Enzyme Science and Technology
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Module - IV
Enzyme Production (Part 2: Over-expression)
Lecture - 19
Screening of Recombinant Clones

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And what we are discussing? We are discussing about the different properties of the enzyme. And in this context so far what we have discussed, we have discussed about the development of the field of enzymology and subsequent to that we have also discussed about the nomenclature and as well as the classification of the enzymes.

And we have also discussed about the structural studies of the enzyme. So, we have discussed about the primary structure, secondary structure, tertiary structure and quaternary structure of the enzyme. And the previous two modules, we were discussing about how you can be able to produce the enzyme in the bulk quantities so that you can be able to use them for various type of applications such as you can use them for you know for you know for understanding the properties of that enzyme or you can actually be able to use them for industrial applications.

So, in this context, we have discussed about the isolation of the gene, we have discussed about the we have discussed about how you can be able to clone that gene into the vector of your interest and so on. And once you got the clone and then you are actually going to transform or you are going to deliver the DNA into the host of your choice, then subsequent to that you are actually going to have a screening assays.

You have to perform the screening assays. So, that you can be able to differentiate between the host which got the DNA and which does not get the host DNA, ok. So, that we are going to discuss in today's lecture. So, in today's lecture, we are going to discuss about how you can be able to screen the recombinant clone.

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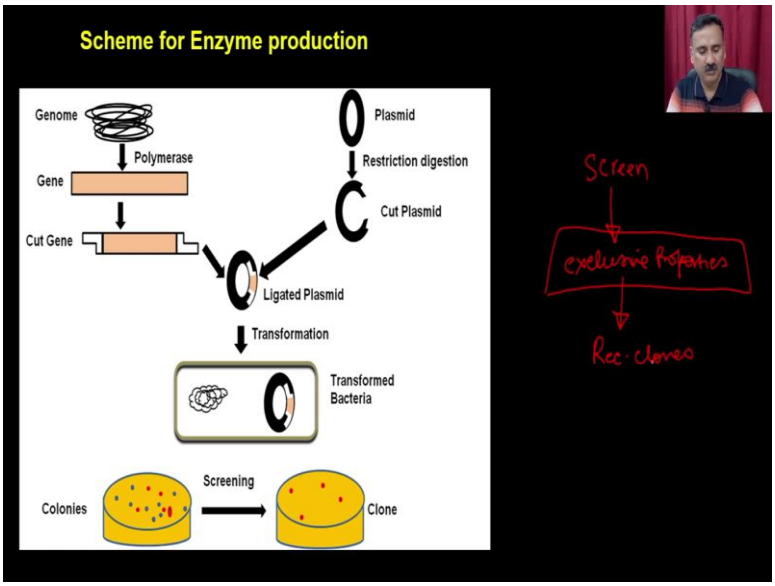

So, now, let us you talk about the screening. The screening is a very important aspect and when you want to screen anything right, when you want to screen, you should actually going to screen a population based on the exclusive properties of and when we talk about the vectors, the vectors are also all the clone is actually going to give you the exclusive properties.

And these exclusive properties can be exploited for screening the recombinant clone. So, let us discuss about this in our subsequent slide.

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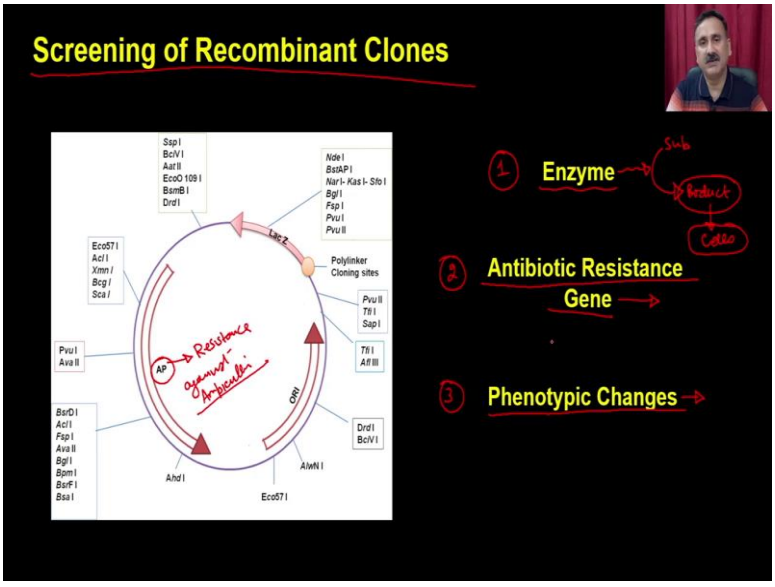

So, screening of the recombinant clone. So, you can imagine that this is a vector right, and where you might have cloned. Now, this vector is actually going to provide you the various exclusive properties. So, that it can be it cannot be exhibited by the plane vector, but it can be exhibited by the recombinant DNA. So, one of the screening criteria is that this particular recombinant clone probably could express some enzyme and this enzyme is going to catalyze reaction.

Where it is actually going to convert the substrate into the product. And this product probably could be colored or it could actually be able to give you some green or red or blue some color. And if this product is actually going to give the color to the cell and that is how you can say ok, blue colored cells are transformed or blue colored cells are actually taken up the DNA of whatever you exogenously added.

The second and the most popular method is that you can actually be work with the antibiotic resistance genes. And this antibiotic resistance gene is actually going to provide the survival of the host cell which actually got the DNA. For example, in this particular vector what you see is it has the ampicillin gene. So, this ampicillin resistance gene is actually going to provide the resistance against the ampicillin. And because the plane vector or the plane host will not actually going to survive.

Because if it not actually going to have the ampicillin resistance and that is going to be the criteria what how you can actually be able to use the antibiotic resistance genes. The third is the phenotype, ok. So, phenotype is where you can actually be able to use that for that when you are when the cells are going to you know take the DNA, they are actually going to show you some phenotypic changes. So, either of these three broader criteria's can be used in different different screening methods.

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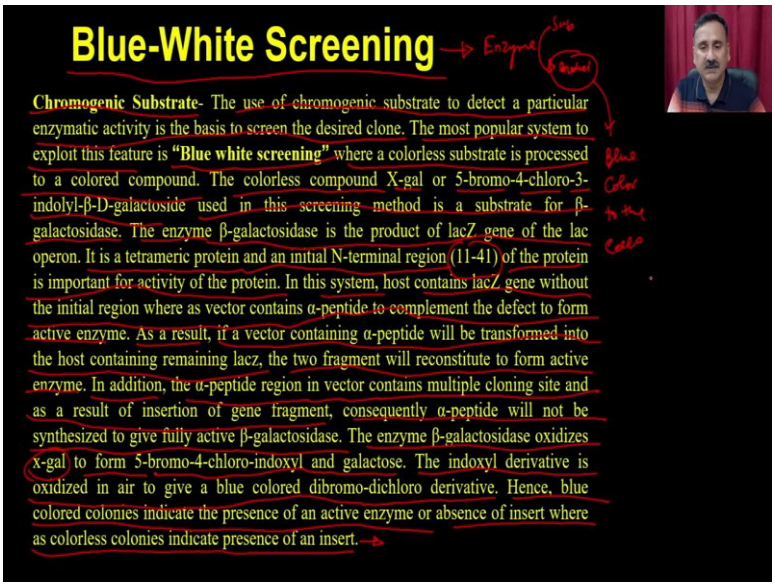

So, the first method what we are going to discuss is the blue-white screening. And blue-white screening is where you are actually going to use a enzyme to convert the substrate into the product and that is how this product is actually going to give the blue colored to the cell, ok. So, it is going to give the blue color to the cell. So, it is actually going to use a chromogenic substrate.

The use of the chromogenic substrate to detect a particular enzymatic activity is the basis to screen the desired clone. The most popular system to exploit this feature is called as blue-white screening where a colorless substrate is processed to a colored compound, right. The colored colorless compound x-gal or its also called as 5-bromo-4-chloro-3-indolyl beta-D-galactosidase is used in this screening method is a substrate for the beta-galactosidase.

The enzyme beta-galactosidase is the product of the lacZ gene of the lac operon. It is a tetrameric protein and it is and an initial n terminal region like the 11 to 41 of the protein is important for the activity of the protein. In this system the host containing lacZ lac gene without the initial region whereas, the vector contain the alpha peptide to complement the defect to form the active enzyme.

As a result, if a vector containing alpha peptide will be transformed into the host containing the remaining lacZ the two fragment will constitute to form the active enzyme. In addition, the alpha peptide region in the vector contains MCS and as a result

of insertion of the gene fragment consequently alpha peptide will not be synthesized to give the fully active beta-galactosidase.

The enzyme beta-galactosidase oxidizes the x-gal to form the 5-bromo-4-chloryl-indoxyl and galactose. The indoxyl derivative is oxidized in air to give a blue colored di-bromo di-chloro derivative. Hence the blue colored colonies indicate the presence of an active enzyme and the absence of insert whereas, the colorless colonies indicate presence of an insert. So, this is actually going to be reversal of what we have discussed, right.

So, if the enzyme is active it is going to convert the X-gal into the blue colored compound, but since we are going to clone the gene of our interest into the alpha region of the protein and once the clone is going, once the gene you are going to insert that it will not going to complement the remaining portion which is present in the host and that as a result the it will not going to show you the activity.

So, the cells which will not show you the activity and remain colorless are actually going to be the transformed cells. So, this is what we have explained here, right. So, the beta-galactosidase is a protein which is actually going to be expressed for lac operons.

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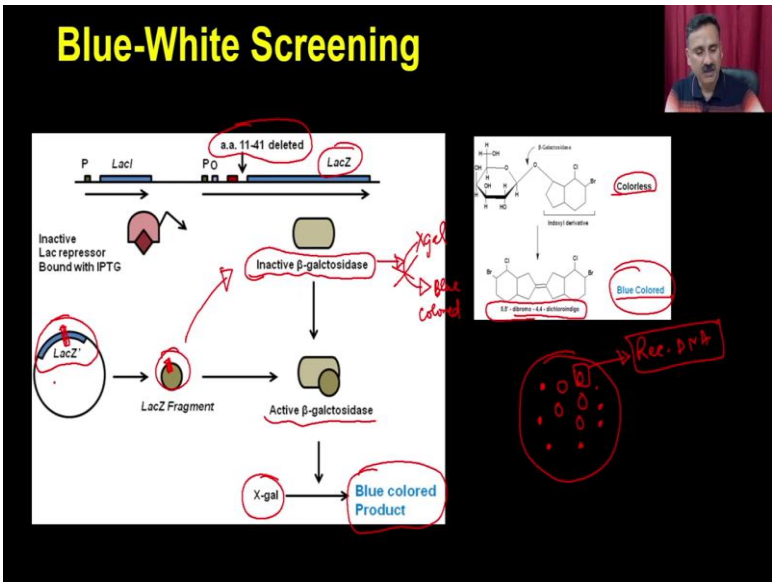

So, what you have is you have a lacZ gene which has a missing 11 to 41 region, ok. So, if you express that it is actually going to give you a inactive beta-galactosidase. Whereas,

this missing region is actually going to be present onto the vector which actually as call as lacZ prime.

So, this lacZ fragment when it combines with this inactive beta-galactosidase it is actually going to give you the active beta-galactosidase. And this active beta-galactosidase is going to convert the X-gal which is a colorless product into a blue colored product. And what reaction it is catalyzing it is actually converting this colorless compound into a blue colored 5-5 dibromo 4-4 dichloroindigo, ok.

So, this is the colored compound, blue colored compound. So, since you are cloning the gene into this particular lacZ area, right? You are even if the fragment is being produced it is also not going to complement the beta-galactosidase. And that is why you are going to have the two ah scenarios. In one scenario one when the only the vector is present it is actually going to give you the active beta-galactosidase and that is how it is actually going to be able to convert the colorless X-gal into the blue colored product.

But if the insert is present, it is actually going to give you the inactive beta-galactosidase and inactive beta-galactosidase will not be able to convert the X-gal into the blue colored compound, ok. And that is why if you see the reaction or if you see the colonies what will happen is that you are going to get the blue colored colonies and you are going to get the colorless colonies, ok.

So, these colorless colonies are the colonies where you are going to have your recombinant DNA, ok. Because of the simple reason that it is actually going to have the inactivation of that lacZ.

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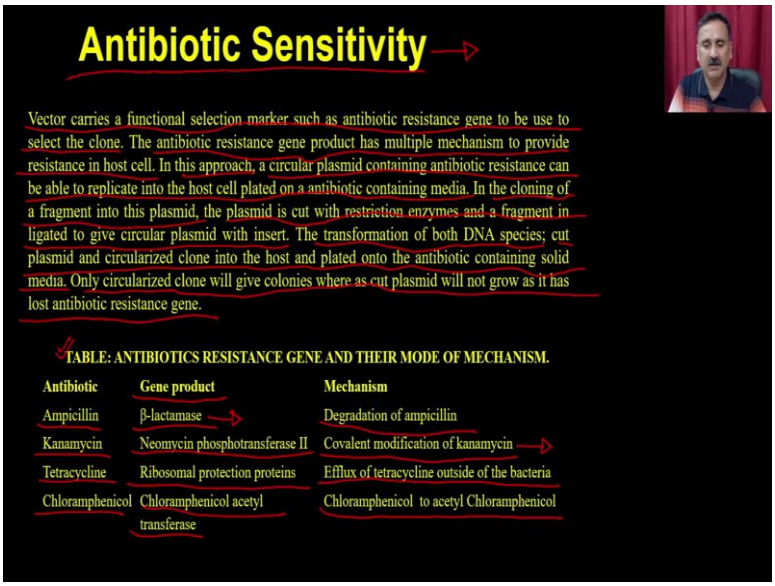

Then the second criteria is the antibiotic sensitivity, ok. So, antibiotics are the drugs which are actually being responsible for inactivation or the killing of the bacteria and it happens because the antibiotics disrupt the some of the you know functioning of the cellular properties, ok.

So, for example, there are antibiotics which are disrupting the translation steps, there are antibiotic which are disrupting the transcriptions and there are other antibiotic which are disrupting the protein synthesis. So, as you add the antibiotic into the media it will not allow the propagation of the normal bacterial cells, right. Because it is going to disrupt the some of these crucial metabolic pathways.

But if you have the antibiotic resistance genes. So, most of these antibiotic resistance genes are actually going to inactivate the exogenously added antibiotics and that is how it is actually going to allow the proliferation of the bacterial cell if they will actually going to have the transform bacteria.

So, in this case vector carries a functional selection marker such as the antibiotic resistance genes and to be used to select the clones. The antibiotic resistance gene product has a multiple mechanism to provide the resistance in the host cell. In this approach a circular plasmid containing antibiotic resistance can be able to replicate into the host cell plated onto a antibiotic containing media.

In the cloning of a fragment into the plasmid, the plasmid is cut with a restriction enzyme and a fragment in a is ligated to give circular plasmid with insert. The transformation of the both DNA species cut, plasmid and the circularized clone into the host and plated onto antibiotic containing solid media. Only circularized clone will give colonies whereas, cut plasmid will not grow as it has lost antibiotic resistance genes.

So, this is the table what I have given and you will see that these are the antibiotics like the ampicillin, kanamycin, tetracycline, chloramphenicol and these are the gene product from the antibiotic resistance genes. So, it will actually going to be a beta-lactamase, neomycin, phosphotransferase, ribosomal protection proteins and a chloramphenicol acetyl transferase.

So, these are the gene product which are going to be responsible for the inactivation of these antibiotics. And what is the mechanism? For example, in the case of beta-lactamase it is actually going to degrade the ampicillin. Similarly, in the neomycin phosphotransferase, it is actually going to make the covalent modification of the kanamycin and as a result the kanamycin will not be able to you know do its action.

Similarly, we can have the ribosomal protection protein which is actually going to have the efflux of the tetracycline outside the bacteria. So, that is how since the tetracycline will not be able to enter into the bacteria, it will not be able to interfere with the protein synthesis. Similarly, we can have the CAD genes and the CAD gene is going to acetylate the chloramphenicol to acetyl chloramphenicol and that also is going to interfere with the action of the chloramphenicol.

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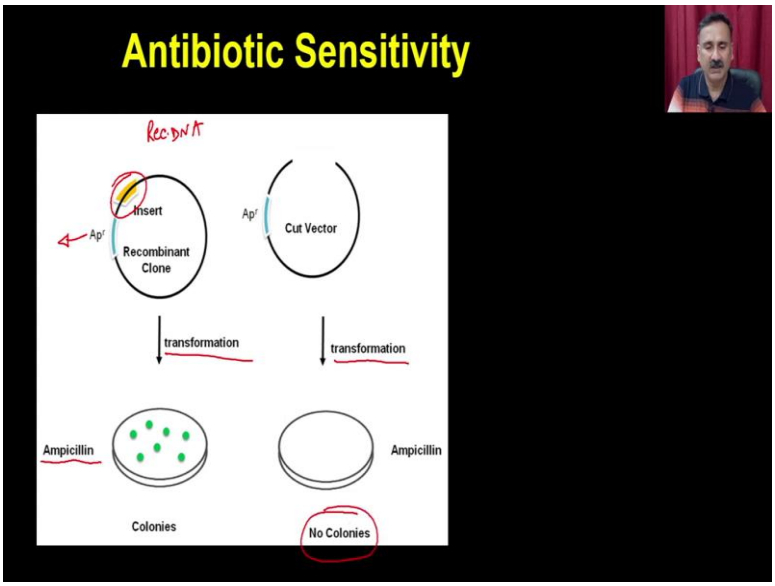

So, what we are going to do in this is we are going to have the two DNA species. One is this is the recombinant clone where you have cloned the DNA into the MCS and it is going to be circularised. Whereas once you have the cut vector it is does not have the you know its circular DNA, ok. So, once you transform and if you put it onto the ampicillin containing plate, this bacteria is actually going to grow because it has the ampicillin resistance gene and that will actually going to degrade ampicillin.

