Experimental Biotechnology

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Module No # 03
Lecture No # 13
Scientific Questions (Part-3)

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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 discussed about the basics of electrophoresis followed by the vertical gel electrophoresis, horizontal gel electrophoresis. And then in the previous lectures, 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 problems related to your work.

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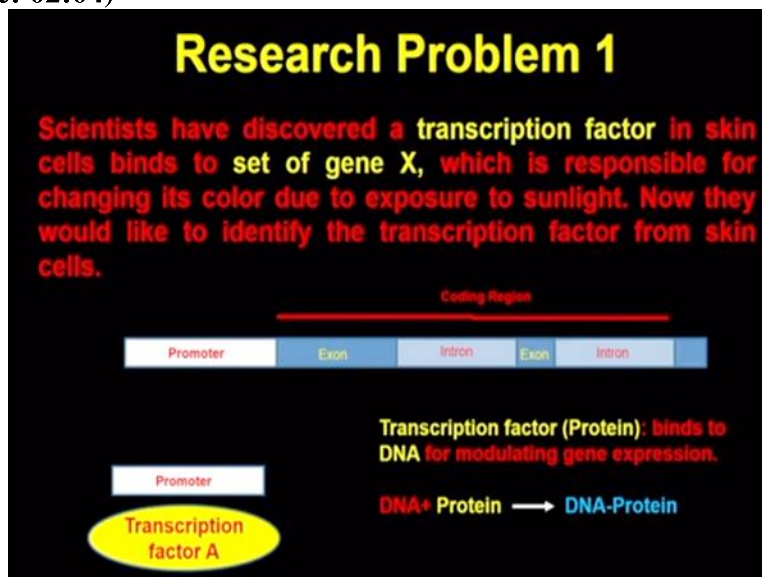

So the first problem today what we are going to see is, and this is a old problem what we have discussed before also that the scientists have discovered a transcription factor, and in skin cells binds to the set of gene X which is responsible for changing its color due to the exposure to the sunlight. Now, they would like to identify the transcription factor from the skin cells. What they want to do is if you remember in the previous lecture what we have discussed that they were interested to identify the transcription factor.

And then on the top they are also interested how the DNA as well as this transcription factor is interacting with each other So as we I think discussed that the gene structure is made up of promoter and then you have the coding region. So this coding region is not responsible for binding of the transcription factor. Only the promoter region is responsible for binding of the transcription factor and in this event, what will happen is that the DNA is making a complex with the protein.

And as the result it is forming a DNA protein complex. Now if you see how it is can be it how this interaction can be mapped with the help of the electrophoresis?

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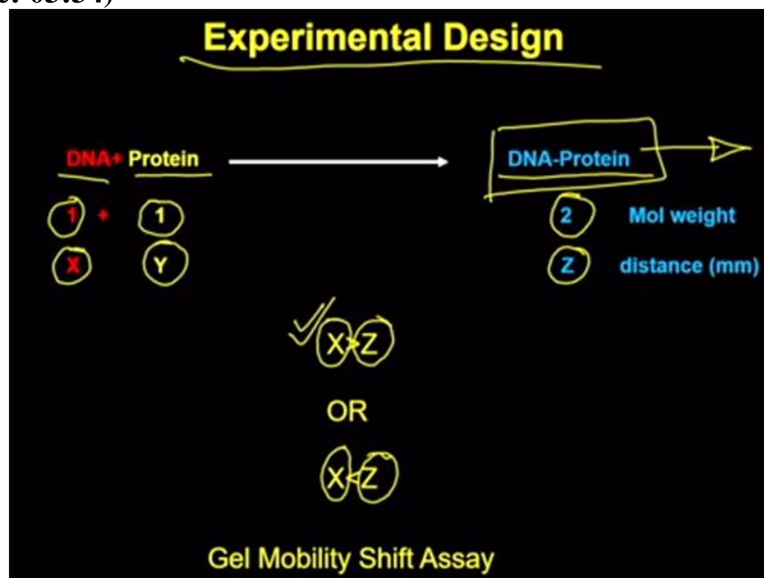

So in the experimental design what we have, we have the 2 molecules. One is DNA the other one is the protein and what they are doing is there interacting with each other to form the DNA protein Complex. Now let us assume that the DNA is of the molecular weight of 1 unit and the

protein is also of a molecular weight of the 1 unit which means the DNA protein complex is going to have the molecular weight of 2 units.

