Experimental Biotechnology
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Module No # 03
Lecture No # 14
Scientific Questions (Part – 4)

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(Video Ends: 01:00)

Hello everybody this is doctor Vishal Trivedi from department of biosciences and bioengineering IIT, Guwahati. And in what we were discussing? We were discussing about the electrophoresis and in this particular module we have discuss about the basics of electrophoresis followed by the vertical gel electrophoresis, horizontal gel electrophoresis. And then in the previous lecture's we have also discussed about the different variants of the gel electrophoresis.

And then ultimately we have also discussed about the some of the research problems where you can be able to utilize the electrophoresis as a tool to answer those questions. And in this particular; series now today we are also going to discuss few more experiments and few more research problems and while discussing these research problems you will be able to understand more and more potential of the electrophoresis to solve the research problem related to your work.

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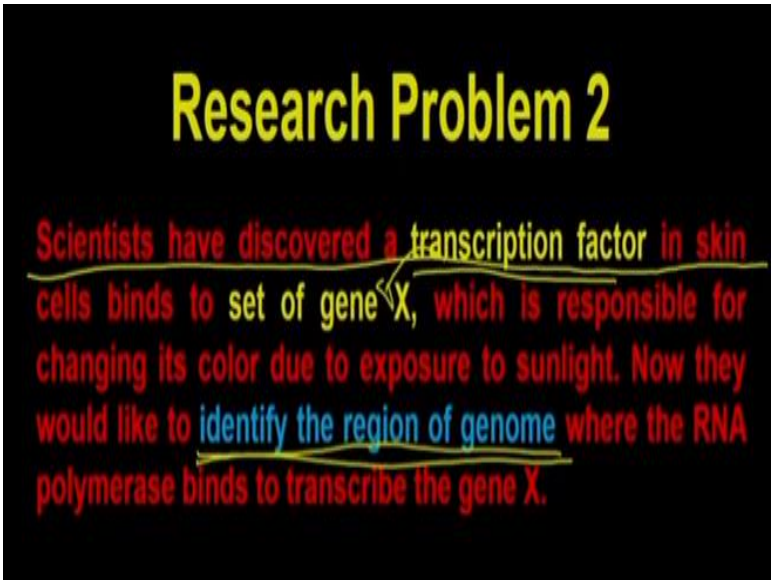

Once you have identified that the transcription factor is interacting the scientist are not going to satisfy there and they are actually more interested to know more and more question. And that is why as I said you know in this course we are not going to only discuss about the one problem what we are also going to tell you know the from one problem you are going to see many more problems.

So what in the previous problem the scientist have identified that the transcription factor is interacting with DNA. And based on that essay then can be able to identify the transcription factor. Now what they want is to know which region of the genome or which region of the DNA that transcription factor is binding. So in this particular question what they want is that? They have identified the transcription factor they know that it is binding to the gene of the gene X.

But what they want to know is they want to know the; identify the region of the genome where this transcription factor is binding. So now this question can be used or this question can be solved by another essay so that we are going to discuss anyway.

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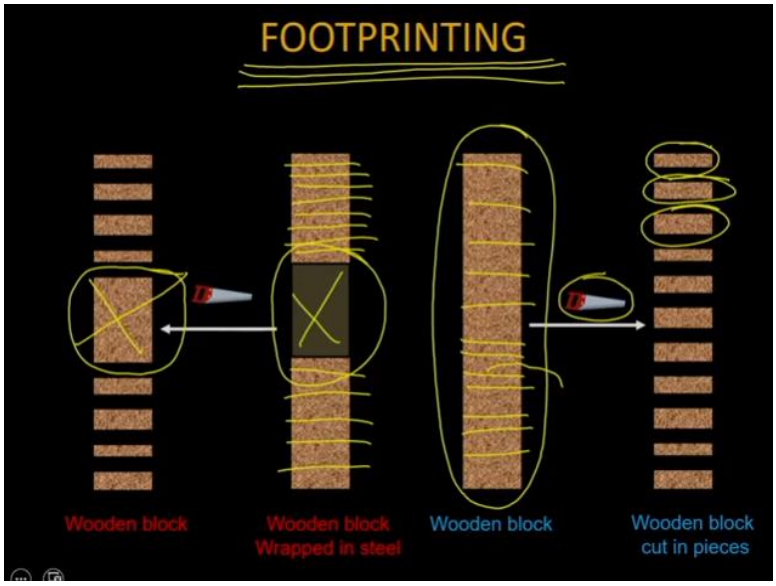

So, that can be done in a foot printing but before discussing for the foot printing let me tell you a how that the idea of foot printing came. So you can have the 2 wooden blocks so 1 wooden block you have 1 wooden block and now suppose you use a cutter and cut this wooden block into the multiple pieces. So what you are going to get you are going to get the multiple pieces. And there

will be no issues you will get you will cut this we will able to cut this wooden piece into multiple pieces.

Because there is no protection present on to this wooden block now if you rap this wooden block with some steel pipe or something. Then what will happen is that you will be able to cut to this wooden block at the same location with the help of the cutter. And you can be able to cut at the lower side as well but you will not be able to cut at this point because this is already been protected from the steel.

So what you will in the pattern is that there will be a intact DNA present or intact wooden block present which is going to have the large chunk of the wooden block. Whereas in this case; everything is going to be cutting into the multiple pieces. And that is the exactly way of so if I ask you where the foot print of the wooden block where is the foot print of the steel block, which you have added you can easy say that this is the place where I have added the steel block because this is the wooden block which is not been cut by the cutter.

And that is what is the basic philosophy of doing the foot printing in the same way you can actually cut the DNA and that is how if you protect some region that region is not going to be cut by your cutters and that is actually is going to be indication that it is the region where that protein is interacting.