So, it will actually allow the bacteria to grow whereas, when you do the transformation of this cut vector. Since the cut vector will not be able to replicate, it will actually going to will not be able to express the ampicillin resistance gene and as a result it will not be able to go in form the colonies.

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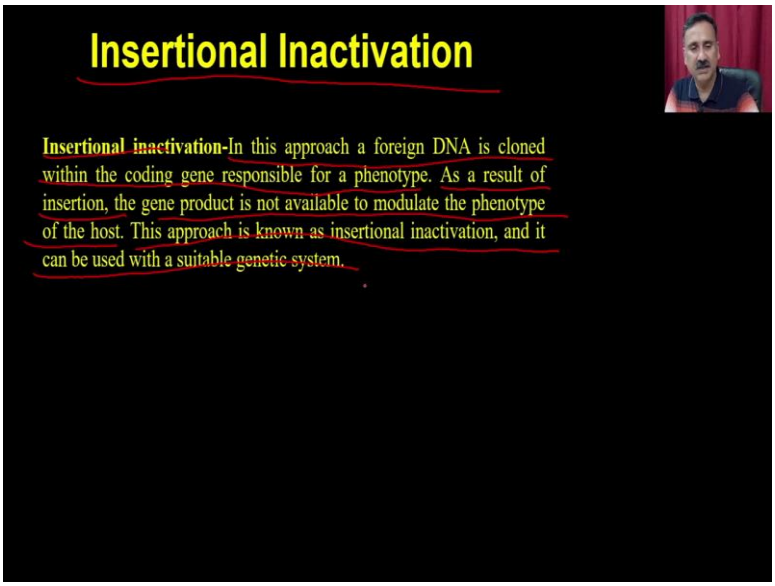

Then the third approach is the insertional inactivation's, ok. So, insertional inactivation in this approach a foreign DNA is cloned within the coding gene responsible for a phenotype. As a result of insertion, the gene product is not available to modulate the phenotype of the host. This approach is known as insertional inactivation and it can be used for suitable gene generic genetic system.

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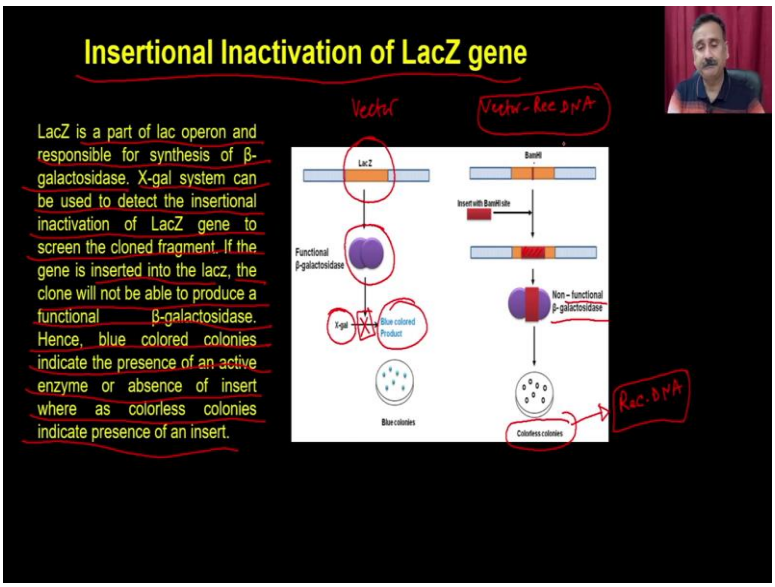

For example, in an insertional inactivation of the lacZ, right. So, lacZ is a part of the lacZ operon and it is responsible for the synthesis of beta galactosidase. And you know that

the X- gal system can be used to detect the insertional inactivation of the lacZ gene to screen the cloned fragment. If the gene is inserted into the lacZ, the clone will not be able to produce a functional beta galactosidase.

Hence, blue colored colonies indicate the presence of an active enzyme or the absence of insert whereas, the colorless colonies indicate the presence of an insert. So, this is what you have a lacZ which is actually going to produce the functional beta-galactosidase and that is actually going to convert the X-gal into a blue colored compound. And as a result, what you are going to see is you are going to see a blue colored colonies.

But if you have the BAMH1 site and if you use this BAMH1 site which is there in the lacZ and if you use that and you will insert then you are actually going to put your insert within the coding sequence of the lacZ. And as a result, what will happen is that it is actually going to give you the non-functional beta galactosidase.

And if you have the non-functional beta galactosidase, it will not be able to catalyze this particular reaction and as a result it is actually going to give you the colorless colonies. So, these colorless colonies are the colonies which are actually going to have the recombinant DNA. So, it is actually going to say that ok, recombinant DNA is present.

So, if you are transforming the vector and if you are transforming the vector which contains the recombinant DNA, the colorless colonies are going to say that it is a vector which contains the recombinant DNA.

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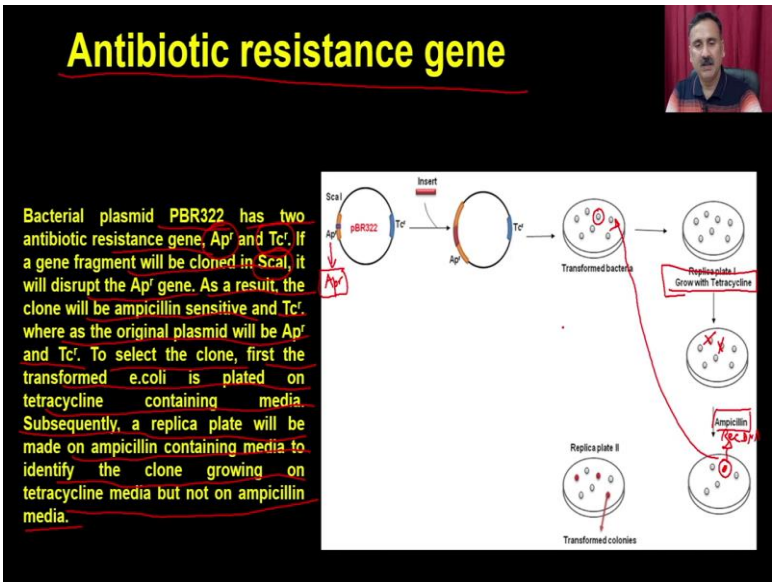

Then we have the insertional inactivation of the antibiotic resistance genes and that we this example I have taken from the vector which is called as PBR322. So, in the PBR322 it has the two antibiotic resistance genes. It has the ampicillin resistance genes and it has the tetracycline- resistance genes, ok.

So, if a gene fragment will be cloned in Sca1 which is a restriction enzyme, it will disrupt the ampicillin resistance gene. And as a result, the clone will be ampicillin sensitive and tetracycline resistance, ok. So, whereas, the original plasmid will be ampicillin and tetracycline resistance. To select the clone first the transformed e.coli is plated onto a tetracycline containing media.

Subsequently a replica plate will be made on the ampicillin containing medium to identify the clone growing on the tetracycline media, but not on the ampicillin media. Which means if you have cloned a fragment into Sca utilizing the Sca as a (Refer Time: 18:33) site. What will happen is that it is actually going to disrupt the ampicillin resistance, ok. So, it is going to disrupt the beta-lactamase gene. So, as a result this particular clone is going to be sensitive for the ampicillin action.

Now, what we are going to do is we are going to transform this onto the in to the bacteria and we are going to get the colonies. Now, this transformed bacteria what you will do is just make a replica plate of this plate and then you grow this first with the tetracycline, ok.

So, when you grow them with the tetracycline both of these clones are actually going to grow, ok. And then if you put them onto the ampicillin what will happen is, that it is this ampicillin resistance is actually going to kill some of the bacteria. For example, in this case if you compare this and that what you see is this particular bacteria is being not present here.

So, and similarly you can have some more bacterial colonies which are going to be present in the presence of tetracycline, but they will not be present in presence of ampicillin. So, these are the clone which are actually containing the recombinant DNA, ok. So, what you are going to do is you can just go back and take out these clones from the master plate and that is how you are going to be able to select the transform plates or you are going to be select the colonies which contains the recombinant DNA.

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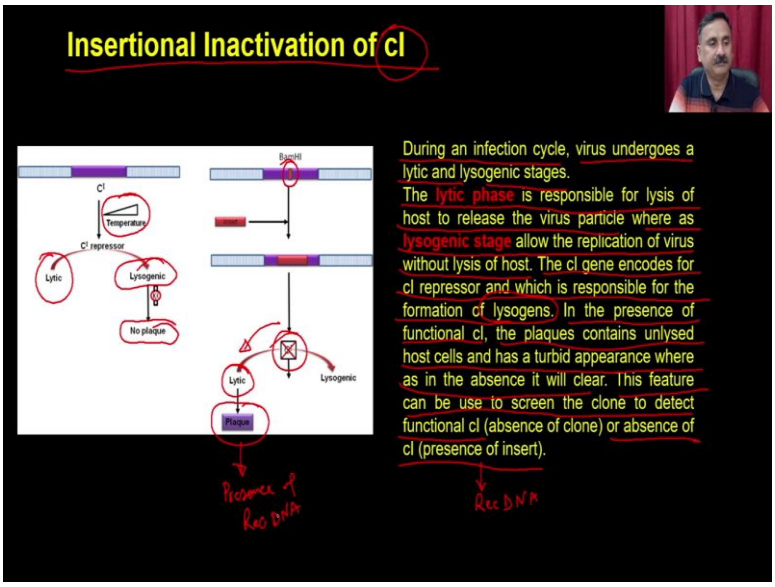

Then we have the third example of insertional inactivation of the cI repressor. So, cI repressor is a protein which is responsible for the shuttling of the virus between the lytic phase and the lysogenic phase. So, during an infection cycle the virus undergoes a lytic and the lysogenic stages and the cI repressor is a protein which is going to you know function as the shuttling protein.

So, the lytic cycle phase is responsible for the lysis of the host to release the virus particle. Whereas the lysogenic phase allow the replication of the virus without the lysis of the cI gene encodes for a cI repressor and which is responsible for the formation of the

lysogens, ok. In the presence of the functional cI the plaque containing unlysed host cells and has a turbid appearance. Whereas, in the absence of it will be clear. This feature can be used to screen the clone to detect the functional cI or the absence of cI, ok.

So, if we have a functional cI it will say that you do not have the recombinant DNA, if you have the non-functional cI then it will say ok, recombinant DNA, ok. So, this is what it is shown here, right. So, cI is a temperature sensitive suppressor repressor. So, if you change the temperature the cI repressor is going to be express and it is actually going to shuttle the virus from the lytic to the lysogenic phase, ok. And if it is in the lysogenic phase, it will not going to allow the formation of the plaques.

But when you clone the protein and you clone it into the cI repressor gene it is actually going to produce a non-functional cI repressor, ok. And when you present the non-functional cI repressor repressor it will actually going to shift the protein shift the cycle towards the lytic phase, ok and. As a result, it is actually going to form the plaque which are going to be you know which will say that it is actually going to be the presence of recombinant DNA.

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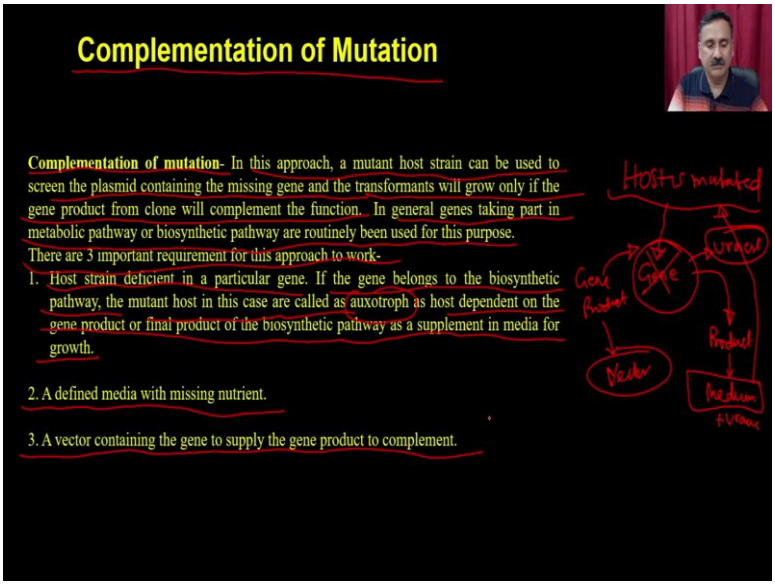

Then the third approach is the complementation of the mutations. So, complementation of the mutation in this approach a mutant gene can be used to screen the plasmid containing the missing gene and the transformant will grow only if the gene product from the clone will complement the function.

In general, the gene taking part in the metabolic pathway or biosynthetic pathway are routinely used for this purpose. There are three important requirement in this approach, ok. So, what is the complementation of the mutation is that the host is mutated for a crucial gene, ok. So, this host will not grow until this particular gene product. So, this it is actually missing with this particular gene.

So, if you supply the gene product right if you supply the gene product which is present on the vector then this is actually going to grow, ok. So, this is called as complementation that the host is mutated in such a way that it will not grow until you provide the gene product and that gene product you are actually going to provide by the recombinant DNA. So, in this there are three requirements of this approach.

The host strain deficient in a particular gene if the gene belongs to the biosynthetic pathway the mutant host in the case are called auxotroph, as host depends on the gene product or the final product of the biosynthetic pathway as a supplement in the media for the growth. So, in some cases this particular gene could be a part of the metabolic pathway and gene could be actually be responsible for providing the some crucial biosynthetic molecule.

So, either you provide the gene product or you can be able to you can be able to provide that product into the media, ok. And as a result, it is actually going to give you the growth of this mutant this mutated host in the presence of this particular product in media. For example, if this gene is responsible for the synthesis of uracil, ok. So, if you do not have this gene the uracil will not be able to synthesize and this particular host will not be able to grow.

But if you provide the uracil into the media then if you add the uracil into this media then this media is actually going to supply the nutrient and that is how this host is actually going to grow. Then a defined media. So, you should have a defined media with a missing nutrients, right. Because while you are doing growing this particular host you actually can use the media which actually contains the uracil, but you should also have a defined media where this particular nutrient is also missing.

So, that when the nutrient is missing the host is looking for that particular nutrient and that nutrient you will get if the vector is going to supply the gene. And then you also require a vector containing gene to supply the gene product to complement.

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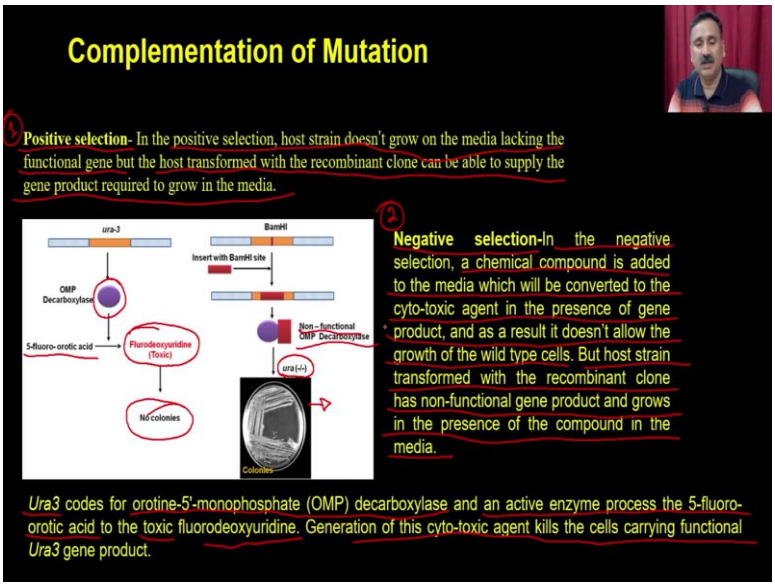

Now, let us see how it works. So, you can actually be able to do the complementation to the mutation in a positive feed selection or you can actually be able to do the negative selection. In the positive selection in the positive selection host strain does not grow on the media lacking a functional gene, but the host transformed with the recombinant clone can be able to supply the gene product required to grow in the media. So, that is called as a positive selection.

Positive selection means you are supplying the gene product from the recombinant DNA and that is how the host is actually going to survive and it will actually grow and it will give you the colony. Negative selection negative selection is that when you are actually going to you know restore the activity of the gene it will actually going to kill the transformed host.

So, in the negative selection a chemical compound is added to the media which will be converted into a cytotoxic agent in the presence of the gene product and as a result it does not allow the growth of the wild type cell. But the host strain transformed with the recombinant clone has non-functional gene product and it will grow in the presence of compound in the media.

For example, in this particular case we have taken an example where we have taken an example of Ura3. So, Ura3 is a gene which codes for the orotidine 5 prime monophosphate or OMP decarboxylase. And an active enzyme process this particular

compound which is called as 5-fluoro-orotic acid to a toxic compound which is called as fluorodeoxyuridine. And generation of the this toxic compound kills the cells carrying the functional Ura3 genes.

So, what you have is we have the gene of Ura3 which actually provides a bro enzyme which is called as OMP decarboxylase. And OMP decarboxylase process this particular compound like 5-fluoro-orotic acid to the fluorodeoxyuridine which is a toxic compound. And when the this called toxic compound is being generated it will actually going to kill the cells.

This means if you have the functional OMP decarboxylase it is actually going to indicate that there is no recombinant DNA. Similarly, if you have cloned the fragment within this particular gene then what you have done is you have done the insertion inactivation of this particular gene. Now, if you have done the insertion inactivation or you have produced the non-functional OMP decarboxylase this non-functional OMP decarboxylase is not going to you know convert the 5-fluoro-orotic acid to the fluorodeoxyuridine.

And as a result, you can allow the growth of these cells and the Ura3 minus minus cells, ok. So, this is actually going to give you cell you that if you got the colonies this means the OMP decarboxylase is inactive. Then these are the; these are the methods are more popular in the prokaryotic system. Let us talk about now how you can be able to screen the clones into the mammalian system.

