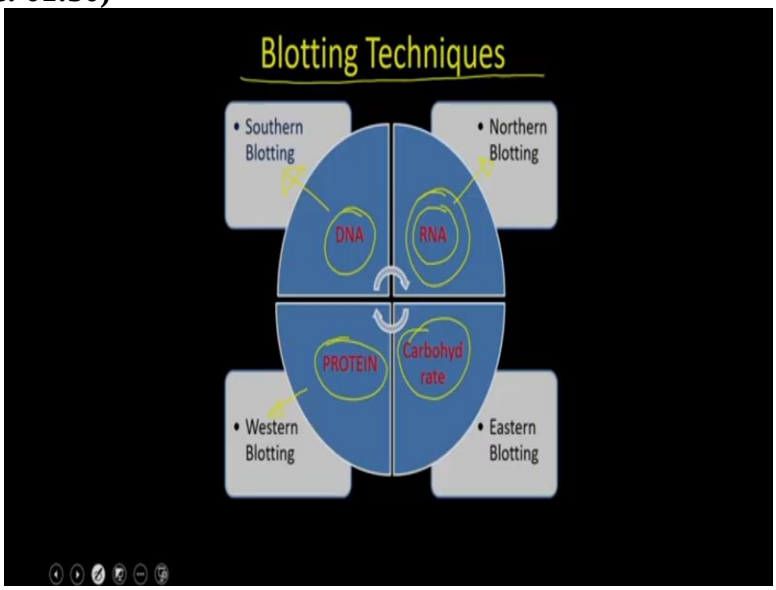

So, as the name suggests the blotting technique means the technique which actually utilizes the blotting of the biomolecules onto the membrane and then you can be able to utilize the different types of analytical reagents to detect these biomolecules. So, depending on the biomolecules, the blotting techniques could be of multiple types. So, if it is a DNA as a biomolecule then the blotting technique is called as the southern blotting, if it is RNA then it is called as the northern blotting.

And if it is a protein then the technique is called as the western blotting, whereas, in some cases the people are also using the term as called as the eastern blotting and that eastern blotting is a

technique which people are normally been referring when you are actually blotting a glycoprotein or the lipoprotein onto the membrane and then you are trying to detect these carbohydrate moieties or the lipid moieties on to the proteins. So, as the name suggests, as you can see that the all these blotting techniques are being directed against one or another kind of biomolecules.

So, in today's lecture, we are going to start our discussion about the RNA as a biomolecule and how you can be able to detect the RNA into the technique which is called as the northern blotting.

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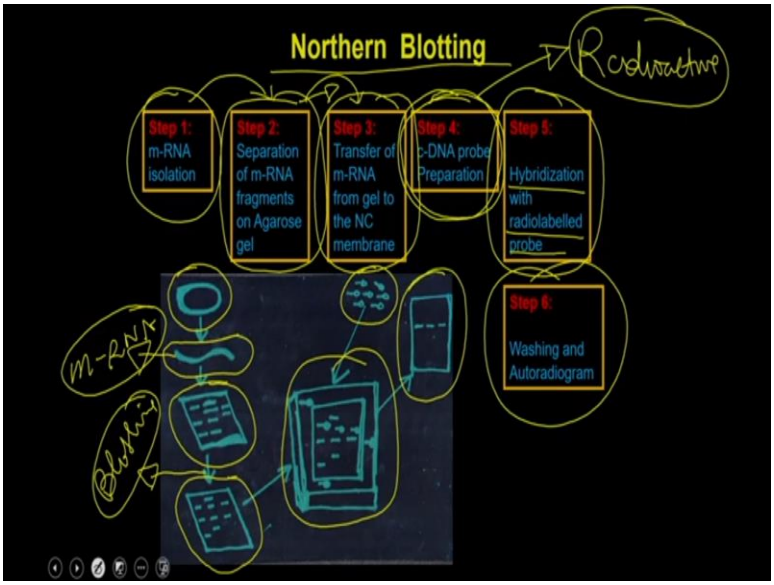

So, the multiple steps during the RNA or during the northern blotting is that the first step you have to do the RNA isolation. So, what you have to do is you have to take the cells, you have to break open the cells and then you have to isolate the total pool of the RNA, when we talk about the RNA we are talking about the messenger RNA because these are the RNA which are being useful to detect the expression level of the particular type of gene.

And once you have isolated the RNA, then you can get into the second step, where you are going to separate these RNA molecules on to a agarose gel and this agarose gel separation and once you are done with that, then you will enter into the third stage you are going to transfer the messenger RNA from to the agarose gel onto our nitrocellulose membrane sheets and that is

being called this step is called as the blotting stage because where the RNA is going to be blotted onto the nitrocellulose membranes.

And then after that, you have to prepare the probe. So, in in most of the northern blotting techniques, you have the flexibility of using RNA probe or you can be able to use a DNA probe. So, if you synthesize a DNA probe, you can be able to use the gene to synthesize the c-DNA and that can be used as a probe. So, you have to ideally you have to synthesize a c-DNA probe, and then you have to also do radioactive labeling of this particular probe.