Now, let us assume that the DNA if you do the electrophoresis of these 3 molecules then the electrophoretic mobility of DNA is X. The electrophoretic mobility of Y is the protein is Y then and the DNA protein complex is Z, then the question comes whether the X is going to be bigger to the Z or X is going to be the smaller to the Z which means whether the DNA is going to be run fastest than the DNA what is present in the complex or whether the DNA what is present is going to be run slower that the DNA what is present in the complex.

As obvious that the molecular weight of the DNA protein complex is bigger. So it is actually going to run on a slower size, which means the DNA is going to have the higher electrophoretic mobility compared to the DNA what is present in the protein. Because as you remember; that the electrophoretic mobility in the gel electrophoresis is directly proportional to the chart and inversely proportional to the molecular weight.

This means you have a smaller molecular weight, you are going to run faster and you are going to have a larger distance, you will cover the larger distance where as if you have a bigger molecular weight then you are going to run the smaller distances.

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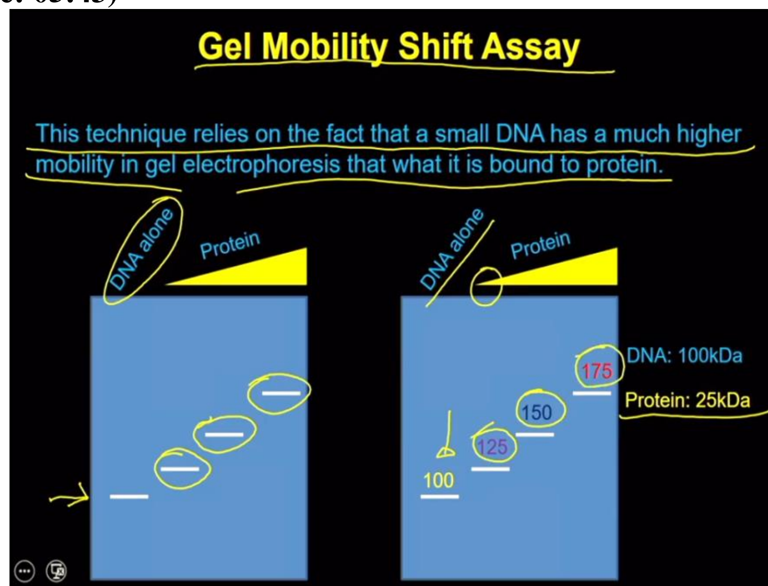

So this interaction of the protein and DNA is been mapped with the technique or with the assay which is called as the gel mobility shift assay. In this technique is relies on the fact that a small

DNA has a much higher mobility in gel electrophoresis then what is bound to the protein. So what you can see is if I am running the electrophoresis or if I am doing the electrophoresis of the DNA alone the DNA will run up to this point. But if I keep adding the protein molecules, the protein molecules are actually binding to the protein to the DNA and as a result it is actually reducing its electrophoretic mobility.

Now Imagine that the DNA what you are putting it for the electrophoresis is 100 kilo Dalton, ok and the protein what we are putting is a 25 kilo Dalton. Now see when you are running the DNA alone, it is actually having a molecular weight of 100 kilo Dalton. But when you are adding the small amount of protein it is actually the 1 molecule of the protein if the 1 molecule of protein is interacting with the DNA, the resultant molecular weight is going to be 125 kilo Dalton.

Similarly if the 2 molecules of the protein will interact then it is going to have the molecular weight of 150 kilo Dalton and if the 3 molecules of the protein are going to interact, it is actually going to have the 170 kilo Dalton as a molecular weight. This means as the more and more protein molecules will bind to this DNA molecule the resultant molecular weight of the complex is going to be keep increasing which means you can be able to map the interaction between the 2 molecules simply by looking at the molecular mass.