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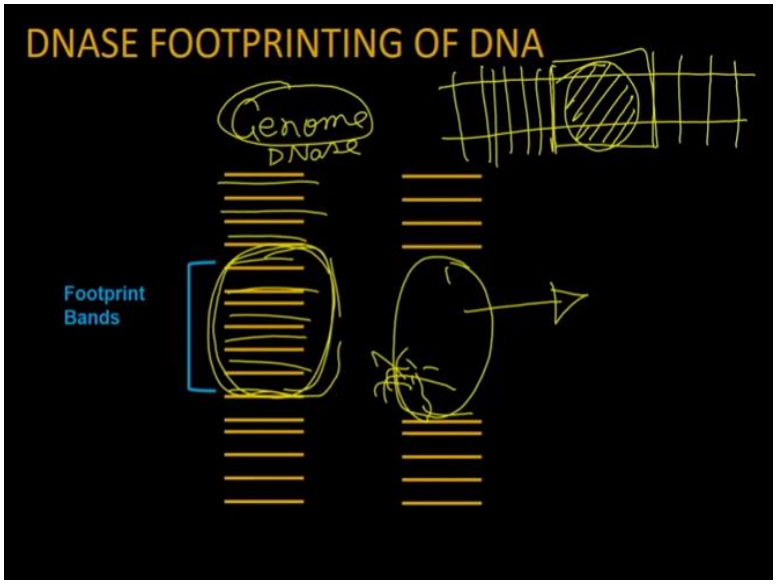

So let us see with the DNA so what will happen is that what you do is? You take the genome and you use a DNAse and ask him to cut. So if you take the genome and ask the enzyme to cut it into multiple pieces you are going to see the multiple pieces okay. But if you protect some region of this DNA or some region you can imagine that at this region we have added a steel block so what will happen is?

In this case because this is a DNA what we have done is we have added a protein blocks so in this case this region is now going to be protected from the action of the enzyme. So as a result this portion is going to be remains uncut and this portion is not going to appear. So the bands corresponding to this portion are actually the site where the protein of your where your protein is interacting and that is what the philosophy of the foot printing of the DNA.

Which means you can imagine that you have a very large DNA and in thus DNA at one point you have a region where the protein is bound. So what happen is if you cut this it is going to give you this pattern because it is not been protected but if it is protected then you will get all other bands except the band which is corresponding to this region. And that is why this region can be identified simply by sequencing these bands and you will be able to know that which region of genome is been bound by this particular protein. How to perform this?

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So to perform you need the different types of reagents, material and equipment's. So what are the reagents are required? You require a buffer A which actually contains the normal buffers and

you know like 10 millimolar HEPES the NaCl Sucrose, the EDTA, Titon X 100. And then you also require the protein inhibitor PMSF then you require the buffer B which is exactly the same except that you need a glycerol ETDA and PMSF.

Then if you require a buffer C which is the same except that it very high consultation of the NaCl and then you require the DNAse binding buffer. So that actually contains the DTT glycerol EDTA MgCl2 and Tris-HClPh 7.6. And then you require the calcium and magnesium solution which is like 5 milli molar calcium 10 milli molar magnesium chloride. Then you require the stop solution which is like 200 millimolar NaCl 30 milli molar EDTA and 1% SDS.

And then you require a loading buffer is NaOH formamide 0.1% xylene cyanol and 0.1% bromophenol blue. Apart from that you require a enzyme which is called as the DNAse and for performing this experiment you require a microfuge as well as the electrophoresis operators.

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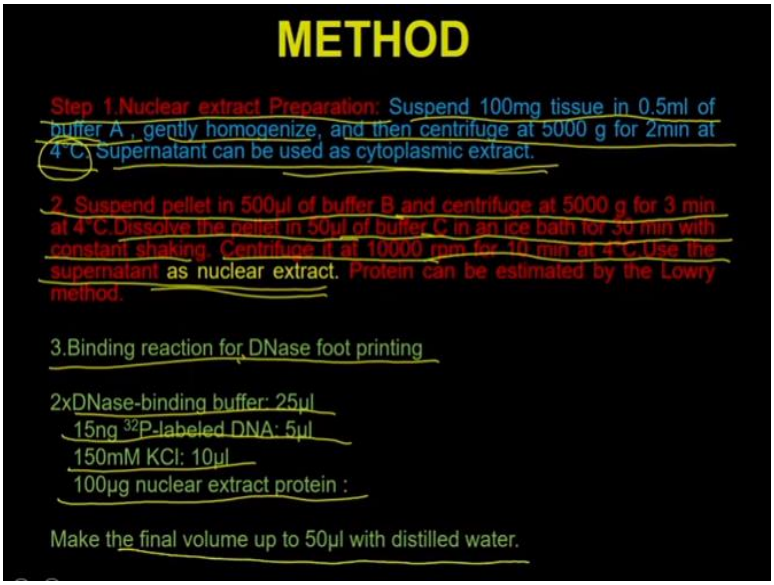

So in the step 1 you are going to prepare the nuclear extract for preparing the nuclear extract you have to suspend the 100 mg tissue. In 0.5ml of buffer A then you have to gently homogenous and then centrifuge at 5000 g for 2 minutes at 4 degree. And remember that all this procedure as to be done in a, cold conditions like 4 degree. So that you will be able to protect the factors what is present in the nuclear extract.

Supernatant can be used as a cytoplasmic extract now once you centrifuge you are going to get a palate and the supernatant can be used as a cytoplasmic extract. Whereas the pellet you will resistment in buffer B centrifuge at 5000g for another 3 minutes. Then you dissolve the pellet so in after this also you are going to get a pellet in a supernatant. Then again you pellet dissolve it into 50 micro liter of buffer C in ice for 30 minutes with the constant shaking.