So that you can be able to detect the binding of this probe onto the RNA what is present onto the membrane and then step 5 you are going to do the hybridization with the radiolabelled probes and in this step itself you are going to do a lot of treatments. So that the probe what you have prepared is going to bind to the main RNA what is present onto the membrane. After that you are in the last step, in the step 6, you are going to do the washing as well as you are going to collect the autoradiogram of that particular gel.

And then you are going to see the pattern wherever the probe is going to bind. So, you have a radioactive probe when you incubate your nitrocellulose membrane with the radioactive probe, the NATO radioactive probe is going to bind to these nitrocellulose membrane and that is how it is actually going to give you the radioactive signal and that you can be able to detect on to the X-ray films in the in the process which is called as the autoradiogram. So, let us start discussing about each and every step and how you can be able to complete the northern blotting and to detect the RNA what is present within the cell.

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So, in the material, what you required if you want to start the northern blotting you require SSC buffers, so SSC buffer is the composition is the 5 millimolar Sodium Citrate and 150 millimolar NaCl, then you require a 5 x buffers which is Tris buffers containing the sodium pyrophosphate 1% PVP, BSA, Ficoll and the SDS. Apart from that you also require the hybridization buffers and that habitation buffer is the Formamide 5 x buffers, water and the NaCl and also you require the Salmon Sperm DNA.

Salmon Sperm DNA is actually being used for the blocking the membrane so it is a blocking reagent, if you remember when we were discussing about the western blotting the blocking of the membrane is very important for that you should not allow the binding of the probe to the nonspecific sites what is present onto the nitrocellulose membrane. So, in the western blotting, we were using the BSA.

Because they are we were more concerned about the binding of the antibodies to the particular type of proteins what is present onto the nitrocellulose. But here since we are more concerned about the binding of your probe to a nonspecific sites present onto the nitrocellulose membrane that is why you are actually going to use the whale sperm DNA as a blocking agent for that all the sites what is present onto the nitrocellulose membrane is going to be blocked.

Then you require the Whatman filter paper the 3 mm thick Whatman filter paper that you are going to use in during the blotting process. And then you are required the labeled RNA or DNA probes then you require the UV lamps shaker water bath you require for detecting the transfer of the messenger RNA onto the nitrocellulose membrane or to do all sorts of incubations and other kinds of processes. And then you also require these instruments for collecting the autoradiograms.

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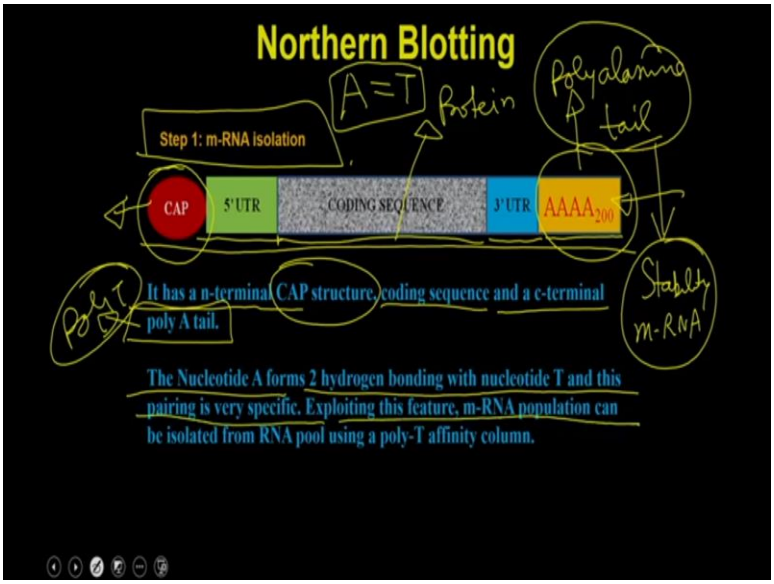

So, in the step 1, you have to do the messenger RNA isolations. So, before we get discussing about the messenger RNA isolation, let us discuss about the structure of the messenger RNA and how the messenger is structured it felt is providing you have flexibility to purify the total messenger RNA present in the cell under specific conditions. So, in a typical messenger RNA, what you have is you have the multiple regions.

So, you have a 5 prime cap, this cap is required to bind the different types of factors and then you have the 5 prime UTR regions apart after just 5 prime UTR region, you have the coding region. So, this is a region which actually codes for a particular type of protein. And then after that, you have the 3 prime UTR region and after that, you have a poly-A tail. So, this is the most important region as far as the purification of the messenger RNA is concerned.

So, this is a poly-A tail, where you can have the poly-A of the multiple lengths like if you can be poly-A of 50 As or you can have the 100 or 200 and all that and depending on the length of this polyalanine tail, the it actually describes the stability of that particular messenger RNA. So is actually this says that what how much does this messenger RNA going to be stable within the cytosol because if you remember that the cytosol is full of RNAs.

So as soon as the messenger RNA is being synthesized, and it is being presented into the cytosol, the RNAs are started chewing this messenger RNA from the 3 prime ends. So because The RNA should not reach to the coding sequence, the RNA is been modified and a poly-A tail is being added. So, that poly-A tail length is going to be used by the RNAs to the messenger RNA.

And the time it the messenger RNAs is going to take the chew the messenger RNA that is the time in until which this particular messenger RNA is going to be functionally active for, you know, for synthesizing the proteins and that is how it actually decides what could be the age of this messenger RNA within the cytosol. So, as I said, you know, the messenger RNA has the n-terminal CAP structure. So, this is a 5 prime cap then you have the coding sequence and a c-terminal poly-A tail.