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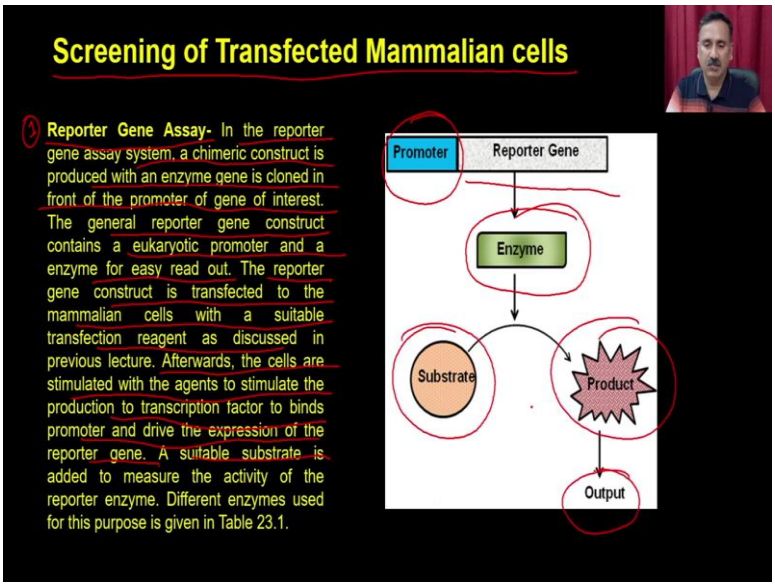

So, a screening of the transfected mammalian cells. First method is the reporter gene assay, ok. [FL] in the reporter gene assay system a chimeric construct is produced with the enzyme gene with the with an enzyme gene which is cloned in front of the promoter of the gene of interest. The gene reporter gene the gene reporter gene construct contain a eukaryotic promoter and an enzyme for easy readout.

The reporter gene construct is transfected into the mammalian cells with a suitable transfection reagent, right. Afterwards the cells are been stimulated with the agent to stimulate the production of transcription factor to bind the promoter and drive the expression of the reporter gene.

A suitable substrate is added to measure the activity of the reporter gene. So, this is what you have the promoter which and you also have a gene a reporter gene which is going to express enzyme and this enzyme is going to convert the substrate into the product and this product readout you can be able to study with the help of the several methods like you can do the fluorescence, you can do the luminescence, you can also be able to do the (Refer Time: 30:08), ok.

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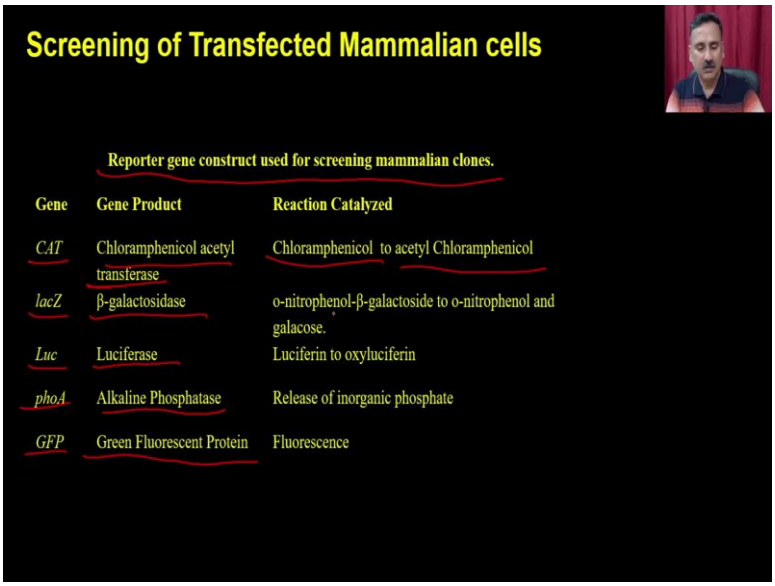

So, these are the reporter gene construct what you can use for screening the mammalian clothes like the CAT gene, lacZ, luciferase, phoA and the GFP and the gene product are cat gene chloramphenicol acetyl transferase then lacZ beta-galactosidase, Luc is for luciferase and phoA is for alkaline phosphatase and GFP is the green fluorescent protein.

And the reaction what you are going to see for catalysing is that when you have the chloramphenicol acetyl transferase it is going to run like chloramphenicol to acetyl acetyl chloramphenicol and so on.

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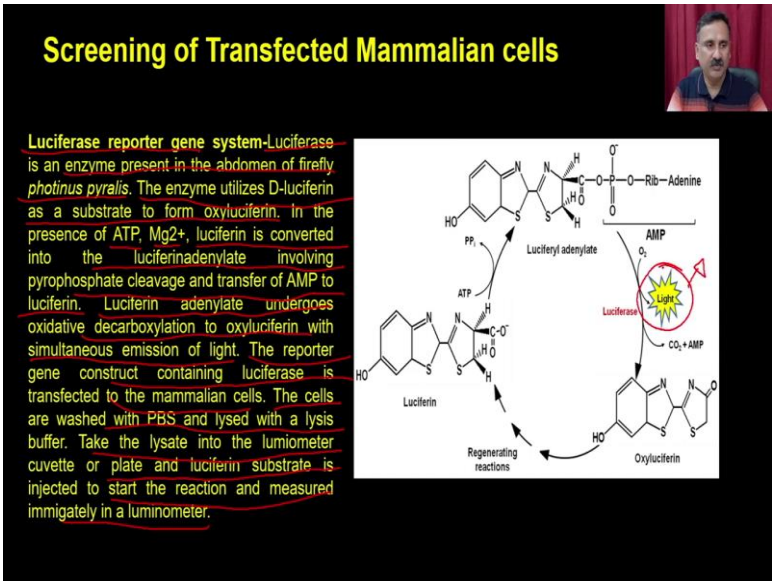

So, in a typical reaction you can actually be able to use like luciferase for example, reporter gene system. So, luciferase is an enzyme which is present in the abdomen of firefly, photinus pyralis the enzyme utilizes the D-luciferin as a substrate to form the oxyluciferin. In the presence of ATP magnesium luciferin is getting converted into the luciferinadenylate involving pyrophosphate cleavage and the transfer of AMP into the luciferin.

And the luciferin adenylate undergoes oxidative decarboxylation to form the oxy-luciferin and simultaneously there will be a emission of light. The reporter gene construct containing luciferase is tranfected into mammalian cell the cells are washed with PBS and lysed with the lysis buffer, take the lysate into the luminometer cuvette and luciferin substrate is injected to start a reaction and measured immediately in a luminometer.

So, these are the reactions what your luciferase is going to catalyze and ultimately it is going to produce the light. And this light can be measured with the help of the luminometer.

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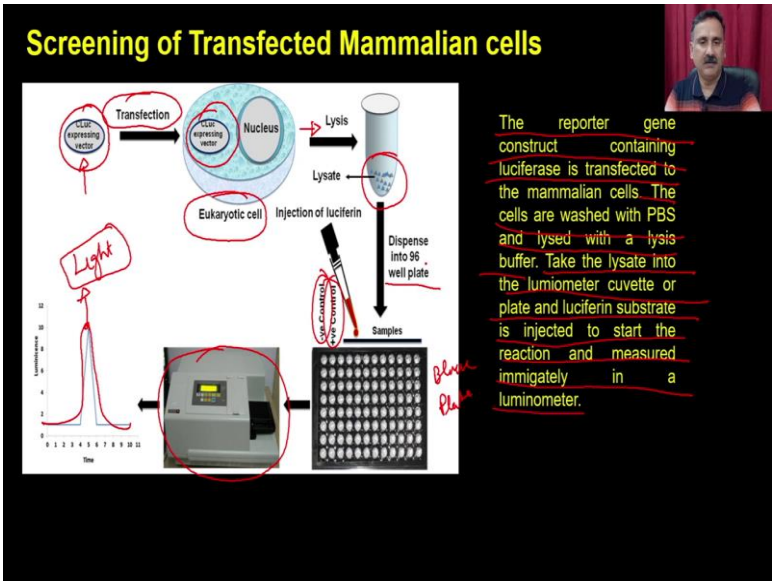

So, what you are going to do is you are going to first take the CLuc expressing vector you are going to clone your recombinant DNA into this, right. And then you are going to do the transfection once you got the transfection you are going to have the eukaryotic

cell which has this recombinant DNA which contains the CLuc gene in front of the promoter.

And then you what you are going to do is you are going to lyse the cells and that is how you are going to have the cell lysate and this cell lysate you can put into the 96 cell plate you can take the black plate, right. And you can take the negative controls, you can take the positive control and so on.

And then you can just put it into the luminometer, right. And what luminometer is going to do is it is actually going to give you the signal for the luminescence and that signal is response is actually a light which is going to come from the activity of the luciferase. The reporter gene construct containing luciferase is transfected into the mammalian cells the cells are washed with PBS and lysed with a lysis buffer take the lysate into the luminometer cuvette or plate.

And you add the luciferrin substrate and it is injected to start the reaction and measured immediately in a luminometer.

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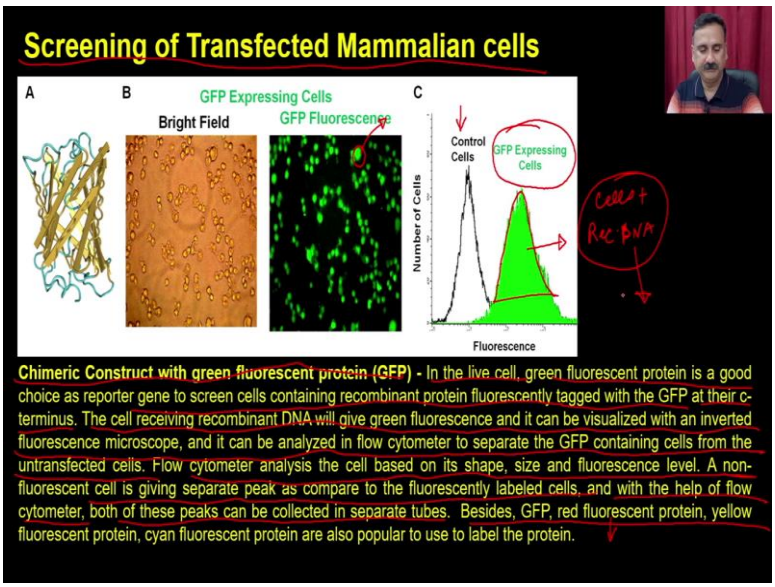

The second method is you can actually be able to use some fluorescent protein to look at the transfection of the screening of the transfected mammalian cells. So, you can take the chimeric construct with the GFP protein for example. So, in the live cells the GFP

protein is a good choice as the reporter gene to screen cells containing recombinant protein plus fluorescently tagged with the GFP at their c or the n terminus.

The cells receiving recombinant DNA will give green fluorescence and it can be visualized with an inverted fluorescence microscope and it can be analyzed in a flow cytometer to separate the GFP containing cells from the untransfected cell. Flow cytometer analysis the cell based on the shape, size and fluorescence a non-fluorescent protein cell is giving separate peak as compared to the fluorescent labeled cell and with the help of the flow cytometer.

Both of these peak can be collected in a separate tube besides GFP the other protein what you can use is RFP and YFP and CFP. And so, what you are going to do is you are going to translate the cells and what you see in the under the inverted microscope that all the cells are showing a green color fluorescence. And if you want be interested to collect these cells what you can do is you can just put into the flow cytometer and flow cytometer is actually going to separate the molecules based on the fluorescence.

So, these are the control untransfected cell, these are the GFP expressing recombinant DNA containing cells. And that is how you can actually be able to select these cells in a separate tube and they will be the recombinant cells or the cells with the recombinant DNA cells with recombinant DNA. And these cells you can separate out and that is how you can actually be able to use them for subsequent experiments.

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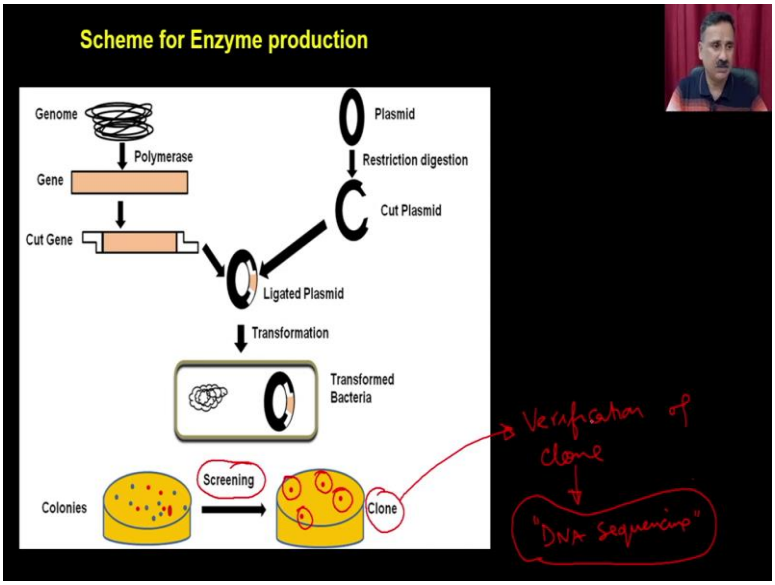

So, this is what we have discussed so far how you can be able to screen the compounds you can get the suitable clones. Now, the question is, if you have done the cloning and you got the suitable clone how you can be able to verify the clone, ok. So, because that is very important, right. So, you can actually be able to verify the clone by several method, ok. So, what we have discussed so far?

We have discussed about the screening of the clone and now what you got is you got the recombinant clone which are present in the LBA plate. Now, the next question is how you can be able to verify the clone, ok. Because the verification of the clone is very important to that. So, that you should not misguide because you know and verification of the clone can be done with the help of the DNA sequencing.

You can actually be able to sequence the clone to know that whether the your the gene of your interest is fragment is also present, ok.

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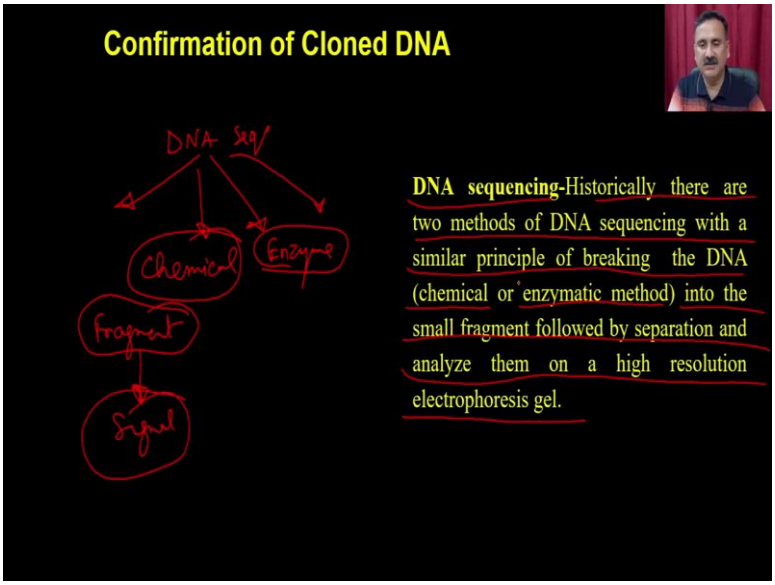

So, the confirmation of the cloned DNA can be done by the DNA sequencing. Historically there are two method of DNA sequencing with a similar principle of breaking the DNA either the chemical method or the enzymatic method into the small fragment followed by the separation and analyze them on a high resolution electrophoresis gel.

So, in a typical DNA sequencing what you are doing is you are taking the DNA sequence, ok. And then you are breaking that into the multiple fragments and all these fragments either you are using the chemical method which is called as Maxam Gilbert method or you are using the enzymatic method which is called as Sanger's method.

So, either with the help of the chemicals or the enzyme you are you know breaking this DNA sequence into the smaller fragment. And all these fragments are then going to be separated in a high resolution polyacrylamide gels and then the signal are going to be analyzed for interpreting the sequences. So, let us first talk about the enzymatic method or the Sanger method.

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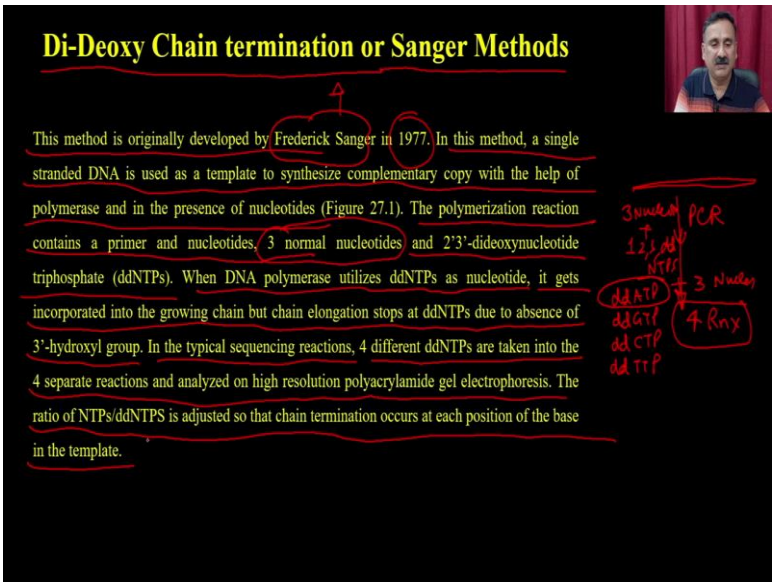

So, the first method what is being discovered is the Di Deoxy chain termination or the Sanger's method. This method is originally being developed by the Frederick Sanger's in the year of 1977 for which the Frederick Sanger's got the Nobel Prize. In this method a single stranded DNA is used as a template to synthesize the complimentary copy with the help of a polymerase in the presence of nucleotide. The polymerization reaction contains a primer and a nucleotide.