So after this when you centrifuge at 10000 rpm you are going to get the supernatant as well as you are going to get the pellet. And that supernatant is known as the nuclear extract you can actually estimate the amount of protein with of the Lowry as well as the Bradford. And that actually can be used even for you know for a basis to see how much activity you have. Then you have to use the binding reaction buffers.

So the binding reaction buffers for DNase binding buffer 25 micro liter then you require the labeled DNA 5 micro liter. KCl and then you have to add the nuclear extract and then you have the make up the volume 250 micro liter with the help of the distilled water.

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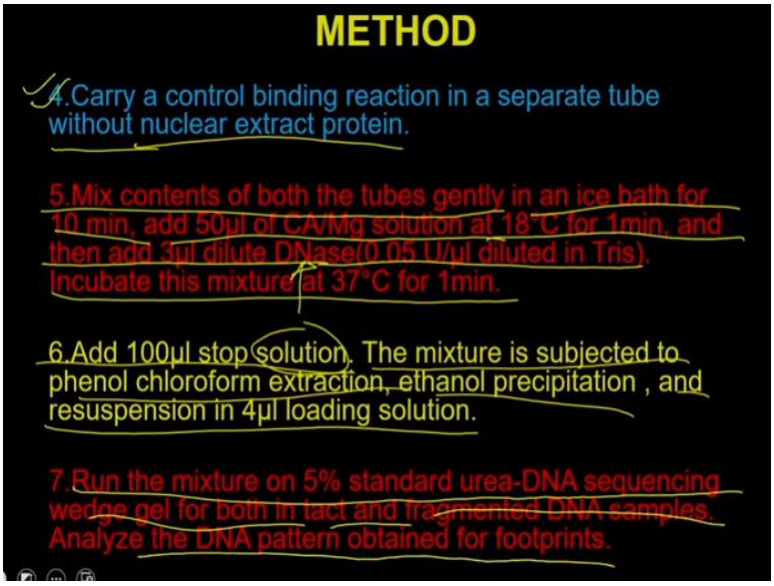

Because the control is very important you can also run a control where you cannot you should not add the nuclear extracts. So without a nuclear extract is going to tell you that you are going to get all, the fragment in that, particular reactions. Whereas when you add a nuclear extract and if the nuclear extract is having some protein which is binding to the DNA that region is going to be protected from getting the cleaved.

Now you mix the content of both the tubes gently in ice bath for 10 minutes and add 50 micro liter calcium magnesium solution; at 18 degree. For 1 minute and then add 3 micro liter of DNAse incubate this mixtures at 37 degree Celsius for 1 minute. Then you add these stop solutions so that you will be able to stop the activity of the DNA so you will just going to do a limited DNase activity.

Which means you are not to allow a DNase to cut the DNA for you know for very long time otherwise what will happen is? The DNase is going to cut the DNA completely then you will not be able to see the fragments. The mixture is then subjected to the phenol chloroform extraction, ethanol precipitation and the re-suspension in the 4 microliter of loading. Which; means once this you are going to add this stop solutions then you have to recover those fragments simply by going through with the DNA precipitation and you know removal of the protein part.

Then you run and this mixture on to a 5% urea DNA sequencing gel for both in the fragmented DNA samples. So you analyze the DNA patterns obtained for the footprints.

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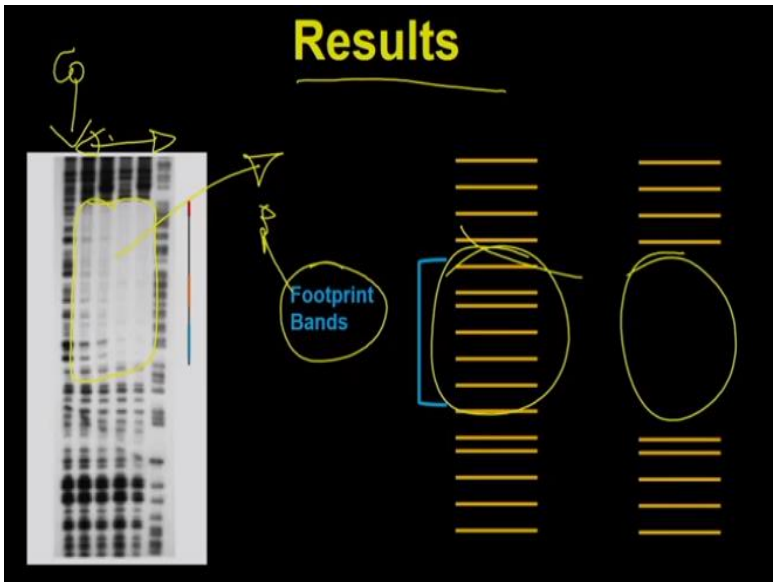

So the results what you are going to see the results you will see that this is the sample for the standards right. So this is your control samples so what you see is you see the bands from top to bottom all the bands what you see is actually corresponding to the different amount different

sizes of the nucleotides. Whereas in this 4 lanes where you have actually added the nuclear extract what you see is that a large junk of the region is not having any fragments.

And these are actually the footprint bands because that is what exactly happens these are the bands which are disappeared from your treated sample means the sample where you have added a extracts.