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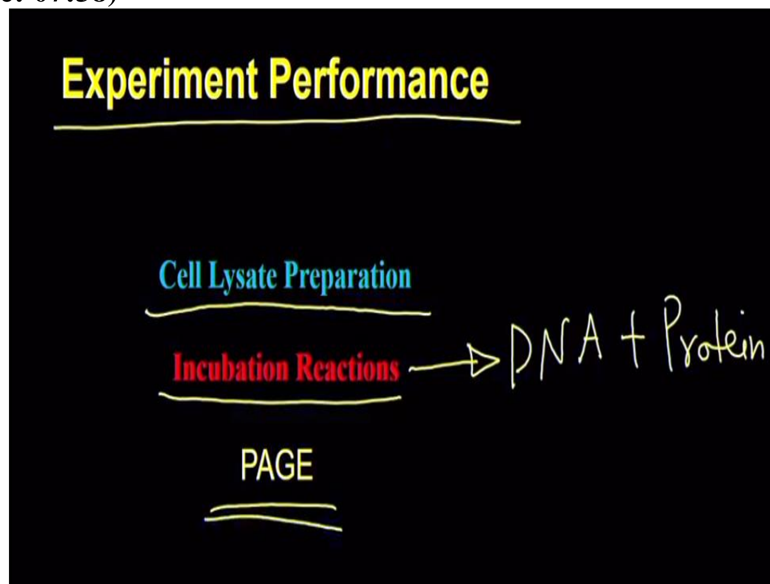

So for performing this experiment what you are supposed to do? You have to first prepare the cell lysate you have to do the incubation reactions where you are actually going to incubate the

DNA plus the protein, ok and then you are going to done the polyacrylamide gel electrophoresis, and that actually is going to give you the resultant that addition of the DNA bands.

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Reagents,Materials,and Equipment

1. ^{32}P-labelling reaction mixture
Oligonucleotide (200ng/μl):1μl
10x polynucleotide kinase(PNK)reaction buffer:2μl
T4 PNK (10 U):1μl
[α-^{32}P]ATP(10μCi,high specific activity):10μl
Double-distilled water:5μl

2. 5x binding buffer
50mM HEPES(pH 7.6)
225mM KCl
10% glycerol
2.5mM DTT
1mM MgCl2
1mM EDTA
0.75mM PMSF

3. 5x TBE
Tris base:54g
boric acid:27g
0.5M EDTA:20ml
Final volume made up to 1L

4. 5% non-denaturing PAGE gel(7ml)
Acrylamide stock(30%):1.16ml
Water:4.38ml
5x TBE buffer:1.40 ml
10% APS:50μl
TEMED:5μl

5. Two specific sense and antisense oligonucleotides

6. Protein gel electrophoretic system, microfuge

The material reagents the material and equipment what your required. So the first reagent what you required is the labeling reaction mixtures so that you will be able to label the DNA. Because, how are you going to monitor the migration of retardation of the DNA molecule is that you actually going to label the DNA with a radioactive material. So in this case we are using the P32 labeled oligonucleotides.

So what you going to do is you are going to take the oligonucleotides you are going to run the 10X PNK reaction buffers and then you are going to add the enzyme that T4 polynucleotide kinase and then you are going to add the alpha 32 labels ATP and the double distilled water. And this labeling reactions is going to be catalyzed and then it is actually going to label the DNA molecule which is the oligonucleotide which you are going to use in the gel shift assays.

You also need the binding assay buffer. So the composition of the binding assay buffer is given here which actually mostly contains the buffers, then you have the reducing agents and then you have the EDTA so that you should take care of the contaminating metals and all that and then you need to resolving the buffer which you are going to use for running the page. So 5X TBE buffer where you are going to have Tris, boric acid and EDTA and then the final volume what you are going to make up to the 1 liter.

And then you need to prepare the recipe for the non-denaturing page gel, so where you going to take the acrylamide, water, TBE then APS and TEMED are going to be added as the polymerization agents. And then you would require the sense as well as the antisense oligonucleotides. So that you can be able to design the oligonucleotides and then in the instrument what you required you need gel electrophoresis system and as well as the microfuge.

Microfuge means you required a small centrifuge so that you will be able to pallet down the reaction mixture. This can be done in a multistep.

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METHOD

Step 1: Preparation of Labeled Probe:

- Complementary strands of the oligonucleotide containing the consensus-binding sequence for a particular gene site of DNA are synthesized. For example, following are the sequences of the sense and antisense oligonucleotides (binding site sequence shown in blue).