So, you can have the 3 normal nucleotides and a 2 prime 3 prime di deoxy nucleotide triphosphate. This means it is actually you are going to take the single standard DNA and you are going to perform the PCR with the help of the three nucleotides which are going to be normal plus one nucleotide which is 2 prime 3 prime ddNTPs.

So, what will happen is. So, you are going to run the multiple reactions of the same for the same DNA. So, but you will actually going to change the ddNTPs. In some cases, you are going to take the ATP, in some cases you will take the GTP, you are in some cases you will take the CTP and in the other case you will take the TTP, ok. So, this means for every DNA sequence you are going to run the four reactions. And in four reactions you will take either the ddATP plus you will take the all other remaining three nucleotides, ok.

So, that is how you are going to you know make the four different types of reactions. When the DNA polymerase utilizes a di deoxy nucleotide as a nucleotide it gets incorporated into growing chain, but the chain elongation stops at the ddNTPs to the absence of the 3 prime hydroxyl group. In the typical sequencing reaction, you are going to run four different ddNTPs are taken into the four separate reaction and analyze on to a high resolution polyacrylamide gel electrophoresis.

The ratio of NTP and ddNTPs is adjusted. So, the chain termination occur at the each position of the base in the template.

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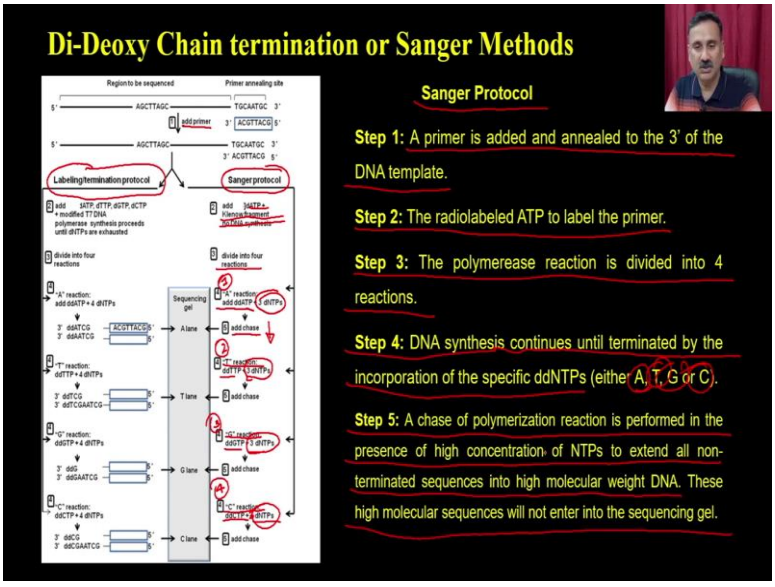

Now, this is what exactly you are going to do. In the Sanger's Protocol what you are going to do is you are going to first take the DNA sequence, ok. And you are going to have the terminal sequence, ok. So, then you add the primers ok, and when you add the

primer ok, and you are going to have the two options either you go with the Sanger's Protocol or you go with the labelling as well as the termination protocol.

So, in the Sanger's Protocol what you are going to do is you are going to add the dATPs and the klenow fragments and you are going to label the DNA and then you are going to divide this DNA into the four reactions. In the reaction A or that will called as A reaction you are going to add the ddtATP and the remaining three NTPs. In the second reaction. So, this is the reaction number 1, in the second reaction you are going which is called as T reactions you are going to add ddTTP.

But and the rest three nucleotides which are normal nucleotides. Then in the reaction number 3 you are going to add the or which is called as G reaction which is called as ddGTP and the 3G dNTPs and in the fourth reactions you are going to take the C reaction which is called as ddCTP plus the four three remaining NTPs. And after every reaction you add, you are going to put them for chase which means you will allow the DNA to be synthesized.

So, in the step 1 your a primer is added and annealed to the 3 prime of the DNA template the radiolabeled ATP is been added to label the primers then the step 3 the polymerase reaction is divided into the four reactions and in the step 4 DNA synthesis continue until terminated by the incorporation of the specific ddNTPs either the A, T, G or C.

And in the step 5 a chase of polymerization reaction is performed in the presence of high concentration of NTPs to extend all non-terminated sequences into the high molecular weight DNA this high molecular weight DNA will not enter into the sequencing reaction.

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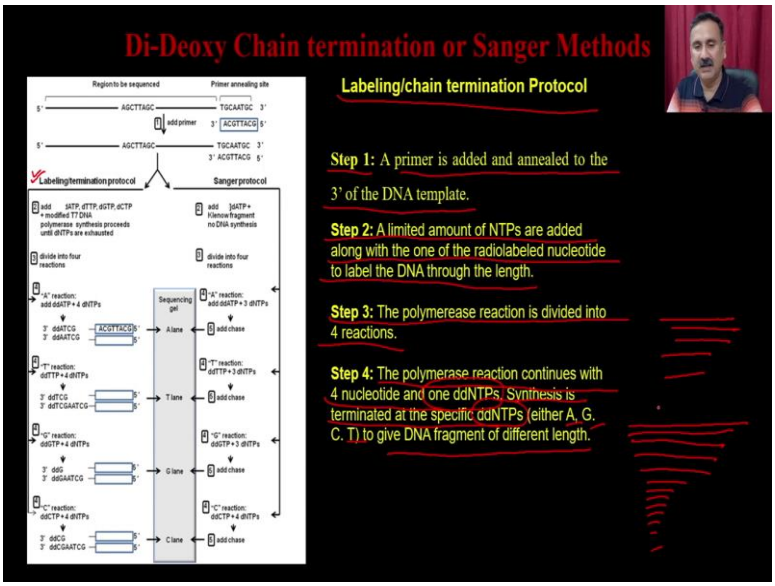

In the labeling or the chain termination protocol this is the labeling and the chain termination protocol in the step 1 a primer is added and a lean to the 3 prime of the DNA template then the step 2 a limited amount of NTPs are added along with the one of the radio labeled nucleotide to label the DNA throughout the DNA throughout the length. Then the step 3 the polymerase reaction is divided into the 4 reactions just like as we discussed here.

And the polymerase reaction continues with the four nucleotide and out of four one of them would be the diodeoxy NTPs. Synthesis is terminated at the specific ddNTPs either A, G, C or T to keep the DNA fragments of the different length. Now, what you got the DNA fragments of the different length right from see for example, from the same DNA you are going to get first big strand then you are going to get this then you are going to get this like this, right.

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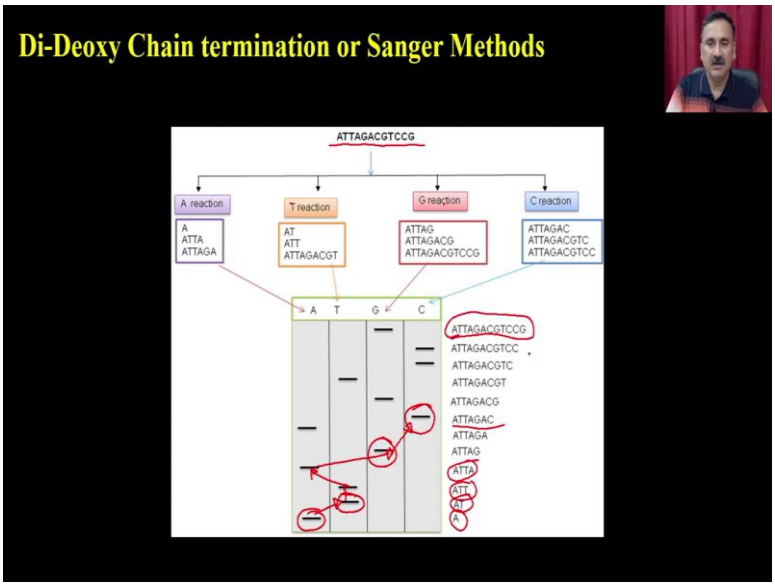

And so, all these fragment has to be analyzed onto a high resolution SDS phage. And when you analyze them, you are going to get this kind of fragments, ok. So, you are going to divide that into four reactions A reaction, T reaction, G reaction and C reaction. Now, imagine that we have started with this particular DNA sequence. So, A reaction you are going to get a fragment here, right. For T reaction you got the fragment here ok, and for G reaction you got this about here and for the C reaction you got the fragment here, ok.

Now, what you have to do is you have to run in a reverse orientation like this. You have to walk when you interpret this you are going to walk like this, ok. And that is how you are going to get first some A then AT then ATT then ATTA. So, that is what you are going to do and that is how you are going to get the complete fragment DNA or the sequence of the DNA what you have started with, ok.

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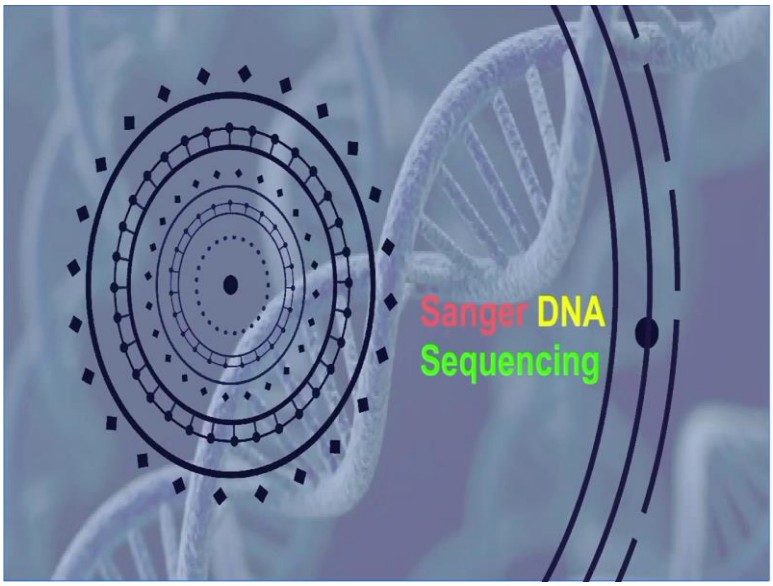

So, in a Sanger sequencing method what you have is you have is you know a you know DNA what you have a target DNA and that you have to sequence.

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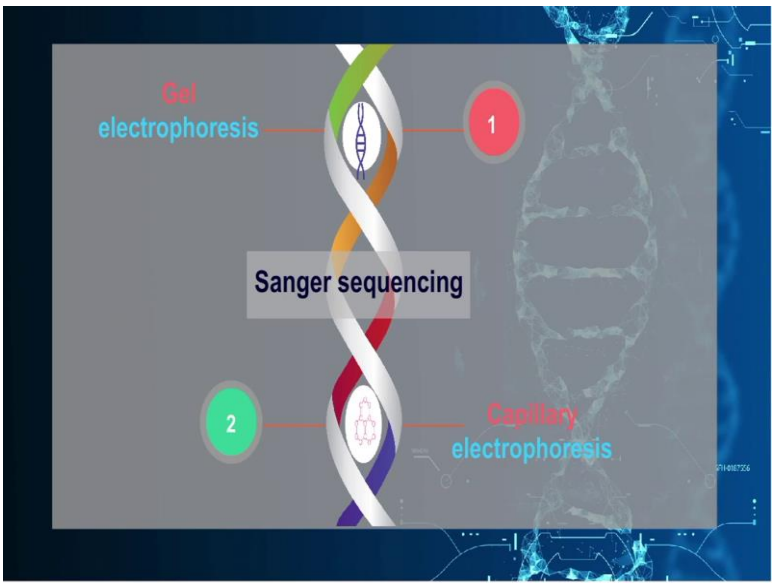

So, what we have you know you know if you want to do a DNA sequencing using the Sanger sequencing method you have the two way either you can go with the gel filtration chromatography, gel electrophoresis or you can do the capillary electrophoresis.

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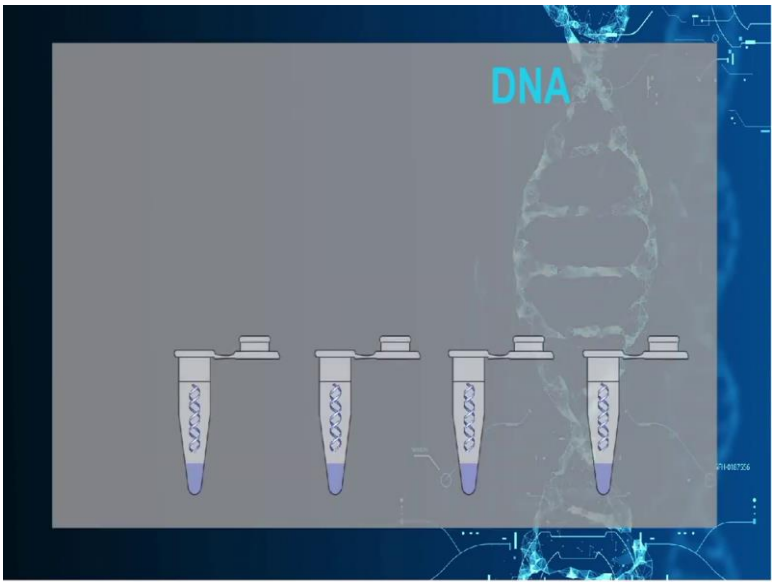

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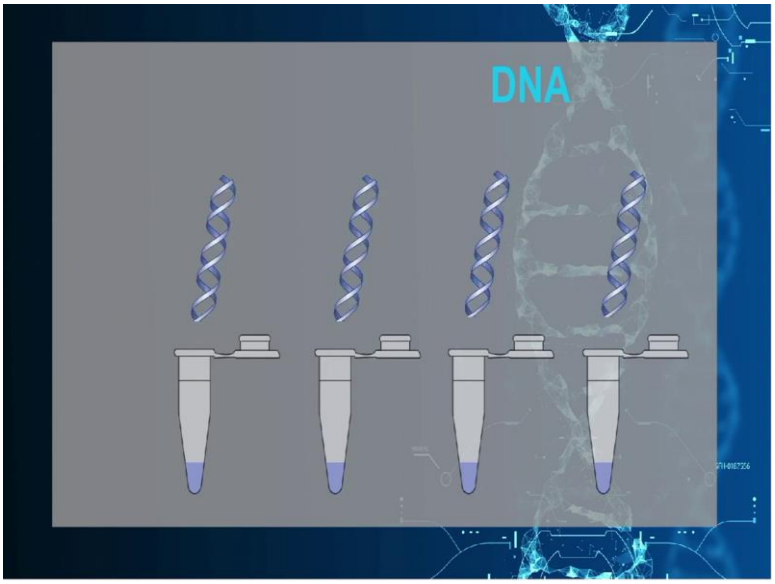

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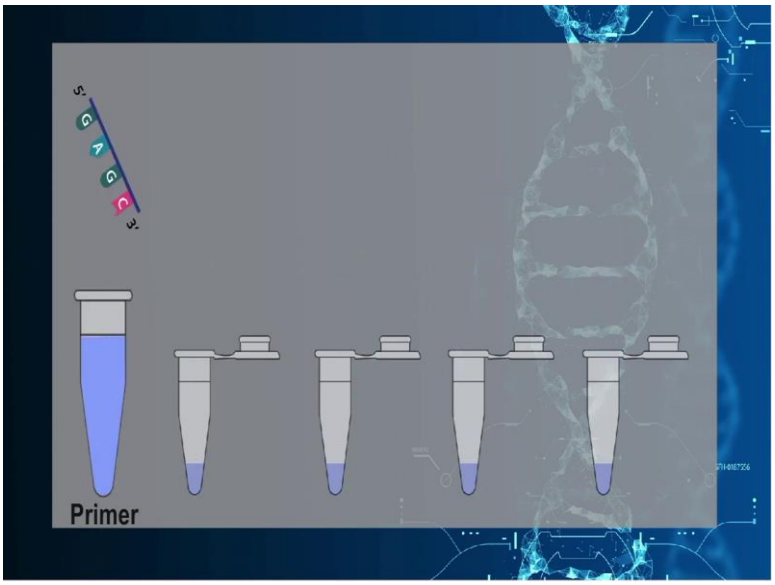

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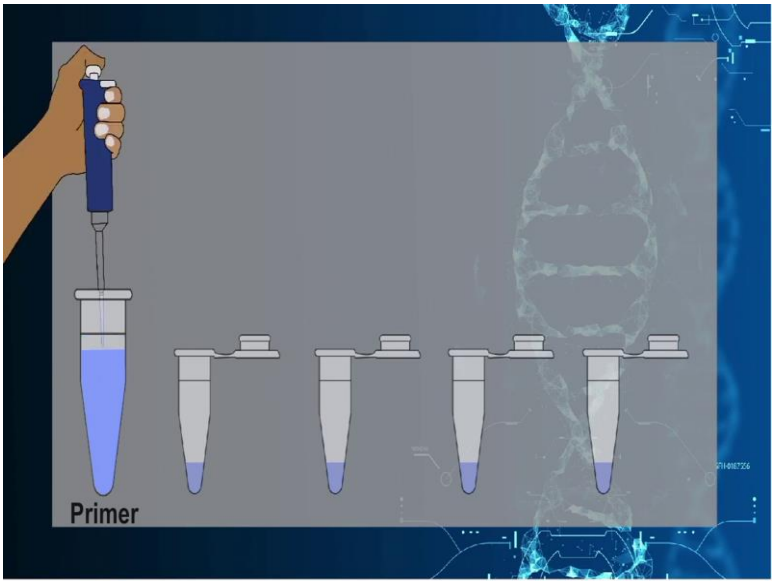

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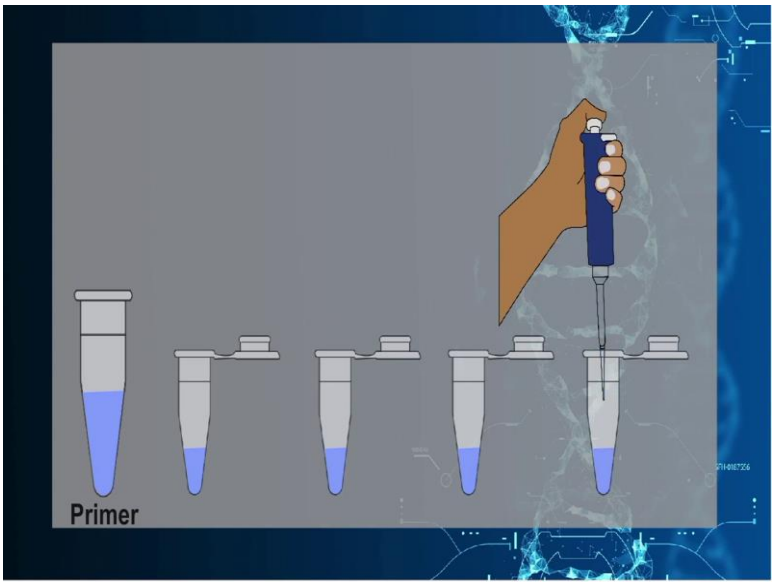

So, the first step is that where you have to take the your DNA into the appendox.