So, poly-A tail means you have a string of the As which can vary from the 50 to 200 and the length of this tail or the number of nucleotide present in this poly A tail is going to decide what could be deep life of this polymer messenger RNA within the cytosol. So, if you want to purify this what you can do is you can easily take the poly-T DNA or the poly-T nucleotides, because you know that the A is always making the 2 base pair with T and this interaction or this complimentary is very specific A is always binds to T.

And so, if you take the poly-T beads like if you take a bead which actually has the poly-T nucleotides attached that is actually going to bind all the messenger RNA where you have the poly-A tail and most of the messenger RNAs are going to have the poly-A tail except the messenger RNAs which are been degraded or which are not been present, but those messenger RNAs are of no use as far as the northern blotting is concerned.

Because the northern blotting is always been used for only to detect the expression of the particular type of RNA species or the level of the RNA of that particular species present in the particular cytosol. So, nucleotide A forms a 2 hydrogen bonding with the nucleotide T and this pairing is very specific exploiting this feature messenger RNA population can be exploited from the RNA pool using a poly-T affinity column.

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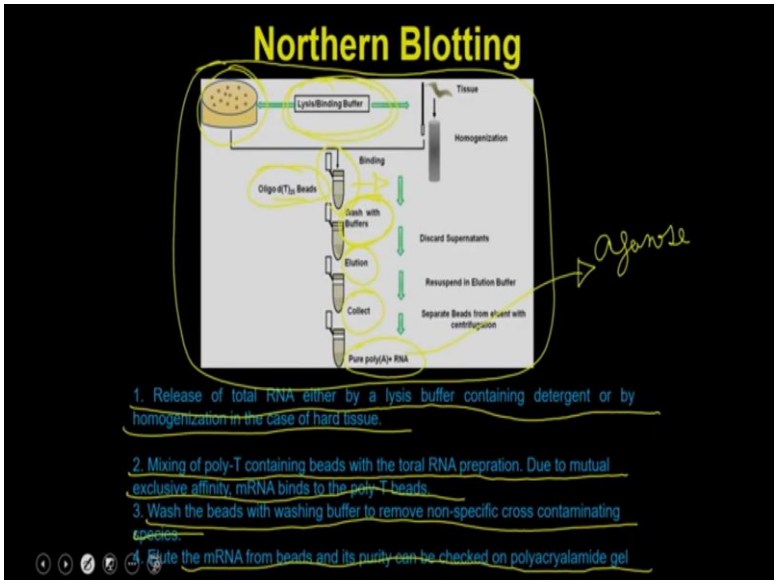

So, this is the complete scheme of how you can be able to utilize a poly-T affinity column to purify the messenger RNA, you have the multiple steps in the first step you are going to take the cells and then you are actually going to incubate the cells in a lysis or buffer and that is actually going to give you the cell lysate. So, the release of the total RNA either by a lysis buffer containing detergent or by homogenization in the case of hard tissue.

So, the lysis buffer you can use in own those conditions where the cell is a very delicate or it is an individual cell. But if you are working with a tissue for example, if you are working with a brain or liver or other kinds of tissues, then in those cases you might have to use some homogenizer or some homogenization techniques. So, that does the tissue is going to be broken down into the individual cells and then you can be able to use the lysis buffer to give you the cell lysate.

And that cell lysate is going to have the mixture of different types of proteins and the RNA actually, so then once you have got the cell lysate what you are going to do is you are going to incubate the cell lysate with oligo d T 25 Beads. So, the mixing of the politic containing beats with total RNA preparations due to the mutual exclusive affinity the messenger RNA binds to the poly-T beads.

So, in this step what will happen is the messenger RNA what is having a poly-A tail is going to bind to all those beads which actually contains the poly-T tail and because of this mutual interactions, the beads are going to bind all the messenger RNA and then you are going to do a washing with the buffers. So, you wash the beads with the washing buffer to remove the nonspecific cross contaminating species.

And after that, you elute the messenger RNA from the bead and you can check the purity of that particular messenger RNA onto the agarose to check whether there will be enough purity or not. So after you watch the beads, you can be able to do the illusions and once you done the illusion you can be able to collect the messenger RNA and then you are going to have a pool of messenger RNA which actually contains a poly-A tail.

And that can be detected on to agarose gel to check the purity of the sample as well as what is the quality of the messenger RNA. So, the quality testing of the messenger RNA what do you have eluted or what you have purified using the poly-T tail polity in beads can be done on the denaturating agarose gels. So, if you remember when we were discussing about the horizontal gel electrophoresis we discuss about how you can be able to perform the formamide gels.

And how you can be able to utilize that gel to run the messenger RNAs and how that is going to give you the individual messenger RNA. So, one of the limiting factor when you are trying to resolve the messenger RNAs onto a normal agarose is, that the messenger RNAs are actually having the secondary structures and these secondary structures are going to allow the messenger RNA to form a compact structures.

And because it forms a compact structures, they runs very fast and because they runs very fast, they do not allow enough time for the agarose to resolve the samples very efficiently and because of that, you are not going to see the separation of the individual messenger RNA. So, avoid that what you are supposed to do is you are supposed to denature the messenger RNA you have to denature the interaction between the nucleotides.