Protein

5'-AGT TGA GGG GAC TTT CCC AGGC-3'
3'-TCA ACT CCC CTG AAA GGG TCC G-5'

- Approximately 30 pmol (200ng/ml) of each oligonucleotide is dissolved in sterile distilled water for further labeling with α-32p-labelled ATP(high specific activity).
- The labeled oligonucleotides are purified by gel filtration on a Sephadex G-50 column(0.5×5 cm).The column is pre-equilibrated by passing 10ml of 10mM Tris(pH 8)through it.

So in the step 1 you are going to prepare the labeled probe. So the labeled probe can be prepared by multiple ways and that all we are going to discuss in detail when we are going to discuss the southern blotting. So for the time being you can just simply understand that you are going to add you are going to take the oligonucleotide you are going to add the radioactivity or radio labeled ATP.

And then you are just simply going to add the PNK and that actually is going to catalyze the reactions to add the ATP to the oligonucleotides. So here you are going to take the 2 oligonucleotide, one is sense oligonucleotide the other one is the antisense onigonucleotide so that you can be able to design a double stranded DNA. So for example, they have given you a sequence of the sense and antisense oligonucleotide.

So this is the sense strand this is the antisense strand which you can use and this is the sequence which is actually going to bind or which is going to facilitate the binding of the protein. So that is the region which you have to keep in the centre of this particular sequence. So that they will be adequate amount of nucleotides will be available on both the sides. So that the protein could be able to fit on top of this particular sequence and will be able to bind very nicely.

Then you have to do the, you know several steps of doing the labeling reactions and then ultimately you have to purify the labeled nucleotide by the gel filtration column using the G50 columns. And this column is pre-equilibrated with the buffers so that you will be able to remove the excess radio activity from the radio labeled nucleotide. So once your labeled probe is ready, then you have to go to the step 2.

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METHOD

STEP 2: Setting up the reactions

For the binding reaction procedure, nuclear protein extracts are incubated with labeled probes . While setting up the binding reaction, the following components are added in the given order under ice-cold conditions.

- Salmon sperm DNA(1μg/μl in DEPC treated water):1μl
- Binding buffer(5x):2μl
- Nuclear extract (10μg protein):x μl
- Labeled probe(5 μl, high specific activity):5μl
- Double-distilled water is added to make the final volume up to 20μl.

After the addition of all the components (over ice),the tubes are spinned briefly in microfuge. They are then incubated for 45 min at 25°C.

For the super shift experiments, 2μg of respective antibody is added to the binding reaction mixture prior to the addition of labeled probe. This mixture was incubated for 20min.Then the labeled probe is added and incubation carried out for another 45 min.

RNase

Protein

label Protein

So in the step 2 you have to set up the reactions, so for the setting of the binding reactions what you have do is you have to take up the nuclear protein extracts are incubated with the labeled probe. While setting of the binding reactions the following components are added in the given order under the ice cold condition. So this is very important that you do all these experiments under the low temperature conditions.

So that there will be no degradation of the DNA or there will be no decoration of protein as well. Because either of these degradations; are going to compromise the overall effects. So what you need is you need the Salmon sperm DNA which you are going to prepare in DEPC treated water.

So DEPC treated water is a water which is free of the RNA so that there will be no degradation of the RNA.

If at all the RNA is present because that actually is going to mask the signal what you are going to get from the DNA. Then you also need the binding buffers so the composition of the binding buffers is already been given the previous slide. Then you have to prepare the nuclear extracts. So you have to add the X microliter of nuclear extract. So that depends what kind of activity is present in your sample and all that.

Then you require the labeled probe. So this nuclear extract is actually going to be the source of the protein because the nuclear extract is going to have the transcription factor. So that you are interested to test and then you have to make up the volume with the double distilled water. So after the addition of the all the components over the eyes the tubes are spinned briefly in microfuge so that you can be able to collect all the reaction mixtures.

And then they are incubated for 45 minutes at 25 degree Celsius so that there will be interaction of the transcription factors, what is present in the nuclear extract with the oligonucleotide which is you are adding and then is it is actually going to form the complex. In this there will be a variation that for the super shift experiment. So super shift experiments are the experiments where are you are also been interested that which protein in actually binding.