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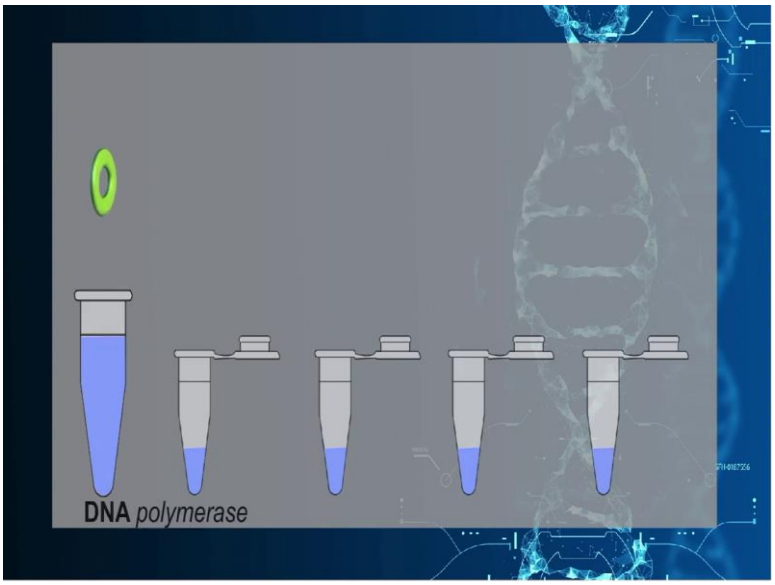

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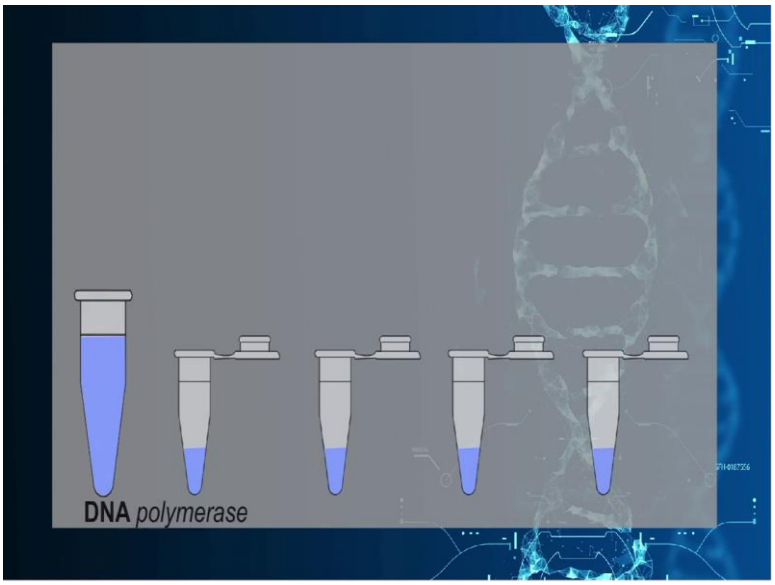

And then you have to add the primers. These primers you have to add into the four reactions.

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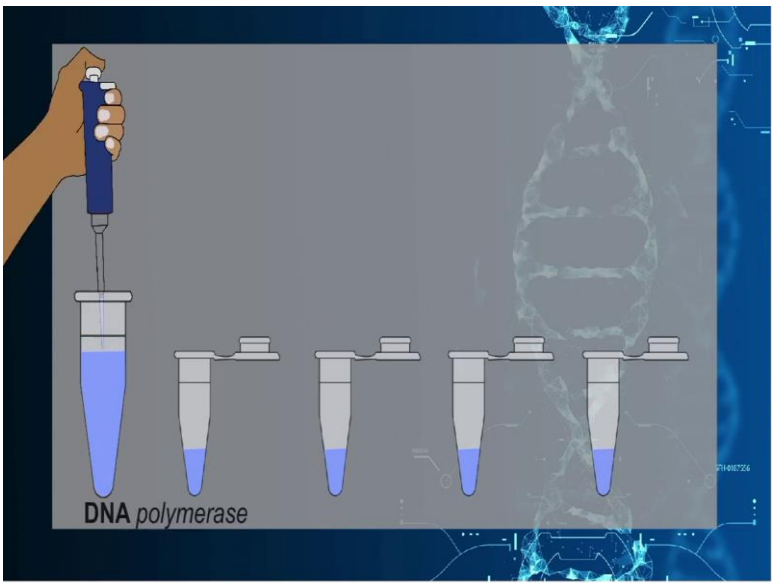

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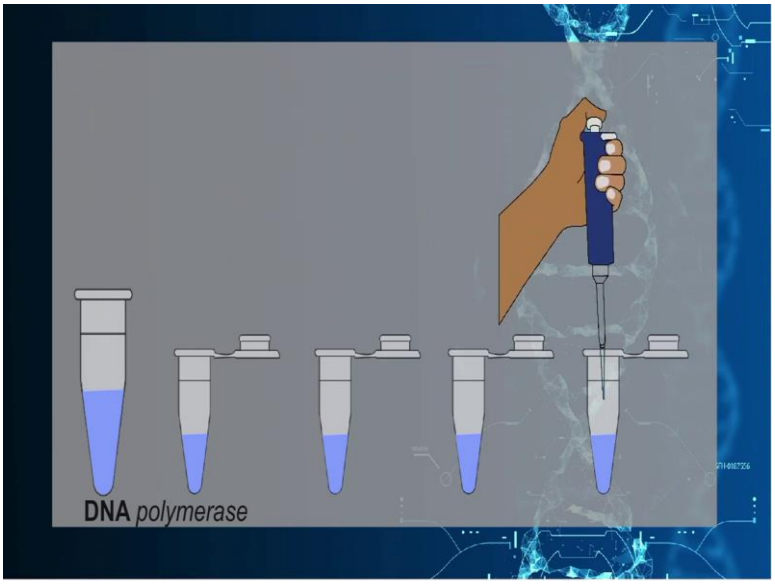

If you remember we have said that you have to divide the reactions into the four reactions and then you have to add the DNA polymerase into the each reactions. So, you have to add the reaction number 1, 1, 2, 3 and 4. And once you added the DNA polymerase into the four reactions then you are going to add the nucleotides.

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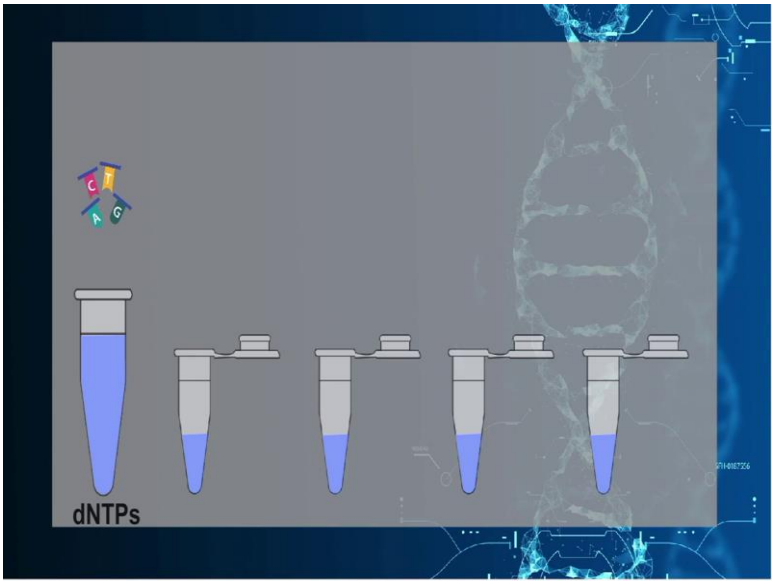

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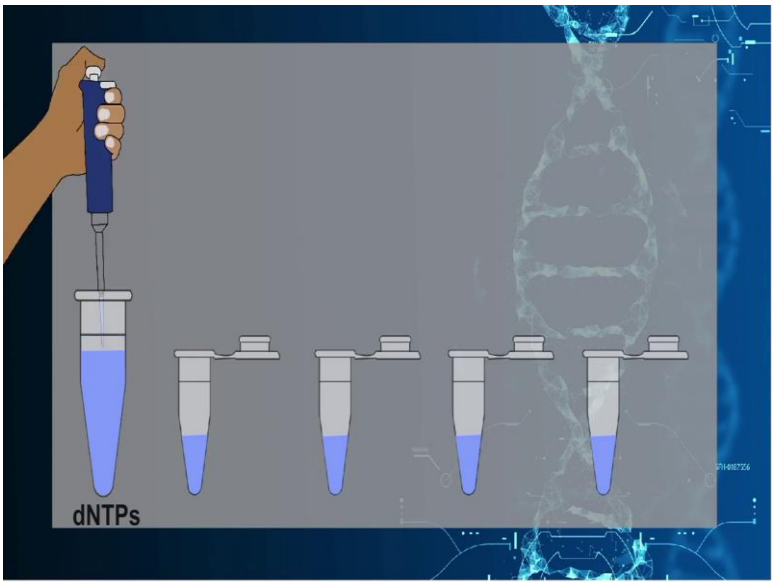

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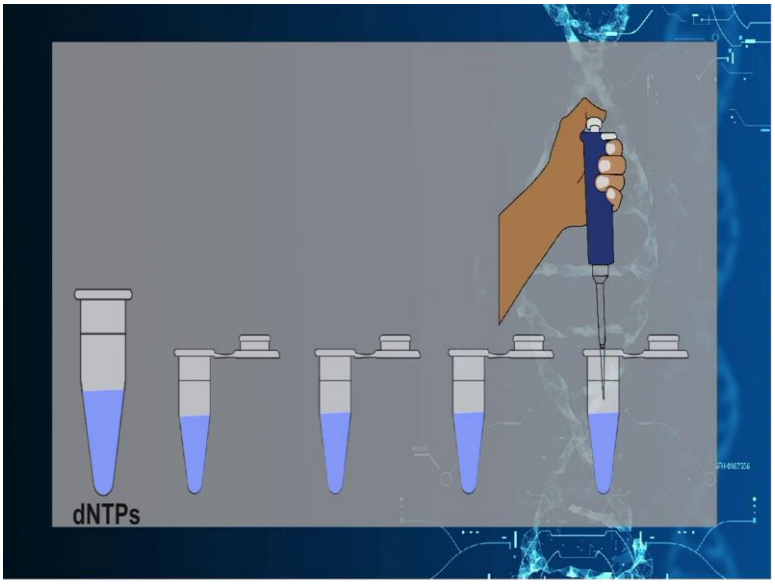

You have to add the all the four nucleotides like C, T, A and G in all the four reactions and in the subsequent step you are going to add the dideoxy- nucleotides.

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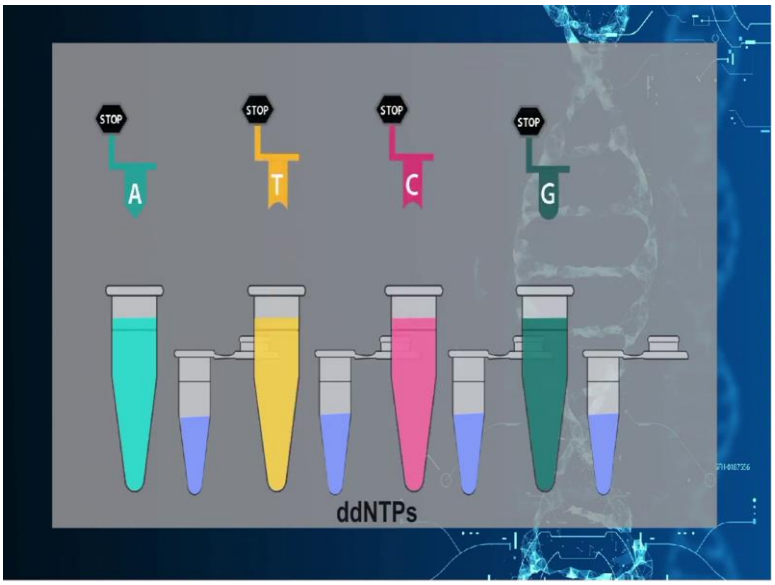

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So, as if you recall you can have the four different reactions A reactions, T reactions, C reaction and the G reaction and in all of these you have added the dideoxy-nucleotides.

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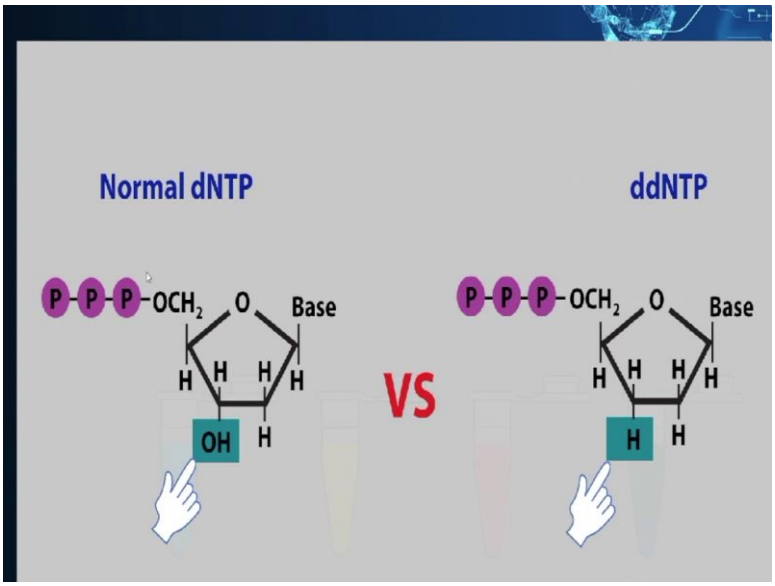

And what is the difference between a normal versus dideoxy-nucleotides? The difference between a normal dNTP is that it has the 5 time 5 prime phosphate and you have the hydroxyl group at the 3 prime. Whereas, in the case of dideoxy-nucleotide you have the this OH is missing and because of this OH is missing it is actually going to do the (Refer Time: 46:37) going to stop the synthesis.

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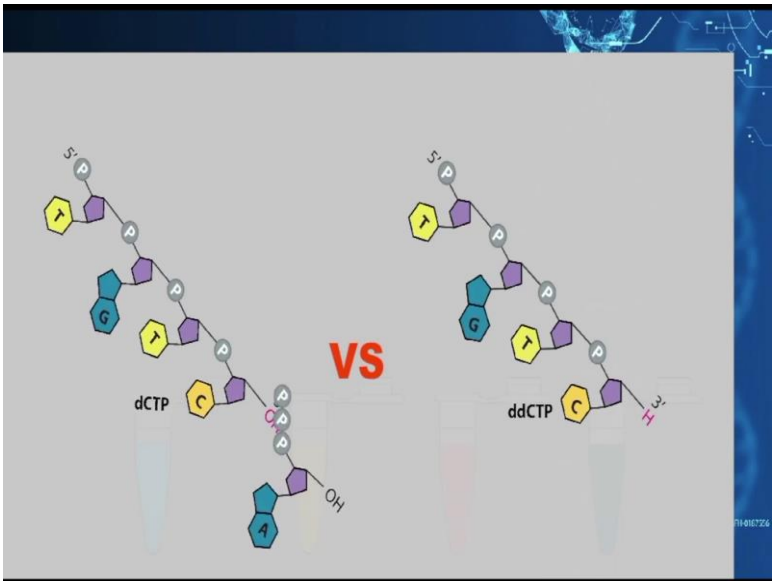

Let us see how it is actually going to stop the synthesis.

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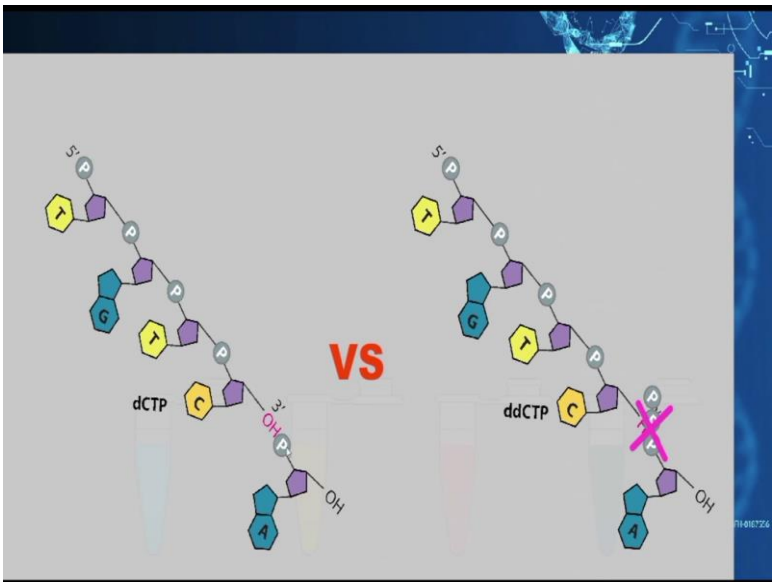

So, you can imagine that if there is a dNTPs it actually will going to form a bond by the phosphodiester linkage. And the OH is still there so that will continue the synthesis. Whereas, in the case of the dideox-nucleotides once the dideoxy-nucleotides is going to use this phosphate and going to form the phosphodiester linkage.