So, that it should not be able to form these secondary structures and that you are going to do with the help of the farmamide gels where you are going to add the denaturing agents. And that is how it is actually going to disrupt all the secondary structures once a disrupt the secondary structures, then the messenger RNA is going to run as per its molecular weight and because of that, it is actually going to give you the discrete bands.

Because that is very important that you should have the discrete bands, so that all the bands are going to be corresponding to the messenger RNA of the respective genes. So that when you do the northern blotting, the probe is going to bind to one of those bands and that is how you are actually going to detect the signal.

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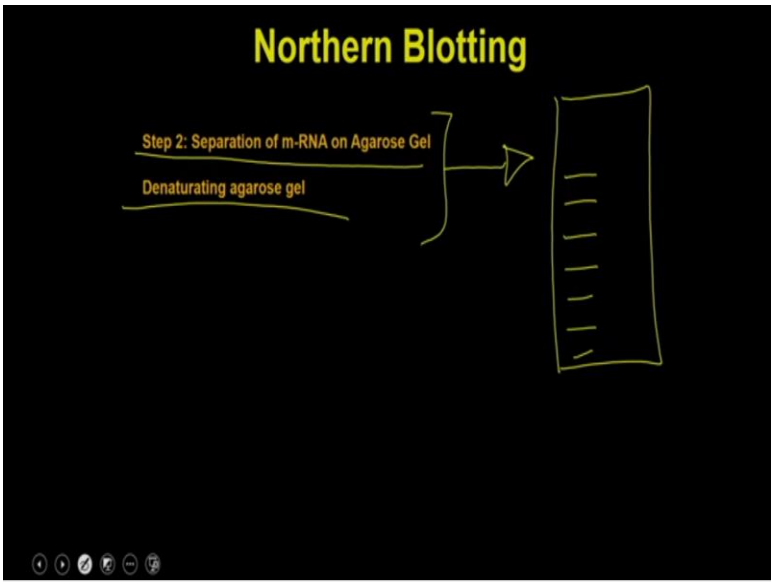

So, that anyway we have discussed when we were discussing about the gel electrophoresis. So, I will suggest you that you should refer to the gel electrophoresis lectures if you are you know if you are do not be able to show or if you want to recall what we have discussed at that point. So,

that we are not going to discuss again here and I suggest you that you should refer to you know the electrophoresis lectures and I think we discuss about the horizontal gel electrophoresis.

And that actually is going to give you the complete idea how to perform the formamide gels to resolve the messenger RNA. So, ultimately, when you are going to be done with these resolutions, what you are going to get is you are going to get the agarose gels and the individual messenger RNA is going to be resolved completely from the top to bottom compared to that if you do not do the formamide gels, you done the regular gels. then you are only going to see the messenger RNA pool you will not get going to see the separation of individual bands.

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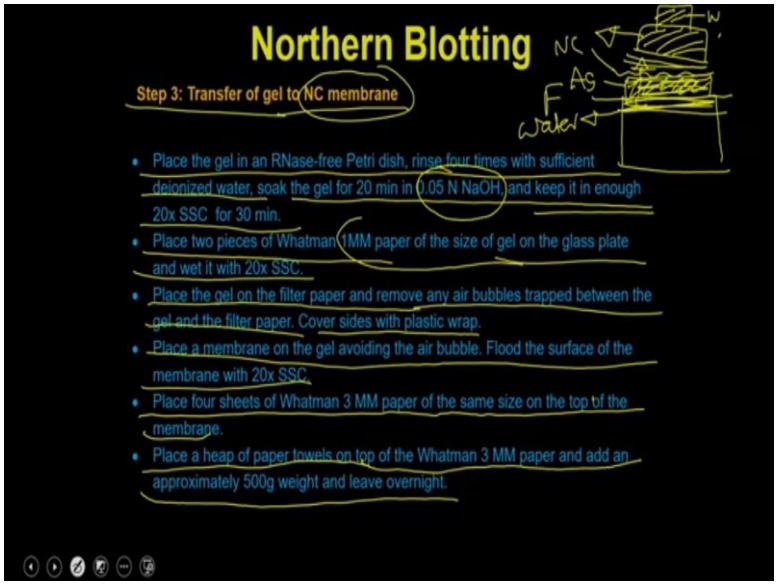

Now, the next step is that you are going to transfer that the agarose gel so you are going to transfer the messenger RNA but it is present onto the agarose gel to the nitrocellulose membrane. So, what you have to do is you place the gel in RNA free petri dish rinse the 4 times with the sufficient deionized water, soak the gel in 20 minutes in 0.05 normal NaOH, and keep it in the enough 20 x SSC for 30 minutes.

Now place the 2 piece of the Whatman 1 mm filter paper of the size of the gel onto the glass plate and wet it with the 20 x SSC the place the gel onto the filter paper and remove any air bubbles trapped between the gel and to the filter paper, cover the sides with the plastic wrapping

paper. So, what you have to do is you have to take a glass slides or the glass base and on this you are going to first going to keep the Whatman filter paper.