So in those cases what you can do is, you can simply add the antibodies directed against a particular transcription factor so that you will be able to not know only that the proteins are binding but also which protein is binding. So those kinds of experiments are called super shift experiments. Because in a normal shift, normal gel shift assay what you are going to do is, you are going to see a gradual retardation of the DNA or gradual moving of the DNA on the upward side.

But in the super shift experiment as soon as you add the antibodies, the antibodies are going to increase the molecular weight of the complex very high. So because of that it is actually going to give you at additional shift into the retardation. So the 2 microgram of the respective antibody is added to the binding reaction before prior to the addition of the labeled probe. Because of that it actually going to label or it is going to bind the protein or the transcription factor of your interest.

So because of that it is actually going to show you the super shift which means in a normal thing what you have is, you have a DNA here and then it will gradually grow actually. Whereas in the super shift what will happen is you have a DNA here and then it will show a very large jump in terms of shift. Because here; you have only the protein whereas here you have the protein as well as the antibody.

So that actually is going to increase the molecular weight in very large quantities. So it will be actually going to add up a lot in terms of the final protein DNA complex what is going to be formed. So this mixture was incubated for 20 minutes and then the labeled probe is added and incubation are carrying out for another 45 minutes. So the only difference between a normal gel shift assay as well as the super shift assay is that you have to add antibodies prior to adding the oligonucleotide.

So that the antibodies will go and bind to their respective target protein and then if those proteins are go and bind into your DNA they are actually going to show you the super shift.

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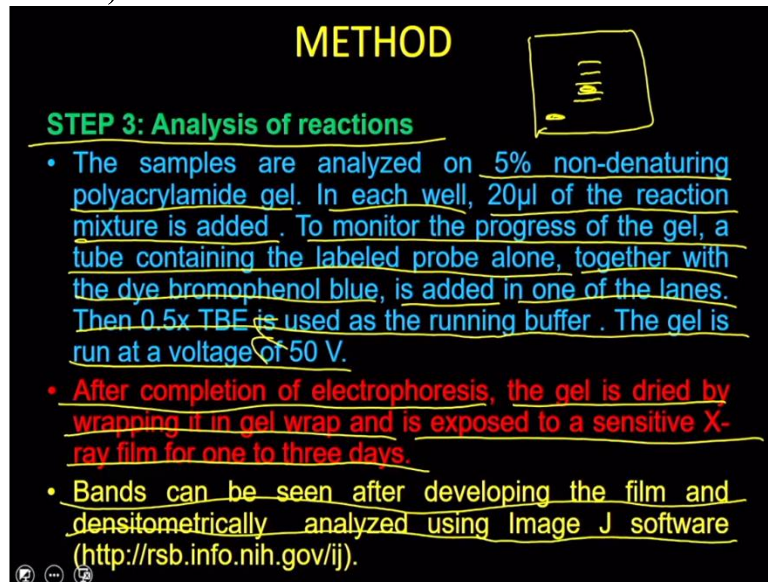

Then the step 3 you have to do the analysis of the reaction. So once the analysis incubation periods are over then what you have to do is you have to take these samples and then you have to analyze them onto the 5% non-denaturing polyacrylamide gels. In each well, you can you can resolve the 20 microliter of the reaction mixture and to monitor the progress of the gel a tube

containing the labeled probe alone together with the dye bromophenol blue is added in one of the lanes.

So that you will be able to do you will be able to see how long the DNA will migrate if the protein component is not available. Because that will be your reference point how much shift of the DNA band you are getting when you are adding the nuclear extracts. Then the 5X TBE is used as a running buffer. The gel is run at the 50 volts constant and it has to be done in a cold condition so that there will be no degradation of the DNA.

Because if there will be any degradation of the DNA that also is going to give you a gradual decrease in molecular evidence so that also is going to give you a mere lie condition. So if there will be a DNA degradation what will happen is the DNA will be here and then you are going to see a lot of bands at that actually is going to mask the actual DNA samples. Even if you have a sample of DNA here, it is actually going to be masked by that degradation of the DNA probe what you have added in this reaction mixture.