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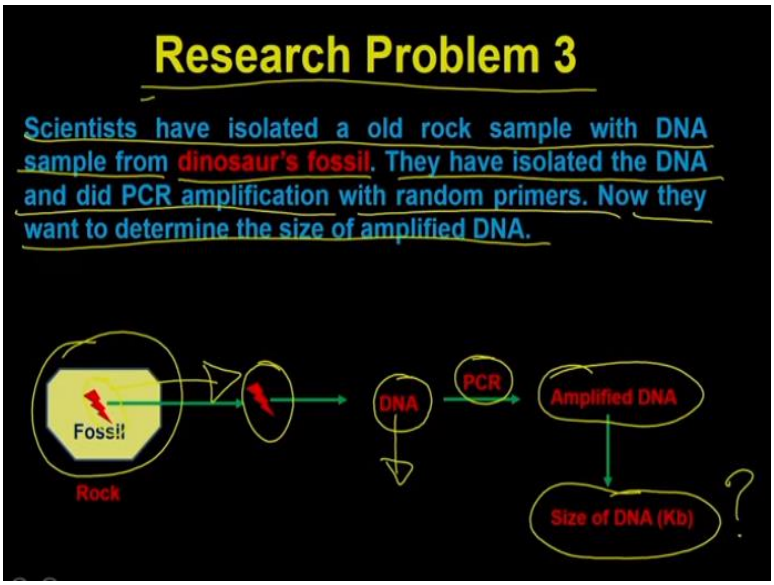

So the next problem is that the scientist, have isolated a old rock sample with DNA sample from the Dinosour's fossil. They have isolated the DNA and did PCR amplification with the random primers. Now they want to determine the size of the amplified DNA. So what they have done is they have isolated a old rock and that old rock was containing a small dinosaur fossil. So what they have done is they have isolated this particular fossil and then by the help of some advance technique they could be able to isolate the very small fragment of the DNA.

But this amount of the DNA was not enough so that you can be able to do any kind of analysis like sequencing and you know the looking at the size of this DNA and what will be the composition of the nucleotides in the particular DNA. So for this purpose they have to amplify so what they have done? They have done a PCR and then they got the amplified DNA so this amount of DNA was good enough.

So now the question is how to determine the size of the DNA so for this they can be able to use the agarose gel electrophoresis. So let us see how to do that.

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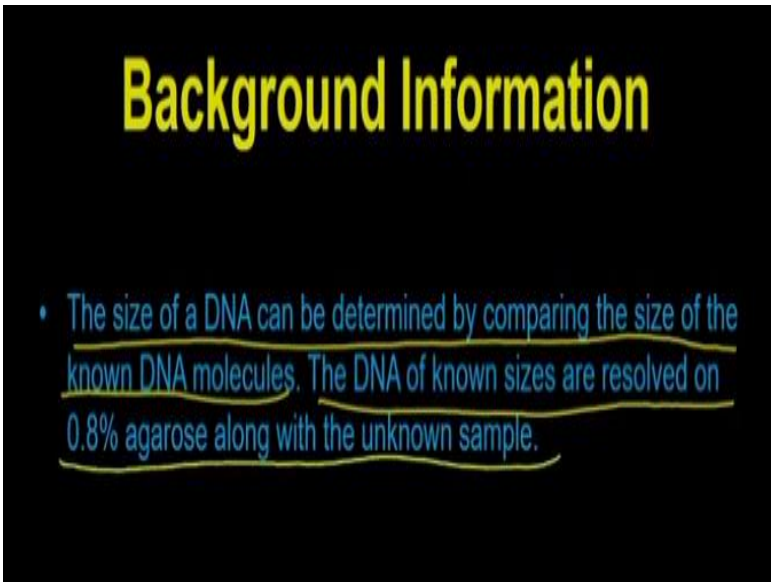

So in the size of a DNA can be determined by comparing the size of the known DNA molecules. The DNA of known sizes are resolved on to 0.8% agarose with the along with the unknown sample.

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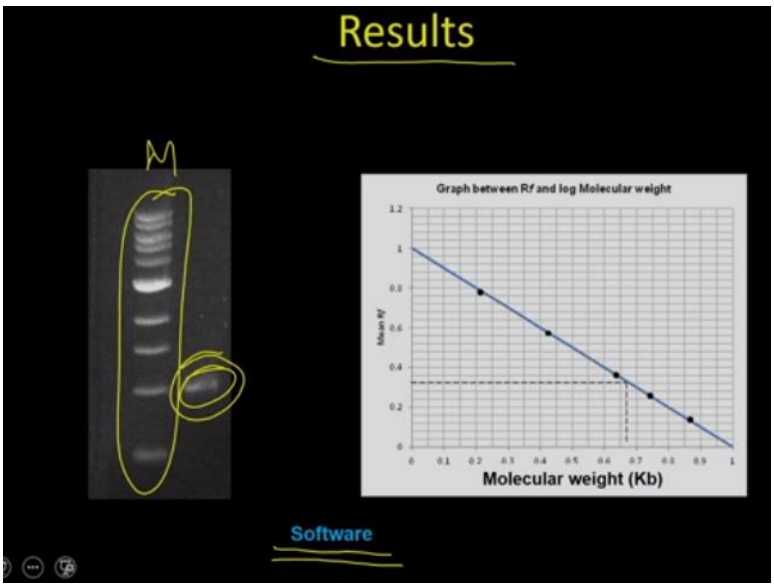

What you have to do is first resolve the DNA on the agarose gel along with the DNA molecule markers. Then you calculate the relative Rf values with the help of the migration of the DNA versus the migration of the DNA dye. So migration of DNA versus the migration of the tracking

dye and then you plot the log molecular weight versus relative Rf value. So that actually is going to give you a standard curve.

Then what you do is you perform a linear regression to calculate the equations and that equation is going to be in the form of y = mx+c so that equation can be used to calculate the molecular weight of your unknown sample. Which means if suppose I have this is a you know if I have the Rf value of this I can just simply go either conventional way of going with the intercept and I can calculate the log molecular weight and then I can easily go and calculate the molecular weight by taking the antilog.