Then on this you are going to keep the agarose block what you are supposed to use for transferring deep RNA from the agarose block and then you place the nitrocellulose membrane onto the gel avoiding the air bubble, flood the surface with the 20 x buffers. So after that, this is your going to be agarose gel and then on top you are going to keep the nitrocellulose membrane and you have to ensure that there should be no air bubble between the agarose block as well as the nitrocellulose membrane.

Because if you have the air bubble that is the place where there will be no capillary actions. So, there if there will be no capillary actions during that particular area, that area that RNA is not going to be transferred onto the nitrocellulose membrane and that is why it is very important that when you keep or when you place the membrane, you should roll the you know the surface with a test tube or with a roller.

So, that you can be able to remove all the air bubble what is been present between this particular agarose block versus the nitrocellulose membrane. After that, you place the 4 sheet of Whatman 3 mm footer paper on same size on the top of the membrane to after that you are going to keep the 3 sheets of nitrocellulose membranes, and then you are going to please a heap of paper towel on top of the Whatman filter and add the approximately 500 grams weight and leave it for night.

So, after that, you are going to keep track of the paper towels and then on top of that you are going to keep the weight. So, what happened is that in this particular glass tray, you are actually keeping a transfer buffer. So, you are keeping the buffer or the water and because of that what will happen is that this buffer is going to go into the upward direction because it is wet at the bottom and it is dry onto the top. So, all these filter papers are actually having the capillary like actions.

So, because of that capillary like actions, these filter papers are going to start sucking the buffer what you have added into this lowered chamber and then while they are sucking the water, it is

also going to provide power to the member RNA also. So, the RNA will also going to run along with the buffer and as a result, the RNA will going to hit to the nitrocellulose membrane, but the nitrocellulose membrane is porous for the buffer, but it is not porous for the RNA molecules.

So, because of that, what will happen is that the RNA molecule is going to stuck to the nitrocellulose membrane, whereas the buffer will be going to pass through and then it is going to you know going to suck by the Whatman filter papers followed by the paper towels and followed by the weight. So, because you have a weight, it always gives water to keep sucking and after some time, you can actually be able to remove this paper towel.

So, once it gets completely wet, you can again remove the paper towel and put the new set of paper towel which is dry actually and because of that you actually going to continue the failed capacity actions and that is how the RNA what is present into the agarose gel is going to be transferred on to the nitrocellulose membranes.

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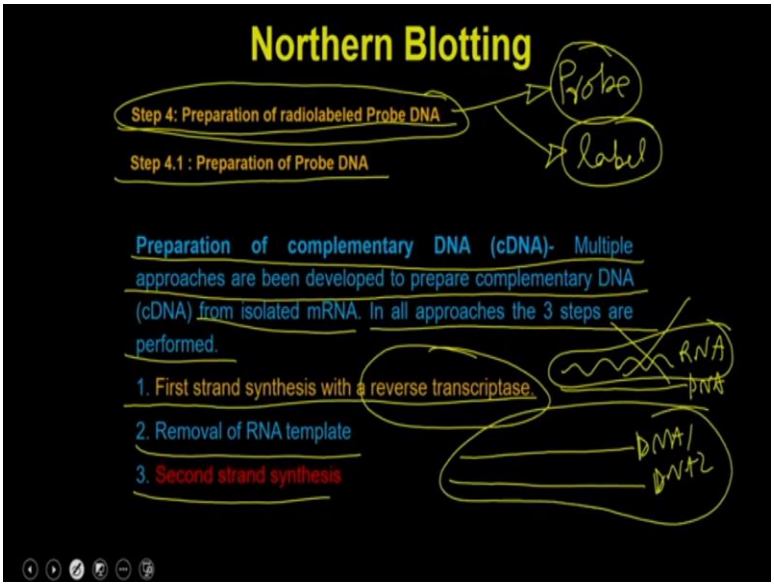

Now, in the step 4, you are going to prepare the radiolabeled probe DNA. So, the step 4 has the 2 events one is that you have to design a probe. So, in this case, we are only discussing about how you can be able to use the probe as a DNA. So, first you have to design the probe and then the second step, you are actually going to label the probe with the radio activity. So, in the step 1 you are going to do the probe.

The preparation of the probe DNA for the preparation of the complementary DNA like the multiple approaches are being developed to prepare the complementary DNA from the isolated messenger RNA in all approaches you have the 3 steps to perform. So, there are multiple ways in which you can be able to prepare the c-DNA from the messenger RNA of your interest. So, suppose you are you interested to look for the level of the expression of a particular gene.

So in that case, what you are supposed to do is you are going to supposed to use the probe, which is corresponding to that particular gene. So what you are going to do is you are going to take RNA sequence and then you are going you are going to isolate that particular RNA, you are going to synthesize the c-DNA with the help of many methods, what has already been explored. And irrespective of any method to use, the processes are different.

But the basic fundamental aspect is that it is actually going to first allow you to do a first strand synthesis with the help of the reverse transcriptase. So in the first strand, what you are going to do is you this is your RNA. So in the first strand what is going to do is with the help of the reverse transcriptase, it is actually going to synthesize the first strand of the DNA, then in the second stage, you are going to do some treatment.

So that this RNA is going to be destroyed by the enzymes and then you are only going to have the DNA species left, then you are going to do a second strand synthesis where you are going to take the DNA. So, this is the first copy of DNA and then you are going to synthesize the second copy of DNA and that is all your complementary DNA is going to be ready for this purpose to be used as a probe.