Since the OH is missing on this side it will not going to allow the incoming nucleotide to bind. So, that is how it is actually going to stop the synthesis of the dNTPs by the DNA polymerase.

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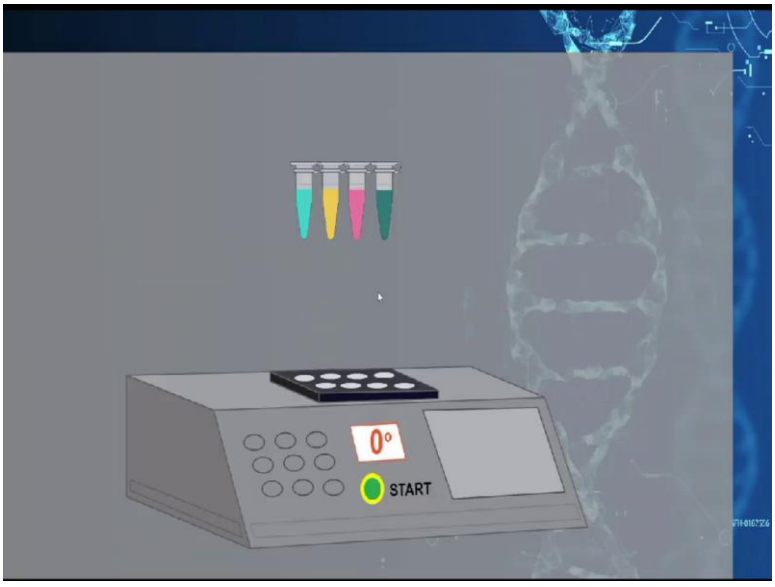

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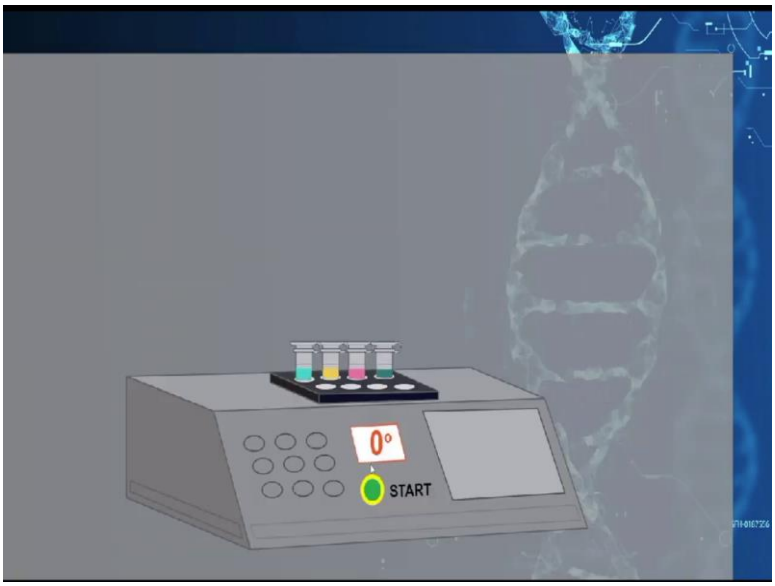

Now, what you have to do is you have to take the 4 reactions.

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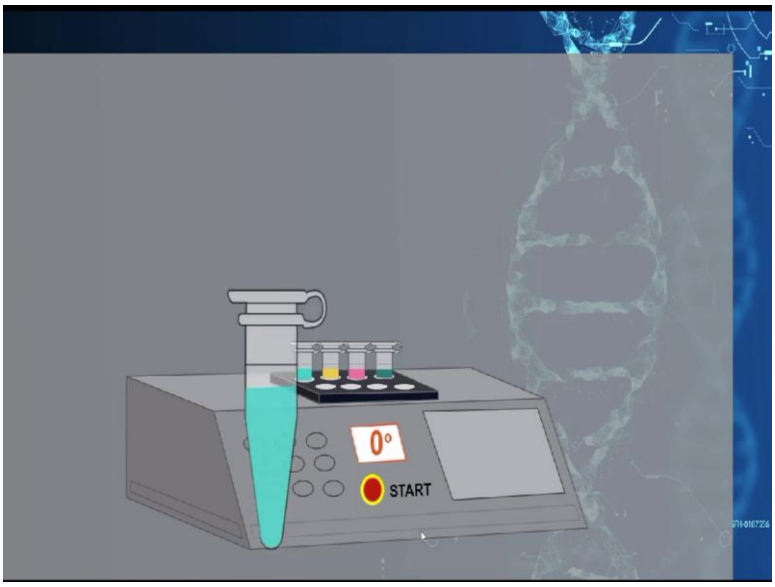

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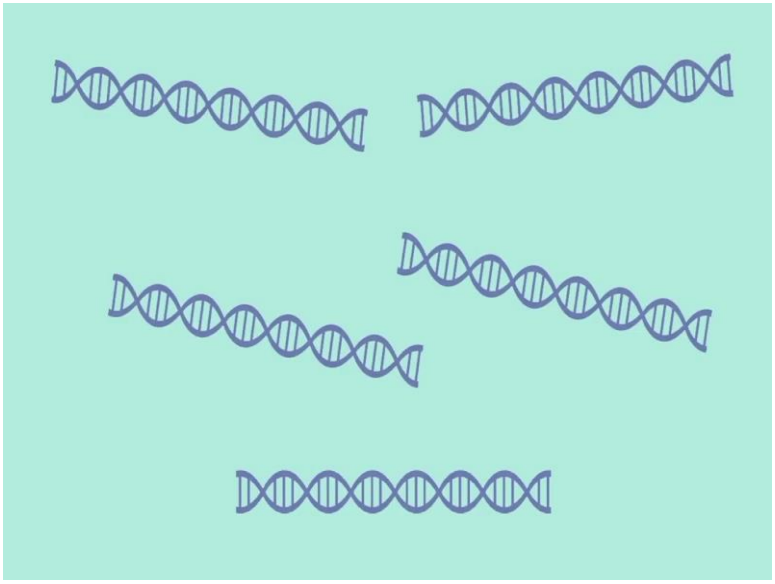

And put it into the thermal cyclers where you have the all the reagents.

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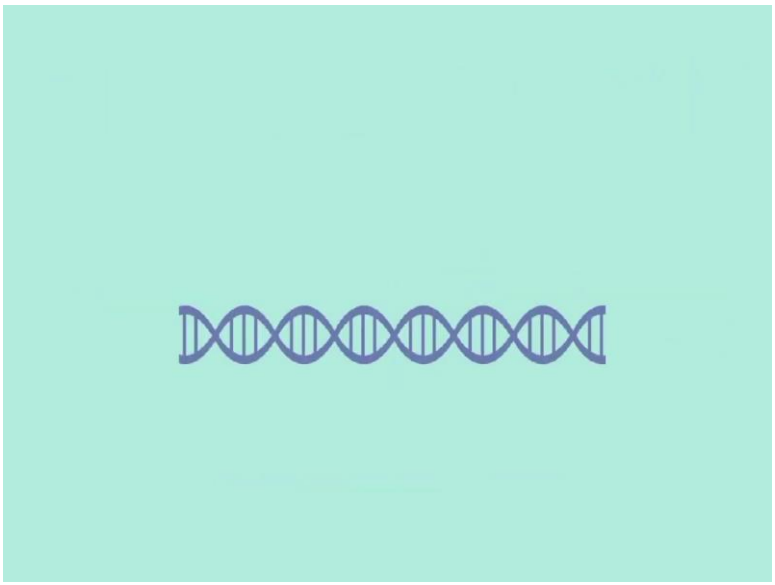

And the thermal cycler you have the different steps like in the first step you are going to do the denaturation.

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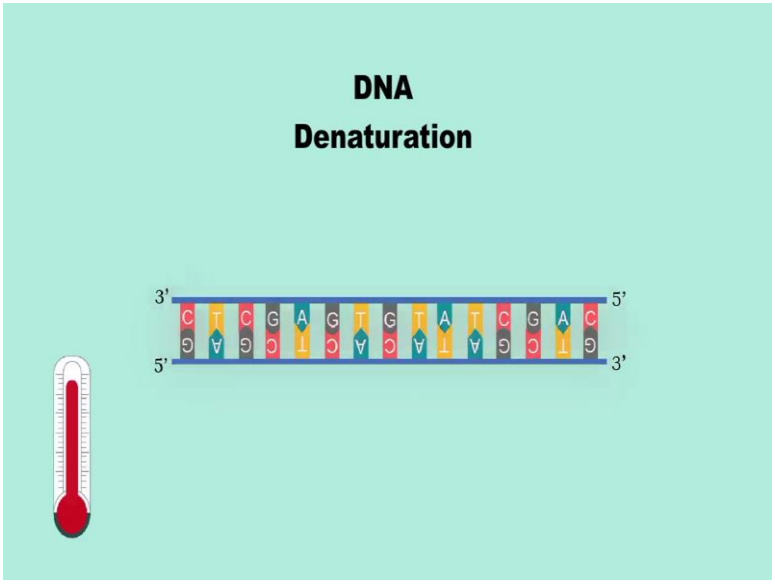

So, in the denaturation step you are going to increase the temperature of the thermal cycler.

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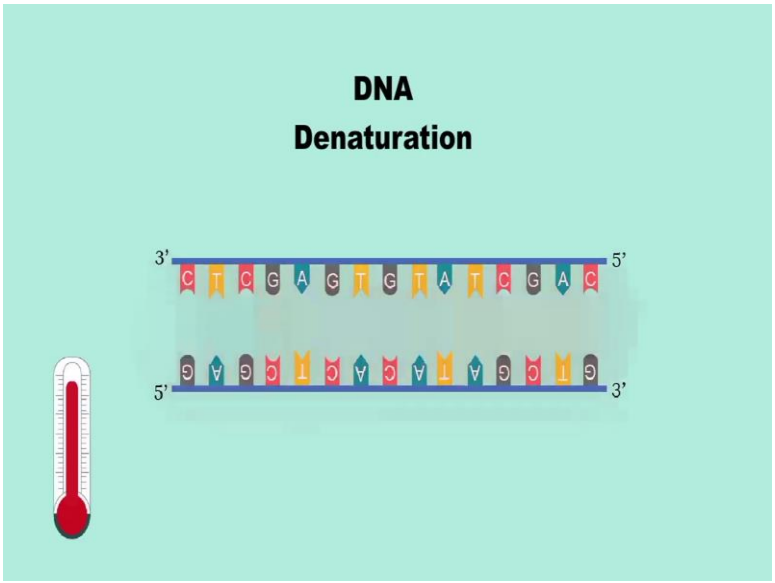

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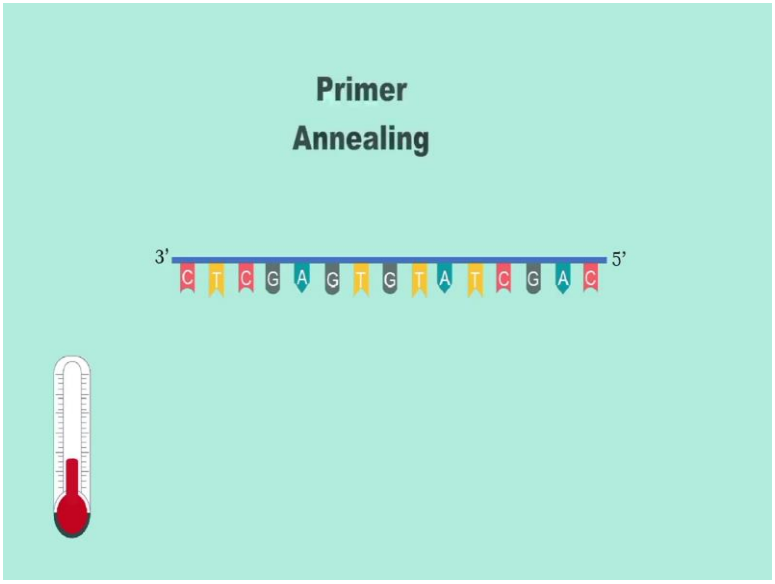

And once you increase the temperature of the reactions the 2 strands of the DNA are going to be removed going to be attached.

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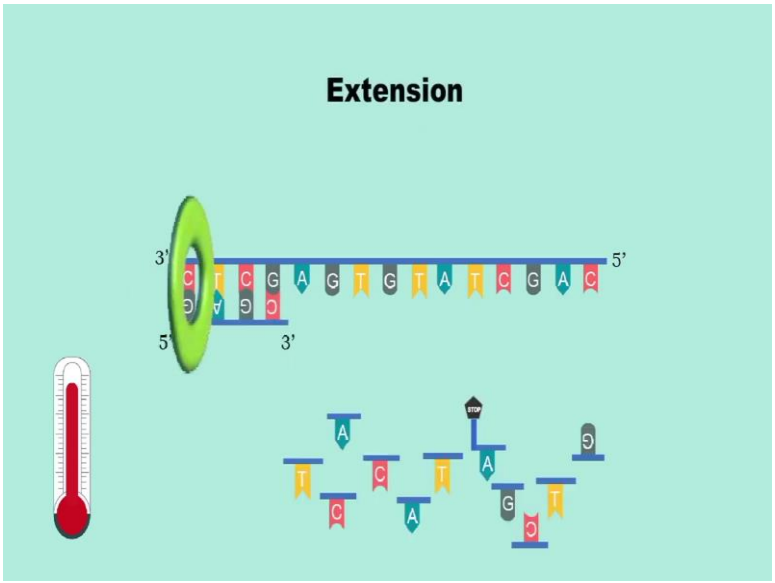

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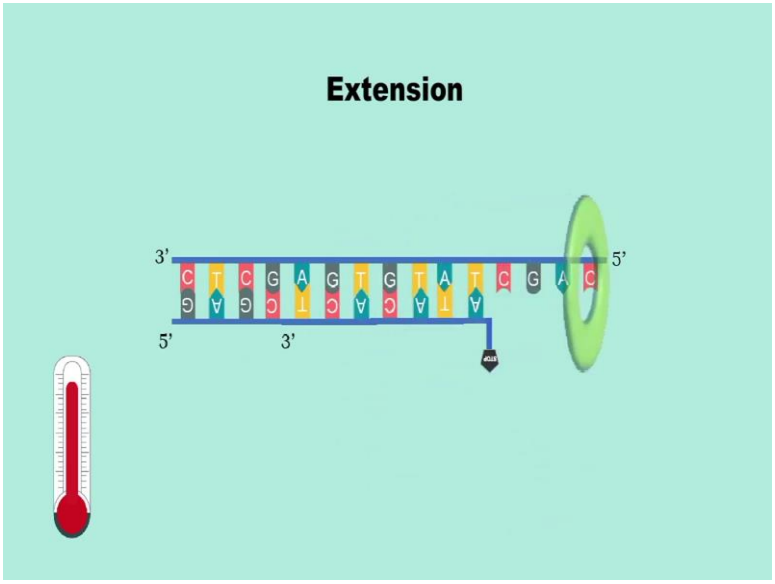

And then you are going to add the primers.

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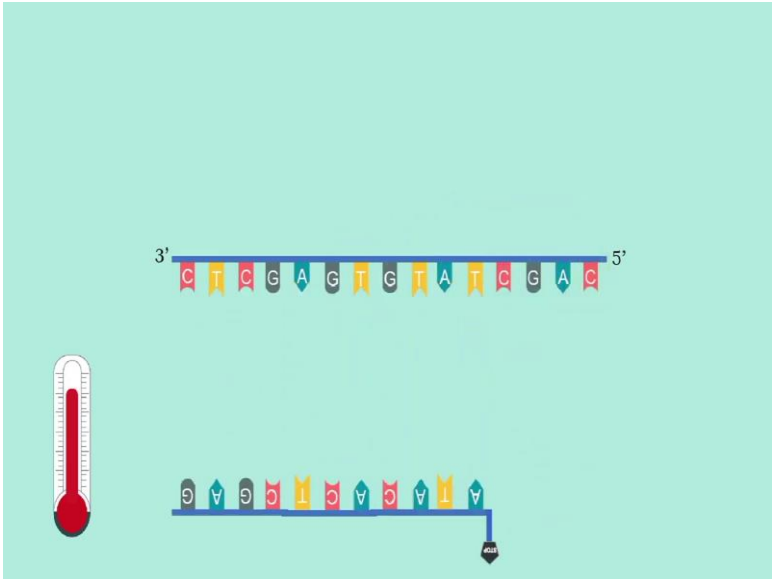

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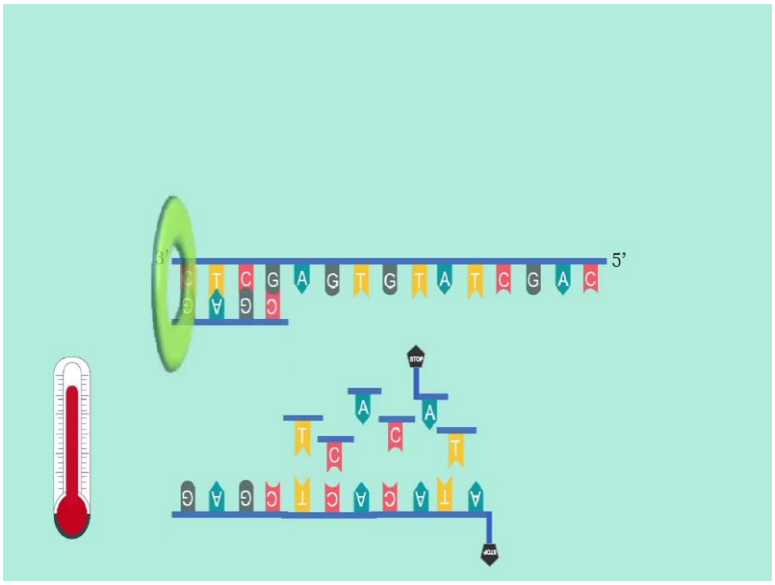

And the primer will anneal and then they will be an extension.