Or I can just simply go with the you know so what will be the result in this essay is that is you have first run the sample with the molecular weight occur and this is your sample. So what you can do is simply calculate the Rf value for this molecule weight markers and calculate the molecular weight of you and then samples. So if you have the software's you have the image analysis software's

This image analysis software can easily be able to trained; with the help of these molecule weight markers. So you know the size of this molecular and then with the help of the software's you can be able to do the calculation automatically without even plotting this. Because this plotting will always been done by the software itself and that actually is going to give you the size of the DNA.

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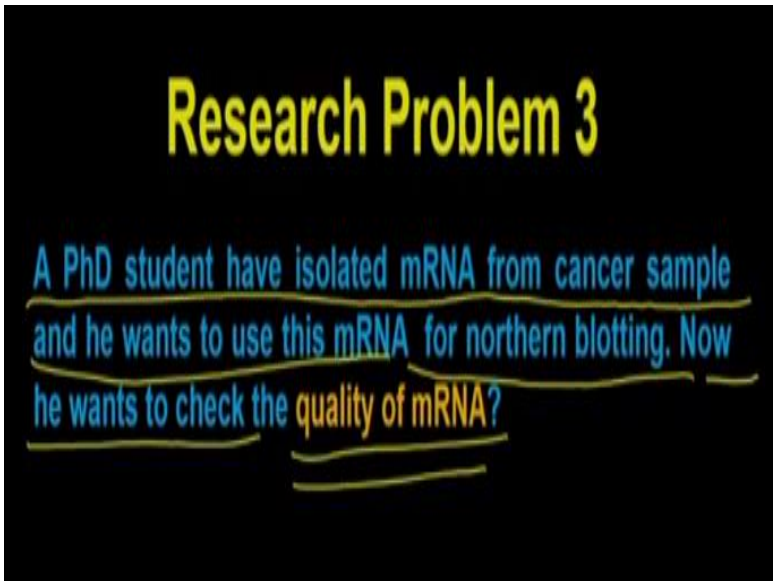

Let us move on to the next problems so the next problem is a PhD students have isolated messenger RNA from the cancer sample. And he wants to use this messenger RNA for northern blotting. Now he wants to check the quality of the messenger RNA. So you know when you want to do the northern blotting or that actually anyway we are going to discuss in our subsequent lectures and you the first thing is you have to understand the; or you have to know the quality of the messenger RNA.

Which means how; the messenger RNA is whether it is intact or whether it is degraded all that. Because if you use the degraded messenger RNA then you northern blotting is results not going to be accurate and they will be not going to be conclusive.

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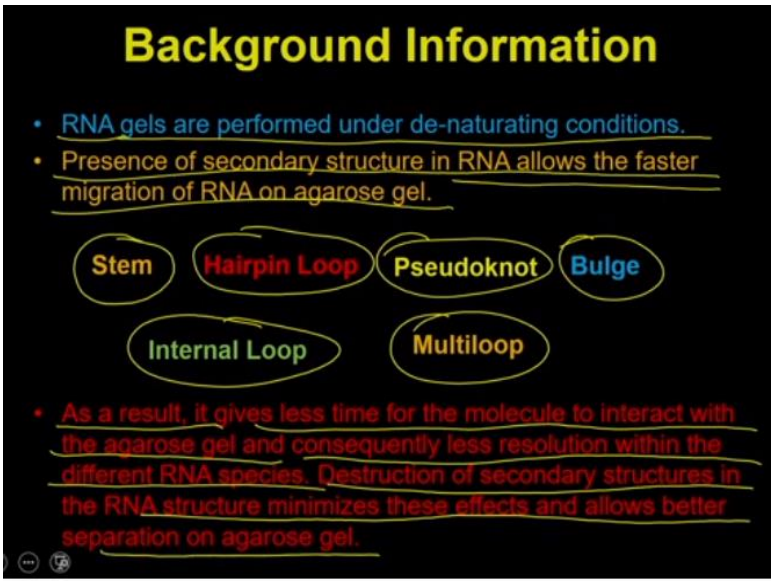

First is that RNA gels are always be performed under the de-naturating conditions. And they are been perform in the de-naturated condition because you have the secondary structures in RNA. You have the different types of secondary structures like stems you have the Hairpin Loop, you have the Pseudo loop, Pseudoknot you have the bulges, you have the internal loops and you have the multiple loops.

And how this secondary structures are not good because they allow the RNA to run fast on to the agarose gel. And if they will fast if they will run fast it will get less time for the molecule to interact with the agarose gel. And consequently there will be less resolution within the different RNA species. Destruction of secondary structures in the RNA structure minimizes these; effect and allow the better separation on the agarose gel.

So because it as a secondary structure it becomes very compact structure and because of that it actually runs very fast within the pores what is present in the agarose gels. And as you know and I think we have discussed in the past also that when you are doing a horizontal gel electrophoresis with the agarose as a matrix. The pores sizes; within the agarose is very big compared to the pores sizes within the polyacrylamide gel electrophoresis.