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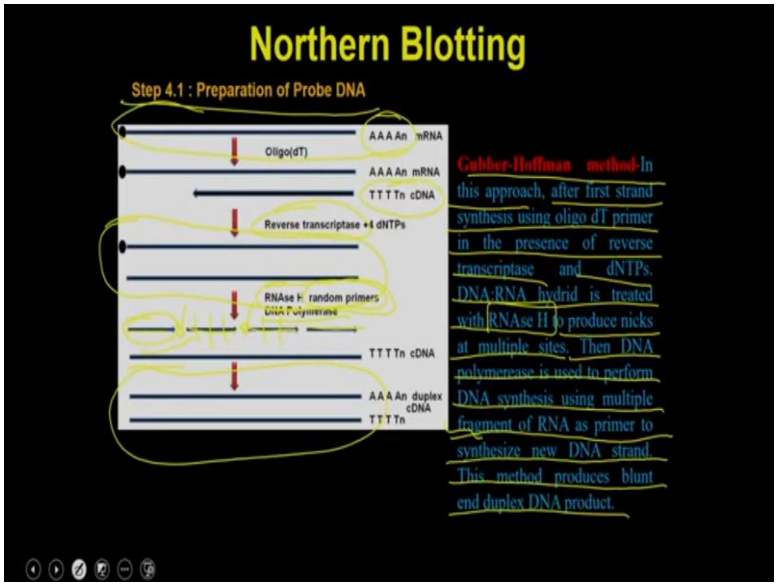

So, one of the methods what we are going to discuss to prepare the probe DNA is the Gubber-Hoffman method. So, in this approach after the first strand synthesis using the oligo dT primer in the presence of reverse transcriptase, and the dNTPs. DNA, RNA hybrid is treated with RNAse H to produce the next at multiple sites. So, in this case, what you are going to do is as you note that all the messenger RNA are going to have the poly-A tail.

So, what you are going to do is just synthesize a poly-T c-DNA or poly-T primers, and then you ask the reverse transcriptase to synthesize. So, it is actually going to synthesize the complementary DNA and then you are going to have you know, the RNA DNA hybrids and then you are going to treat this RNA DNA hybrid with an enzyme which is called as the RNAse H. And what is the RNAse H is going to do is it is actually going to cleave the RNA molecules at the multiple places.

So, because of that, it is actually going to have the multiple RNA stretches been formed. So, it is actually going to generate the nicks at multiple places. Now, what you are going to do is you are just simply going to add the primers and DNA polymerase. So, you know that the DNA polymerase is also utilizing the RNA as the primer. So, what DNA polymerase is going to do is it is going to utilize these RNA molecules as a primer and then it is actually going to synthesize the second strands.

But in this process, what is going to do is it is actually going to destroy this RNA molecules and it is actually going to synthesize the second strand of the DNA and that is how your complementary DNA is going to be ready for the gene what you are going to interested to synthesize. So, then; the DNA polymerase is used to perform the DNA synthesis using the multiple fragments of RNA as a primer to synthesize the new DNA strand.

This method produces the blunt end duplex DNA from your gene of your interest. So, now, you have generated the particular DNA fragment what you can be able to use as a probe. Now, the second stage is that you want to do the radio labeling before that you can be able to detect where this probe is going to bind.

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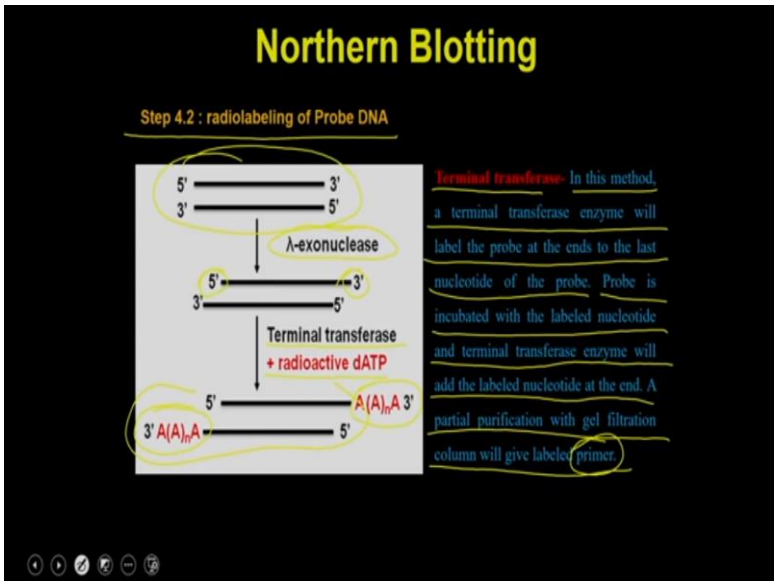

Now, the radio labeling of probe DNA can be done with the multiple approaches what we are going to discuss is the terminal transferase method. So, in a terminal transferase method in this method, a terminal transferase enzyme will label the probe at the end to the last nucleotide of probe is incubated with the labeled nucleotide and terminal transferase enzyme will add the labeled nucleotide at the end, a partial purification with the gel filtration column will give you the labeled primer.