So because of that, it is important that you should keep the DNA in a very stable condition. And you should when even when you are resolving the samples and also should resolve it in a cold condition so that there will be no degradation. After completion of the electrophoresis the gel is dried up by wrapping it in the gel wrap and it is exposed to a sensitive x-ray film for 1 to 3 days. Band can be seen after developing the film and densitometrically analyzed using image J software.

So the image analysis anyway we have discussed in a detail. So that you can be able to have the digital image you can be able to utilize many software's what we have discussed before also. That you know either you can use image J or any other commercial software what are available from the different companies.

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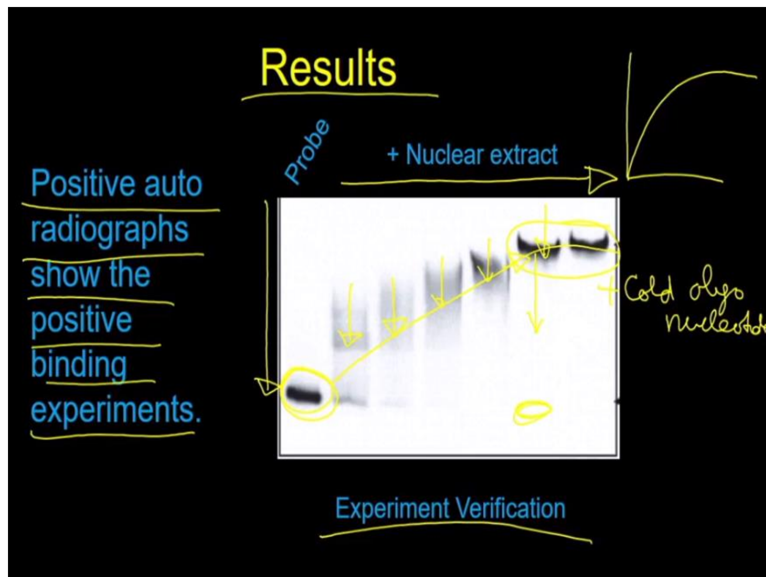

Let us see what results you are going to see so the in the results section the positive auto radiographs shows that the positive binding experiments which means what you can see in a typical experiment that when you are running the probe alone, it is actually going to run in a very far away from your lane. But as you increase or as you put the nuclear extract what will happen is that band is going to be keep moving towards the upper side.

Which; means the proteins are now binding in a very low quantity and then ultimately the whole protein is bound. And what you see is actually there will be a separation which means what you see is the kind of the curve which means at this stage the whole DNA, what you have added in been saturated by the proteins which are present in the nuclear extract. Now how you can be able to verify this? You can be able to verify this by adding the cold oligonucleotides.

So what will happen if you add the cold nucleotides? The cold nucleotide is going to compatite with the protein what is bound here. And because of that it is actually going to release the bound oligonucleotide which is the labeled oligonucleotide. So what will happen is that you will be keep observing the band at the bottom. So if you are going to form the protein complexes and then if you add the cold oligonucleotide.

What is mean by the cold oligonucleotide is the unlabeled oligonucleotide. So that unlabeled oligonucleotide is going to compatite with the complex and it is going to release the radio labeled oligonucleotide. And in that process, you are going to again going to see the re-appearance of the

radio labeled oligonucleotide. Because that the protein is making a complex with DNA is giving you the protein DNA complex.

But if you add the probe which is not radio labeled so this probe is actually radio labeled, that is why you have a radio labeled complex. So if you add the probe or the DNA which is not radio labeled so what will happen is in that this protein is going to be fractionate between the labeled probe versus the unlabeled probe. So ultimately what will happen is it is going to start forming the complex with the unlabeled probe and the labeled probe is now going to be released from the reactions.

And this labeled probe is again going to be electrophorate or it is going to have the similar kind of electrophoretic mobility what a free probe is having. So that is why you are actually going to see the reappearance of the labeled probe. So that is a kind of a verification that this is a specific interactions on the top if you are more interested. What you can do also is you can simply block the sides; what is present on to the DNA as well with the help of different types of antibodies and in those cases the protein what you are going to add will not be able to interact.

So these are the different ways in which you are going to get the result and in which you can be able to verify the results. So with this I would like to conclude our lecture here in our subsequent lecture we are going to discuss some more problems related to electrophoresis.