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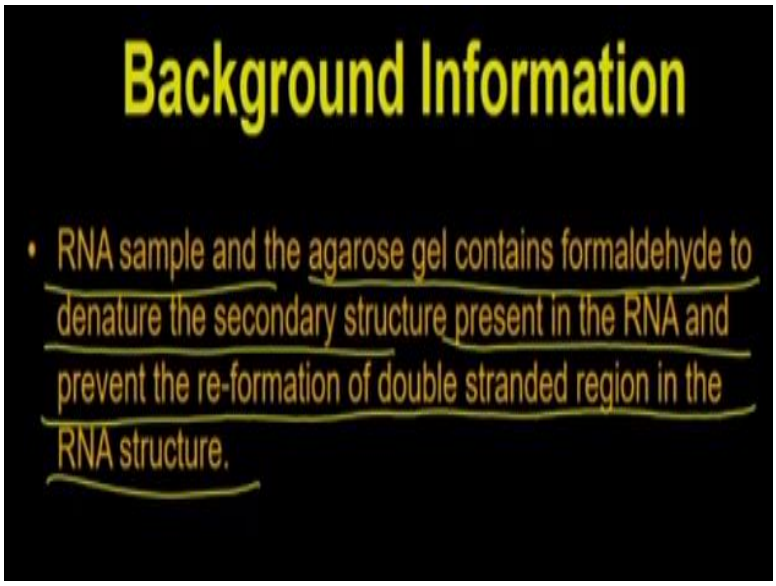

How to do that the RNA samples and the agarose gel contains formaldehyde to denature the secondary structure present in the RNA and then that prevents the reformation of the double standard region in the RNA structures. And that is actually going to let you to run these RNA into a linear structure's and that is how they will be able to get resolves nicely from the other RNA species and it will give you the better pattern.

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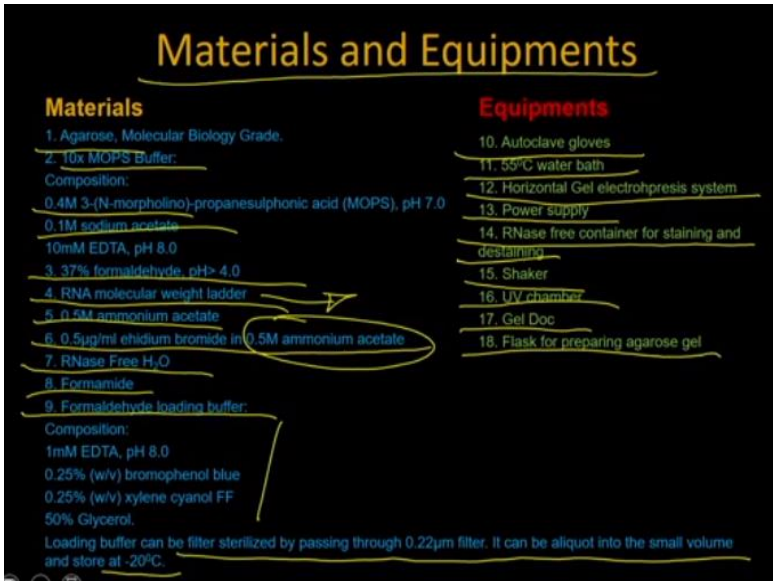

The materials and the equipment's what you required to run the RNA gels what require is you need a agarose you need the you require 10X mops buffer the composition of the mops buffer is given. Then you required the 37% formaldehyde solutions then you require a RNA molecular

mark ladder. So this is different from the DNA ladder because here you are going to have the different sizes of the RNA.

Then you required the 0.5 molar ammonium acetate then you require the staining dye which the 0.5 micro grams per ml ehidium bromide and that is prepared in the 0.5 molars ammonium hesitate. Then you require the RNA's free water formamide formaldehydes loading buffers and that is the composition of the formaldehyde loading buffer. And the loading buffer can be filterized by the 0.2 micro filter and then it can aliquoted into a small pieces and store at -22 degree.

What you require the autoclave gloves you require the water bath you require the horizontal electrophoresis system. You require the power supply then you require a container where you can be able to store the RNA's free staining and de-staining gels. Then you require the shaker's you require the UV chamber's you require a gel Doc and then you require a flask for preparing the agarose gel.

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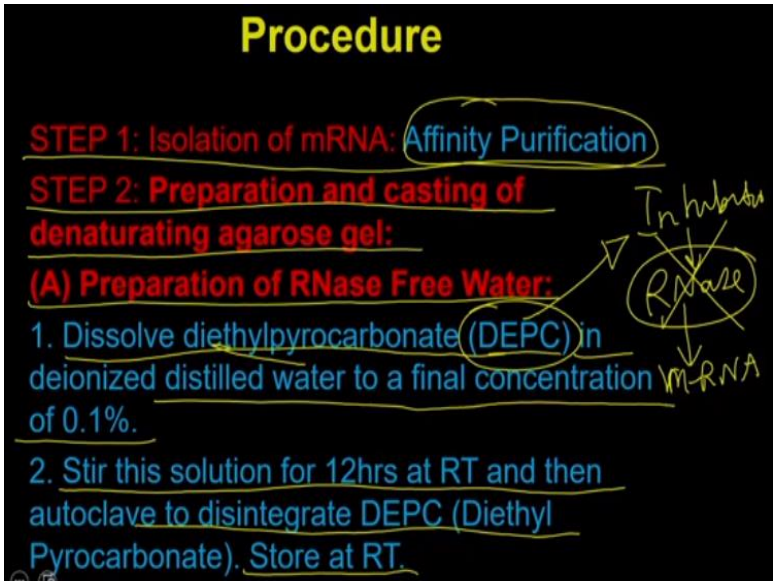

It is a multistep process so in the step 1 itself you have to do the isolation the messenger RNA. So that you are going to do with the help of the affinity purification and that anyway we are going to discuss when we will discuss about the northern blotting. Then you have to prepare the de-naturating agarose gel and that is also a multiple steps. So in the step 1 you have to do the preparation of RNA's free water.

So the RNA free water is prepared by simply dissolving the diethylpyrocarbonate or DEPC in deionized still water to a final concentration of 0.1%. And DEPC is a strong inhibitor of strong RNA's so that actually is a inhibitor of RNA which means it is actually going. If you prepare or use the DEPC treated water it is actually going to protect your RNA from degradation because it is going to inactivate the RNA's what is present in the buffers or in the reaction mixtures.