So what do you have you have the complementary DNA, what you can do, you can just treat it with the alpha-exonuclease, that is actually going to create a nick so it is actually going to create

and then what you are going to do is you are just add the terminal transferase and radioactive ATP. For example, in this case, we are trying to radio label with the ATP. So, if you add radiolabeled ATP, what will happen is that it is actually going to start adding the ATP onto this overhang.

And it is going to add some more nucleotides so on both ends, it is actually going to generate the ATP. So, now you have generated the radiolabeled probe. Now, what you can do is you can simply run a gel filtration column so that if you run the gel filtration column, what we will going to do is it is actually going because the DNA is of the big size whereas, the DNA all other things are going to be present in this particular reaction like you are going to have the terminal transferase.

You are going to have the non-reactive ATP and you are also going to have the unlabeled probe as well. So, all these things are going to create a lot of confusion. So, what you can do is you can just inject the whole reactions into a gel filtration column. So, what the gel filtration column is going to do is because gel filtration column normally separates the molecule based on the size, it is actually going to separate the DNA polymerase, it is going to separate the ATP which is going to be a very small size.

So, that will come separate and then the label probe versus the unlabeled probe, it is actually going to be different because you are using the radioactivity as a method to detect. So, that is how you can be able to purify your labeled probe from these reactions and that you can use into the subsequent reactions for the hybridization as well as for detecting the RNA onto the nitrocellulose membrane.

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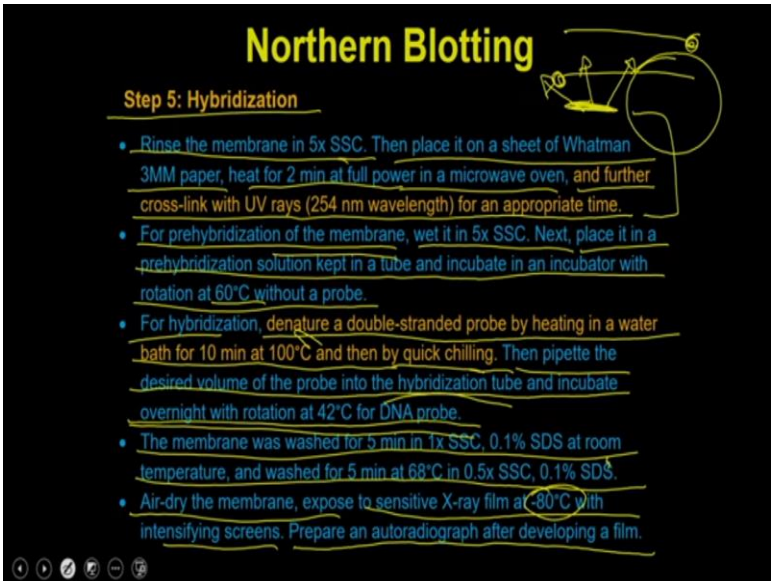

So, the next step is the hybridizations. So, you rinse the membrane in the 5x SSC, then you place it on a sheet of Whatman 3 mm filter heated for 2 minutes at full power in a microwave oven and further cross linked with the UV rays for an appropriate time. So, once your RNA is going to be transferred on to the nitrocellulose membrane, then you are actually going to use the UV light so, that it is actually going to be cross linked.

So, that; during this procedure, the RNA should not come off from the nitrocellulose membrane for the pre hybridization of the membrane wetted in a 5x SSC. Next, place it in a pre-hybridization solution, keep it in a tube and incubate in an incubator with rotation at 60 degree without a probe. So, then after that you are going to prepare the membrane. So, you are going to prepare the membrane a pre-hybridization, you know events like you are going to do membrane, you are going to heat it at 60 degrees without a probe.

So that even if you have some secondary structures, which are still be remained on to the RNA, those also could be broken down at this particular temperature. Then for hybridization, you denature the double stranded probe by heating in a water bath for 10 minutes at100 degrees Celsius. And then you are going to do a quick chilling then pipette the desired volume of the probe onto the hybridization tube.

And incubate overnight with the rotation at 42 degrees Celsius for the DNA probes, which means once just before the hybridization you have to bring the probe so you know; the probe is the double standard DNA where you have the radioactivity on the both ends. So then what you are going to do is you hit this DNA at 100 degrees Celsius. So once you hit it at 100 degrees Celsius, the both strands are going to be separate and then you quickly chill.

So, if you do a quick chilling, you are not allowing the DNA to align properly and because of that, there will be a mismatch. So it is you are going to have the single standard stretches which are going to be overhangs from the double stranded DNA and that those stretches can be used for hybridization. So then you are going to keep this desired amount of the probe into the hybridization tubes and incubate at overnight with rotation in a hybridization chambers and at a 42 degree Celsius.

So you are going to keep the temperature slightly warm, so that you do not allow the nonspecific interactions of the DNA with the other messenger RNAs the membrane was washed 5 times with the next offer at room temperature and then washed at 68 degrees Celsius. So, all these temperatures, what we just discussing is a general understanding, but these temperature has to be optimized as per the signal what you are getting.

So, once you done first time, you may get you will see more nonspecific signal or you may see low signal in those depending on those parameters, you have to optimize the washing time you have to optimize the SSC considerations, you have to optimize the temperature and all that parameters. And you can also you know optimize the interactions time like how long you should do the interaction of the probe with your another blots like the nitrocellulose membranes.