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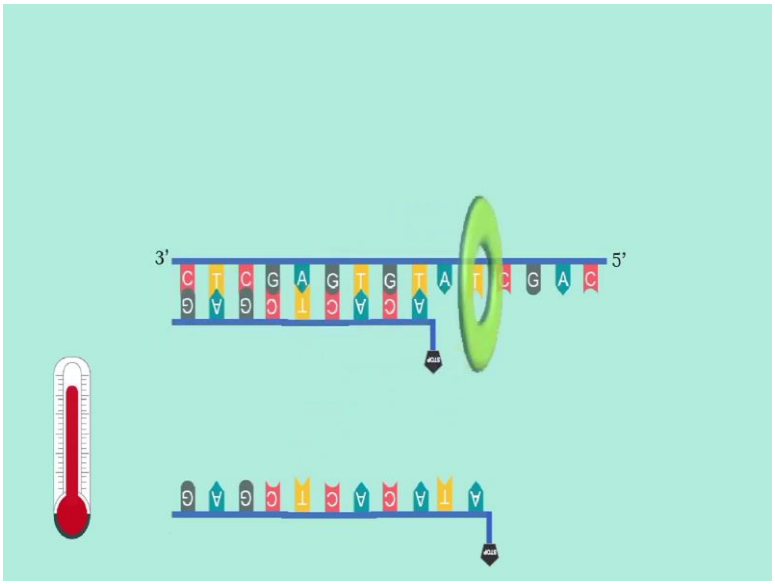

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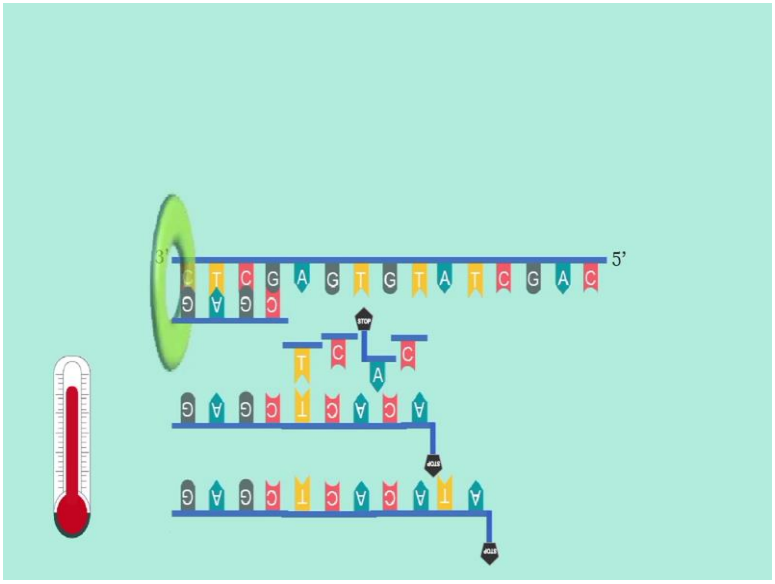

But what will happen is if there will be a dideoxy terminate TPs then it is actually going to terminate the reaction wherever the dideoxy will and the enzyme will find the dideoxy.

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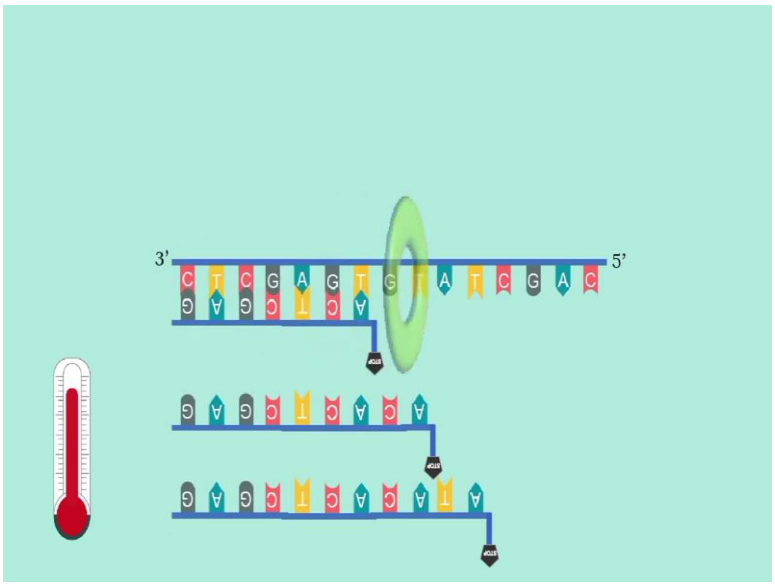

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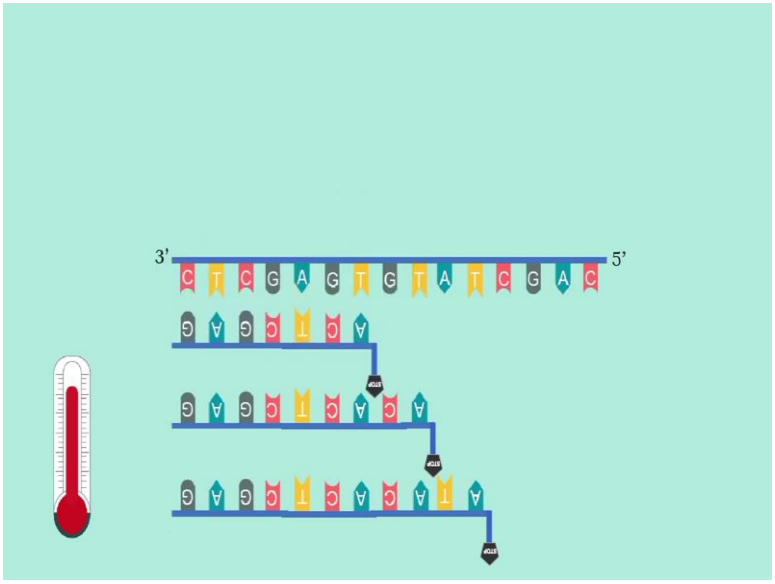

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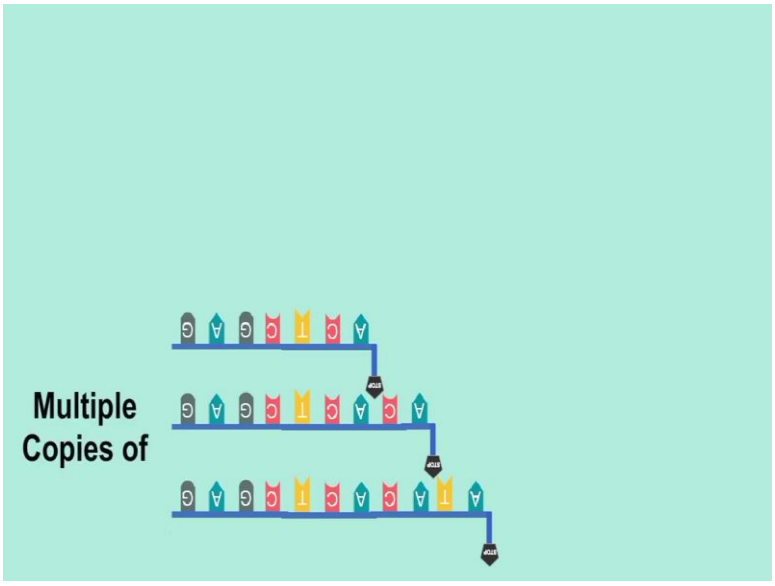

So, what you see here is that the termination is happening at every A.

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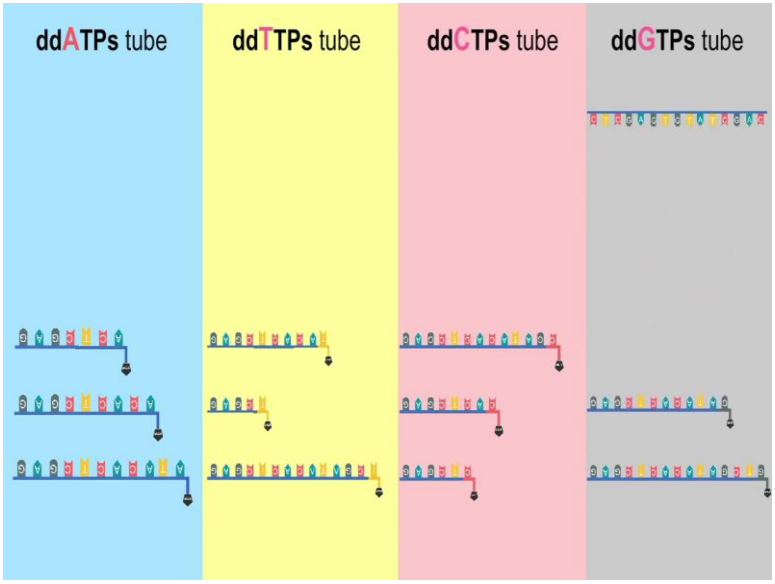

So, if it is going to find the A you it will be going. So, it will be going to give you the DNA of different reactions same is going to happen for the dideoxy T reactions. So, all the T wherever you have the T it is actually going to terminate the reactions same is true for the C reaction. So, wherever you could find the C it is actually going to terminate like here it is going to terminate and so on. So, same is going to happen even for the G reactions that wherever you could find the G it is actually going to terminate.

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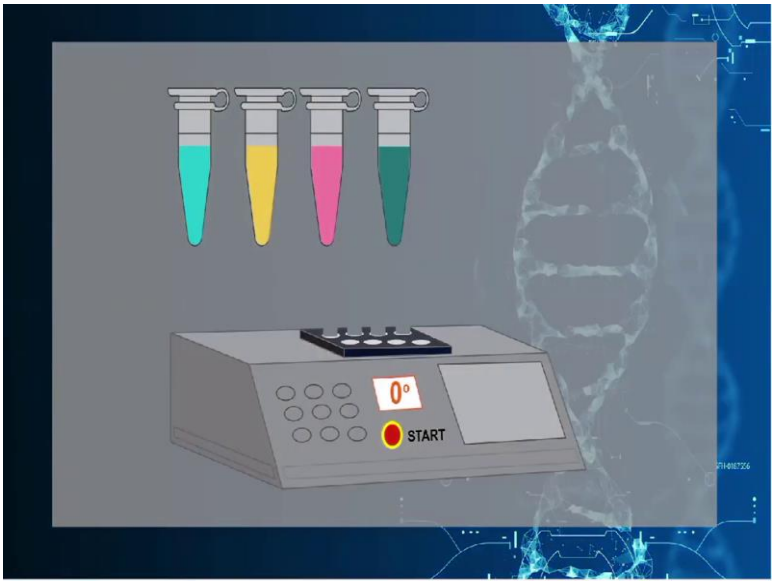

So, for example, in this stage we have find the G at the end. So, it is going to terminate at this point then it is going to terminate at this point and that is how you see they are actually going to give you the different reactions. Now, what you have to do is you have to take out these reactions from the thermal cycler.

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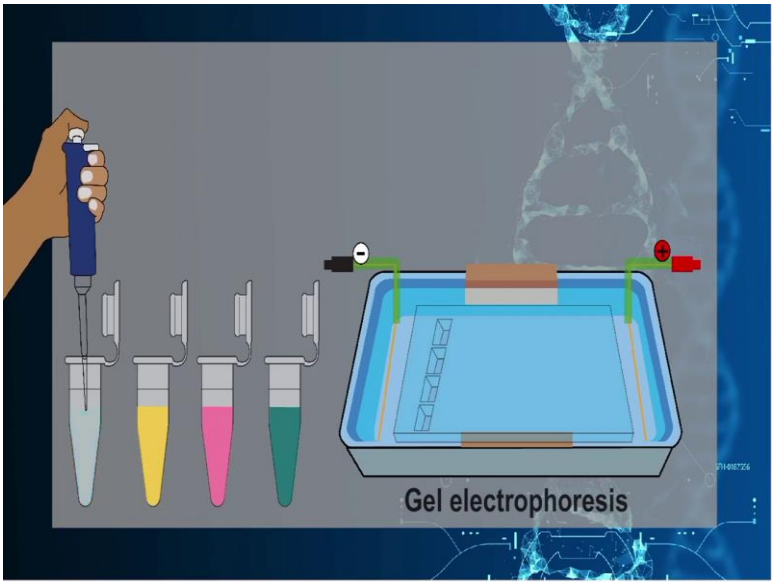

And then you have to resolve these samples on to the gel electrophoresis.

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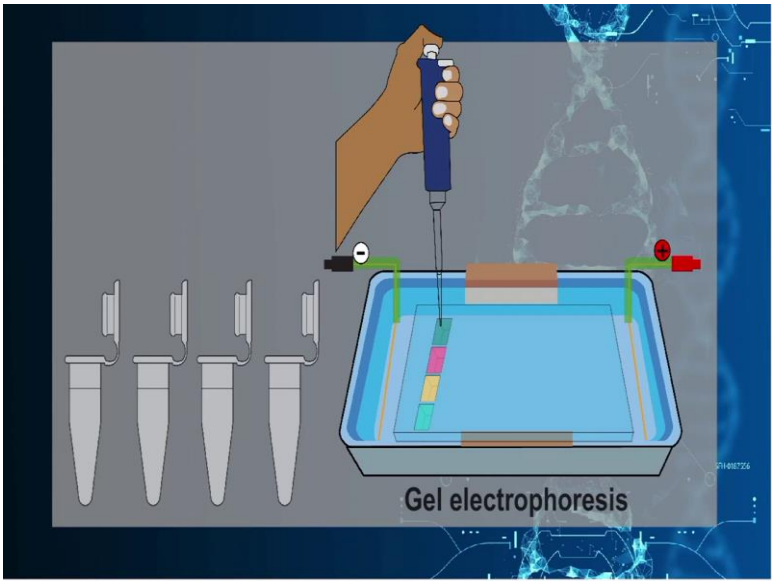

So, you have to take the all the four reactions and load it into the four different wells and you know that the DNA is negatively charged. So, it is actually going to resolve on to the gel electrophoresis and you load the fourth reactions.

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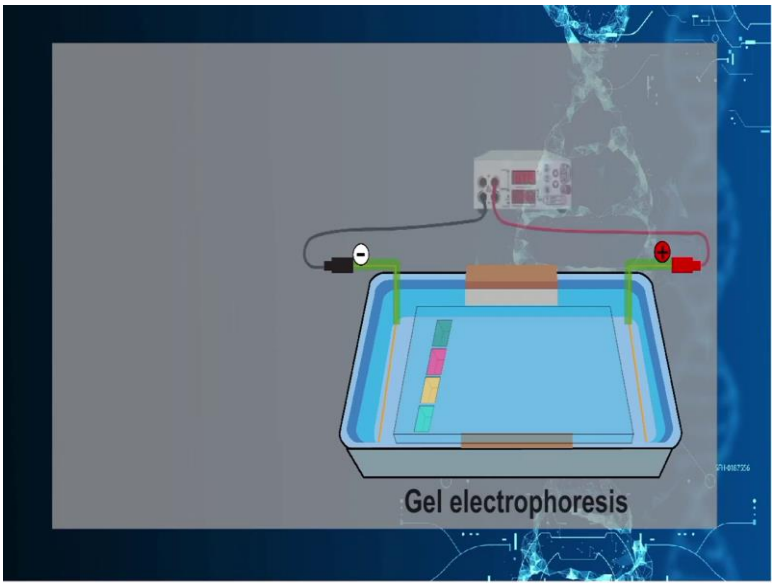

And then you connect it to the power pack.

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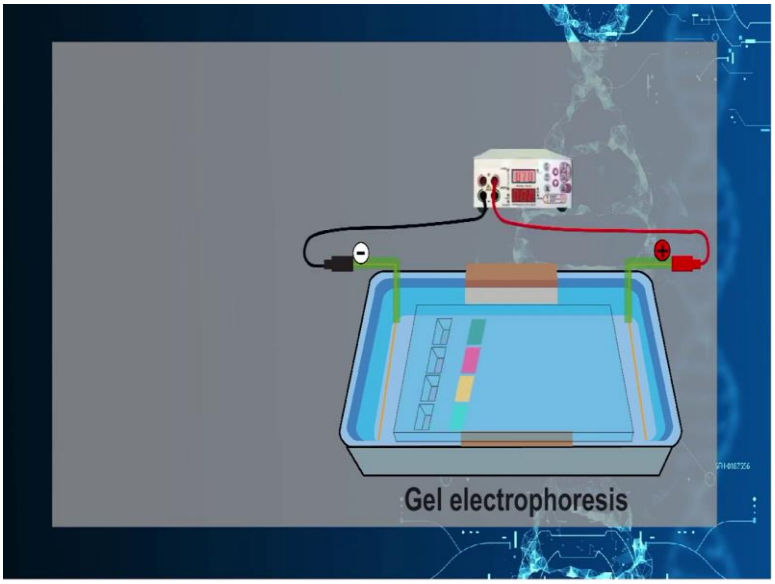

And you turn on. So, when you turn on the DNA is going to run from the negative to positive.