You stir the solution for 12 hours and then autoclave to disintegrate the DEPC and then you can store this solution at room temperature for very long time.

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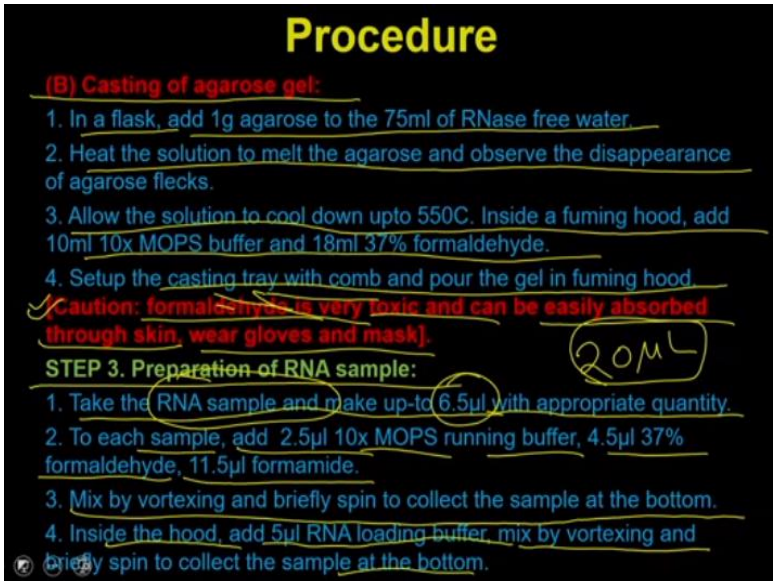

Then the third step you have to do the casting of the agarose gel so in a flask at the 1 gram of agarose to the 75 ml of RNase free water. Heat the solutions to melt the agarose and observe the disappearance of the agarose flask this I think we have already discussed when we discussing about the agarose gel formation, when we were discussing about how to resolve this for the DNA as well. Then you allow the solution to cool down up to 55 degree Celsius inside a fuming hood at 10 ml of 10 X more buffer and 18 ml of 37% formaldehyde.

Setup the casting tray with a comb and pour the gel in the fuming hood at this stage you have to keep the very care that formaldehyde it very toxic and can be easily absorbed through skin wear glass and you have to use the flask. Because formaldehyde is very evaporate very easily so that it

actually get into your body through the breathing. So that is why you have to use the mask as well as you have to wear the cloves so that you have not get a, absorb through skin as well.

Now in the step 3 you have to prepare the RNA samples so take the RNA samples and make up to the makeup to the 6.5 micro liter with a appropriate quantity. So whatever the RNA sample you have you just prepare a 6.5 micro liter of sample. And to each sample you add the 2.5 micro liter 10X mops running buffer 4.5 micro liter or 37% formaldehyde and 11.5 micro liter of formaldehyde formamide.

So that actually is going to make a reaction mixture of 20 micro liter mix it by vortexing and briefly spin to collect the sample at that bottom. Then you inside the hood at 5 micro liter of RNA loading buffer mix it by vertexing and briefly spin it to let the sample at the bottom. As you can see we are doing all this procedure under the fuming hood so that you should not get exposed to the fumes what is coming from the formaldehyde as well as the formamide.

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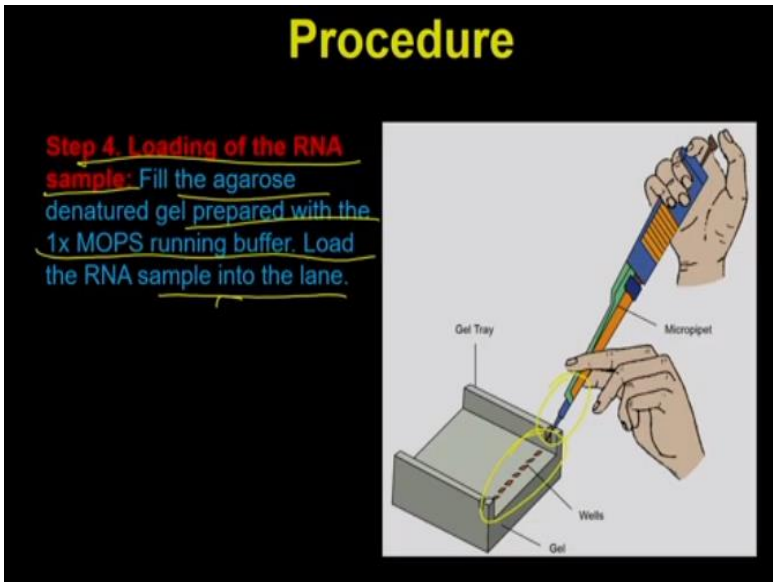

In the step 4 you do the loading of the RNA samples so fill the agarose denatured gel prepared with the 1% 1X mops running buffer and load the RNA sample in to the lane. So the loading is exactly the same as what we have discussed of the loading of the DNA into the agarose gel. Except that you have to be little careful that all these tips and all other instruments and things what you are using should be sterilized.

Because RNA is very sensitive for the degradation so because the RNA is present everywhere so that it actually get degraded with very fast. So you have to sterilize everything and then you have to use the filter tips instead of the normal tips.