So, all these parameters are just we are giving slightly you know guidelines, but as per the protocol, you cannot just get the desired result for getting a desirable result, you have to do the optimization of all these parameters. Then you air dry right the membrane exposed to the sensitive X-ray film at minus 80 degrees Celsius with the intensifying screens to prepare an autoradiogram after developing the frame.

So once you have done the hybridization, you can do the deployment and that is how it is actually going to give you the autoradiogram. The autoradiogram has to be done or at a very low temperatures. The reason of doing it at a minus 80 degree Celsius because you do not want beta particles to emit very far away from the signal because what will happen is if you do it on a high temperature.

So suppose this is a place where your probe is bind, then it is actually going to emit the beta particle very high, if you do it at a at a room temperature or at 37 degrees Celsius. So, if that happens, then you are going to see a signal of this height, because it is not the reactivity which is giving the signal it is a beta particle which is giving you the signal so, beta particles scattering or the emitting from the signal has to be minimized. So, there is only occurs at site where the probe is binding, not beyond that, so, otherwise your signal is going to be having a very no big signal and it is not going to have a very high resolutions.

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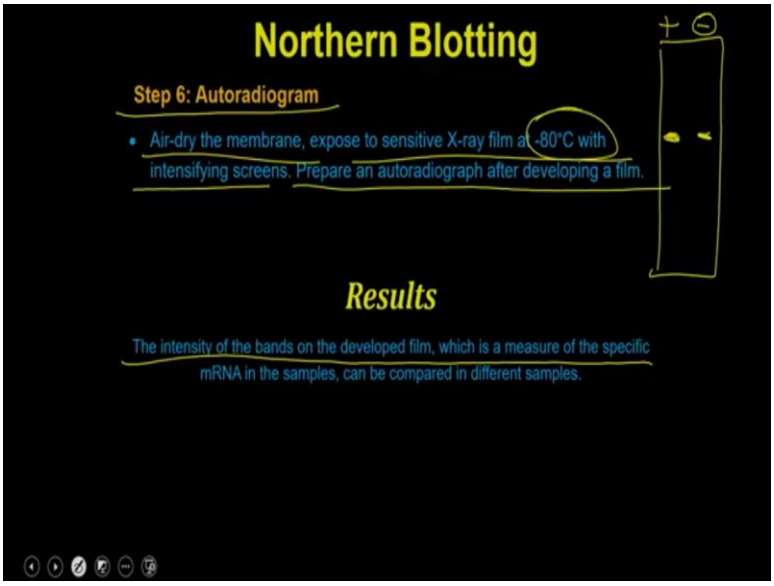

In the step 6, you are going to do the autoradiograms so you air dry the membrane exposed to the sensitive X-ray film at minus 80 degrees Celsius with the intensifying screens prepare an autoradiogram after developing a film. And once you do that, you are going to see the results so what you are going to see in the result is you are going to see the result of the band of your interest. So, it suppose this is your treated sample and this is a untreated sample.

So, it could be possible that the untreated sample does not have that band or there will be a change in intensity. So, that is the kind of result what you are going to see after the blotting. So, the northern blot is always been used to detect the level of gene expression of that particular gene at that particular level or at that particular moment. So, it is actually been used to measure the activity of that particular gene add in under the particular conditions.

So, this is all about northern blotting. And in these particular blotting techniques, you are using the RNA as a target molecules, where you are first separating the RNA onto the agarose gel. So, you are using the denaturing agarose gels and then you are using you know DNA as a probe. And then you are detecting the RNA of your species or your choice with the help of the multiple steps and then ultimately you are doing the hybridizations.

And then you are doing the deployment of the blot with the help of the autoradiodiagramming and then ultimately it is going to give you the pattern where it is actually going to give you the multiple bands corresponding to that particular gene or it is actually going to give you a single band which is corresponding to that particular gene and then you can be able to do the image analysis of that particular autoradiogram and then it is actually going to give you the signal of that particular gene.

And depending on that particular signal, you can be able to say whether the gene is you know expressing more or less one thing you have to always remember that when you are doing this kind of experiments, you always have to take the control reaction. For example, if I am doing a gene, I am looking for the changes in this expression, then I will also have to include the control experiment like I have to use also do LDH primers like I have to detect the LDH messenger RNA.

So, that is actually going to be used to equalize the level. For example, if I am taking the different amount of material like in one case, I have taken 1 gram cell in other case I have taken the 2 gram cell then in that case definitely after the northern blotting, you are going to see the double amount of messenger RNA into the 2 gram sample. But if I use the LDH sample, and if I

use the LDH gene as a probe as well and I will do the double probing, then the LDH gene is also going to be 2 x.

So that is actually going to be used for equalizing. So what I will do is the image analysis for my sample, I will compare the sample 1 and sample 2 and I will also compare the sample the LDH signal in sample 1 and sample 2. So if the LDH signal in sample 1 and 2 is almost same, and then the gene signal is different, then I can be able to conclude that there is a change in the expression of that particular gene. So with this brief discussion about the northern blotting, I would like to conclude my lecture here in our subsequent lecture, we are going to discuss about the southern blotting. Thank you.