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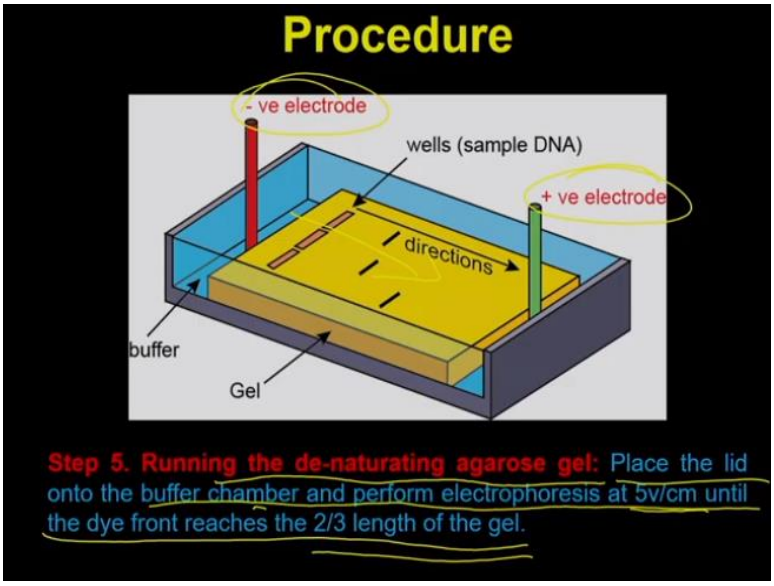

Now in the step 5 you have to do the running of the de-naturating agarose so place the lead on to the buffer chamber and perform the electrophoresis and 5 volt per centimeter until the dye front reaches to the 2 third of the length. So this is exactly the same like you have to do the negative electrode positive electrode and then you run the RNA. And you have to run up to the 2 third length of the gel. After this you have to do a staining and destaining to visualize the RNA.

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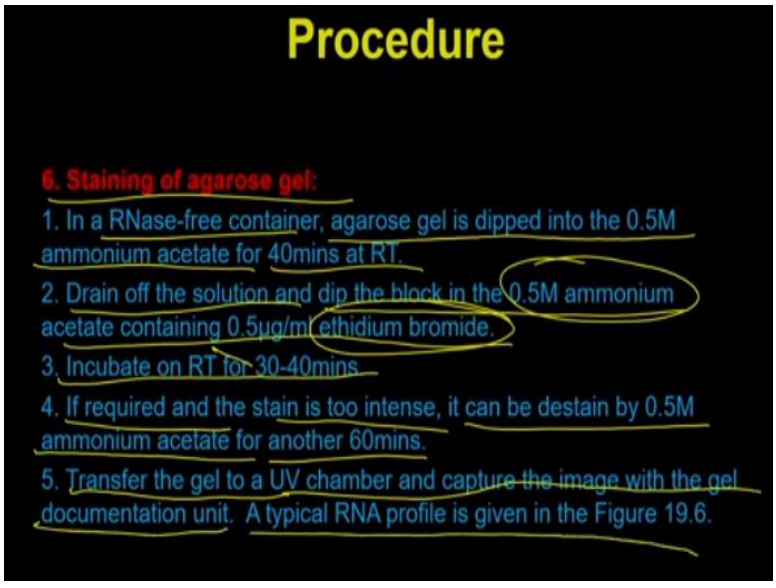

Then you have to do the staining of the agarose gel so in a RNAse free container agarose gel is dipped into the 0.5 molar ammonium acetate for 40 minutes at a room temperature. This is for followed by so you remove the solution and dip the block in a, 0.5 molar ammonium acetate containing 0.5 micro gram per ml ethidium bromomide. So this is the staining dye which is prepared in the 0.5 molar ammonium sulphate.

Then you incubate the gel in room temperature for 30 to 40 minutes and if required and the stain is too intense which means if you are not been able to see a better background or better contrast between the RNA as well as the background then what you can do is you can simply do the de-stain by the 0.5 molar ammonium acetate for another 50 minutes to 2 hours. Then what you do is the transfer the gel to a UV chamber and captures the image with a gel documentation unit. A typical RNA profile is look like this.

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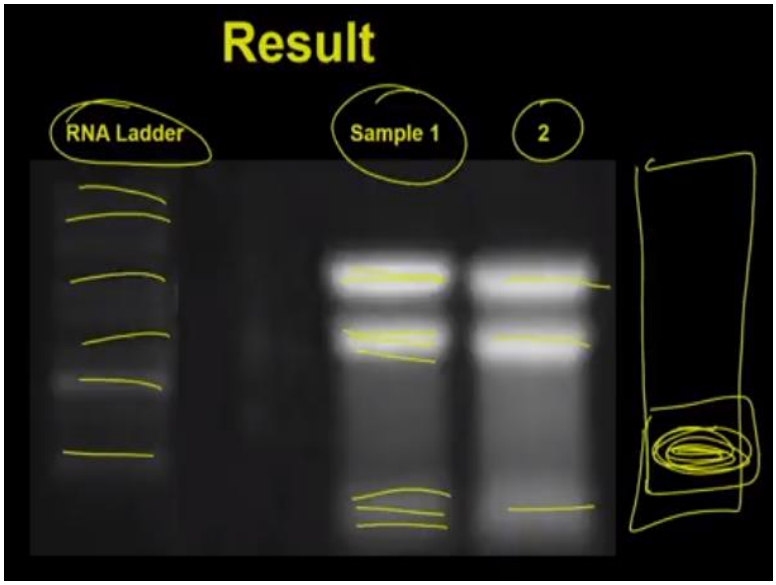

So here what you see is this is the RNA ladder so what you see is the bands of the different sizes and the typical sample will look like this where you have the RNA of different sizes. And this is going to be resolved so these are the messengers RNAse of different sizes. Whereas normally when you run the DNA or agarose del which is not the denaturating gel what you see is that the full RNA is present at the bottom of the gel and not been resolved.

So this is what you see is actually a resolution of this which is where all the RNA bands are been separated into the multiple messenger RNAse. And that is how you can be able to perform the

northern blotting by transferring into a membrane and with probing with the radioactive probes. So with this I would like to conclude our lecture here, thank you.