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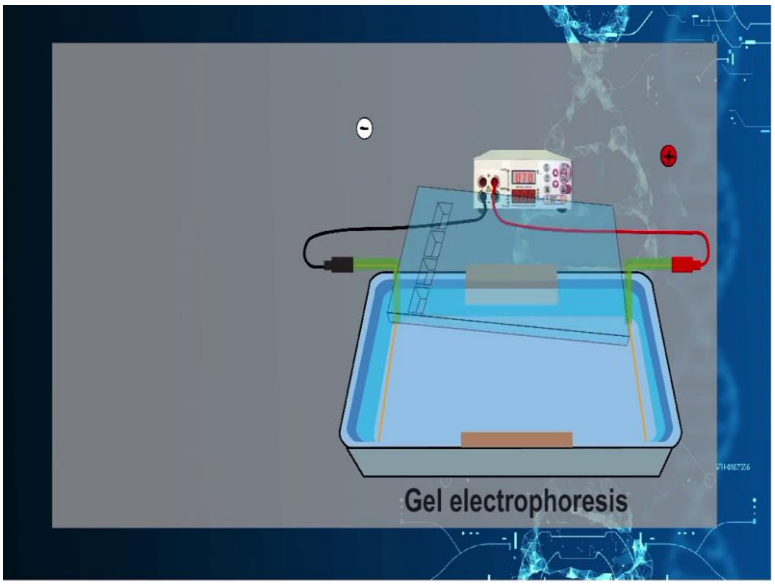

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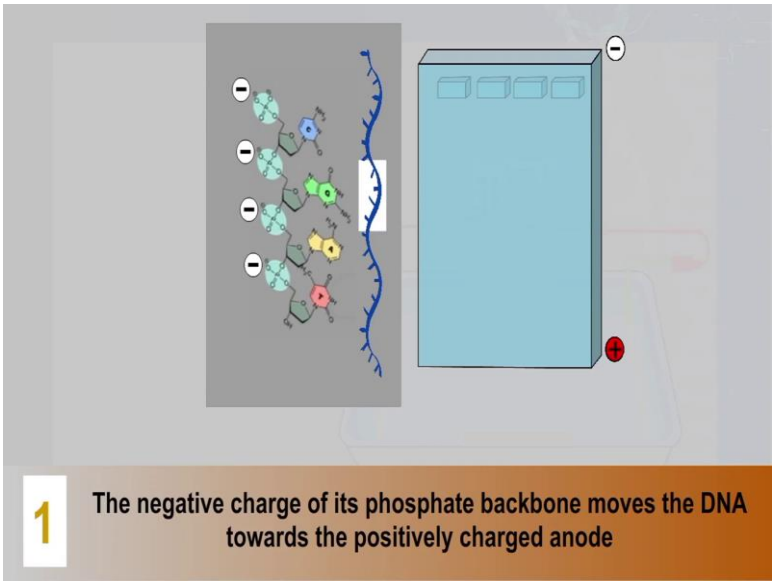

Because the DNA is negatively charged because the DNA is having a phosphate phosphate backbone.

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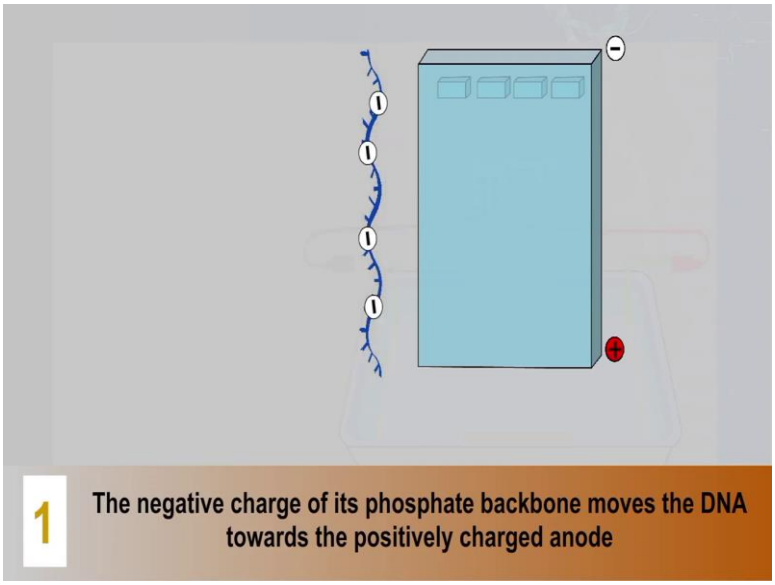

And that actually gives the negative charge to the DNA.

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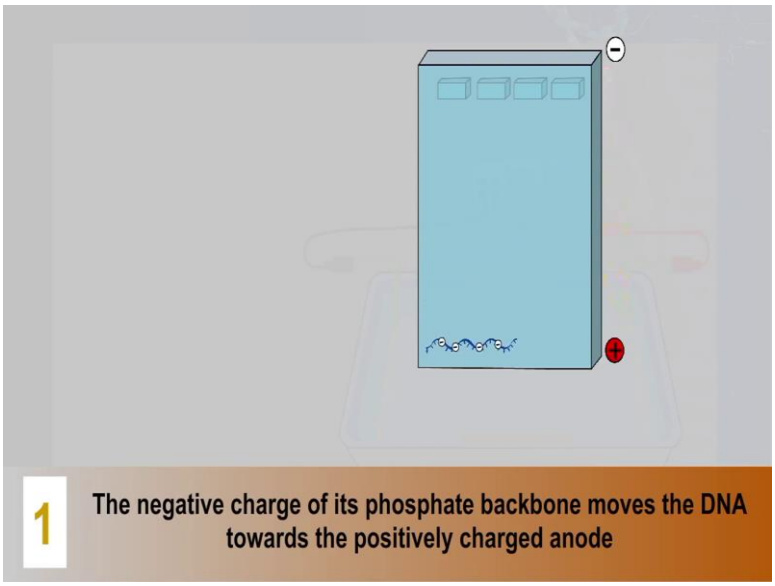

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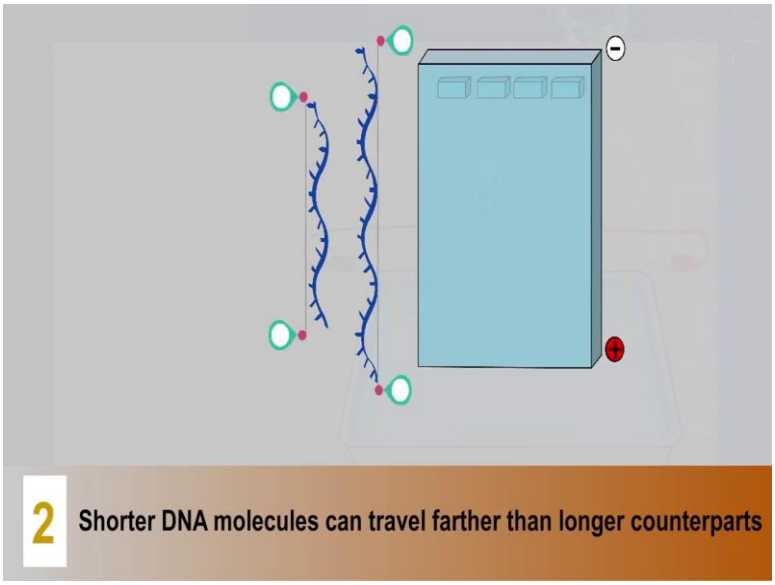

Because of the negative charge it goes towards the positive electrodes in the gel electrophoresis.

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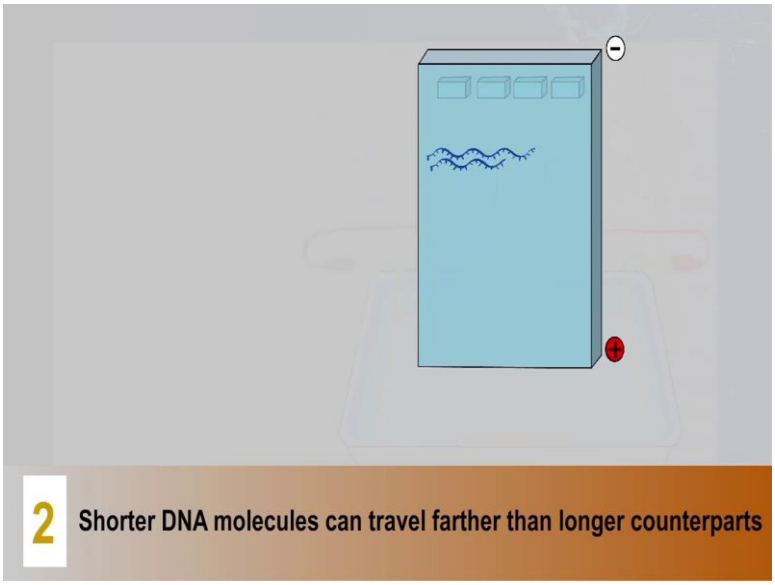

And you know that the this migration is in inversely proportional to the size of the DNA.

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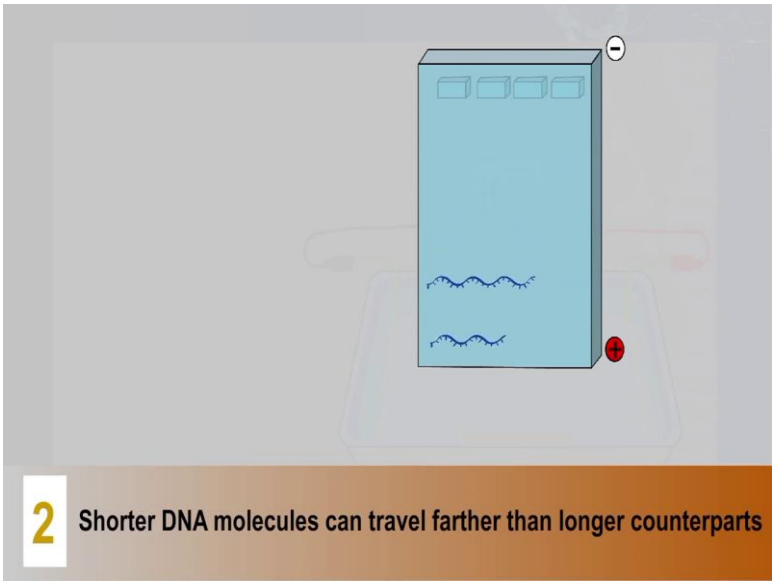

So, the larger smaller DNA will run faster.

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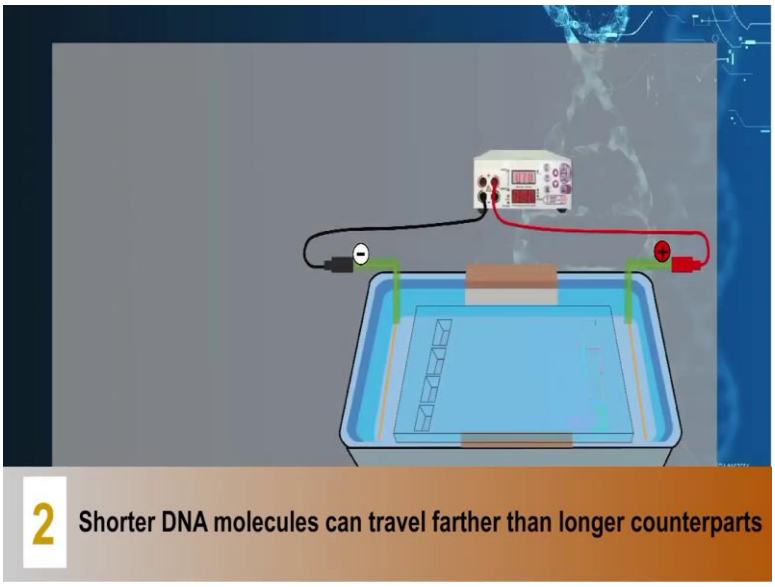

And the larger DNA will run slower. Now, what you have to do is once you have resolved the DNA.

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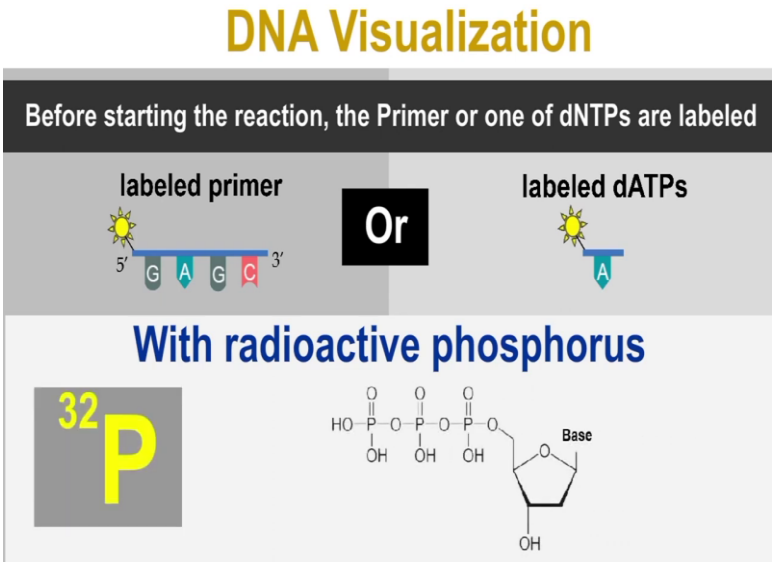

You have the two ways in which you can be able to visualize this DNA, you have the either utilization of the radiolabeled primers or you can use the labeled dNTPs like that labeling you can do with the radioactivity.

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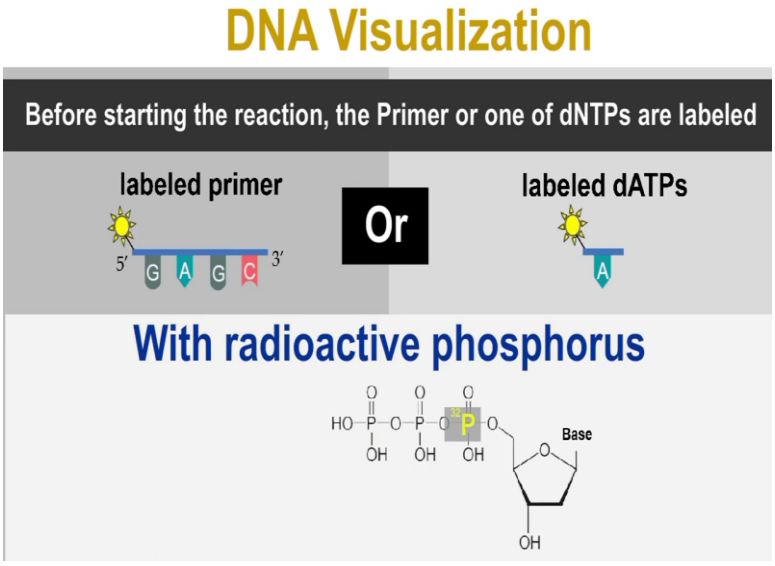

So, what you can do is you can use the P 32 labeled DNA labeled basis and that is actually going to label the DNA when it is actually going through with the synthesis.

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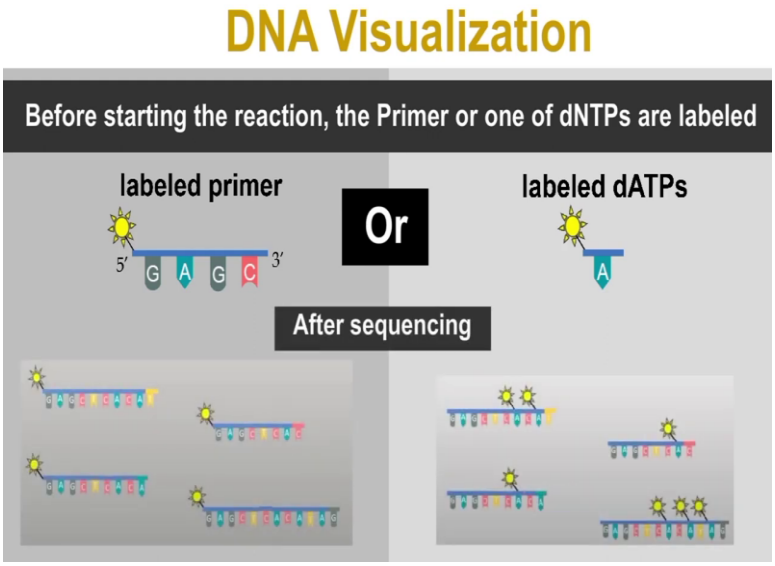

So, irrespective of whether you use the labeled primer or the labeled ATPs.

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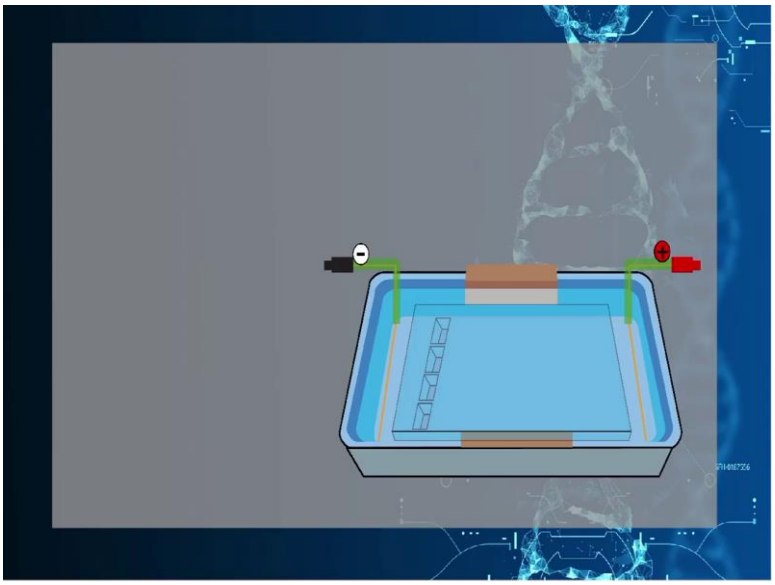

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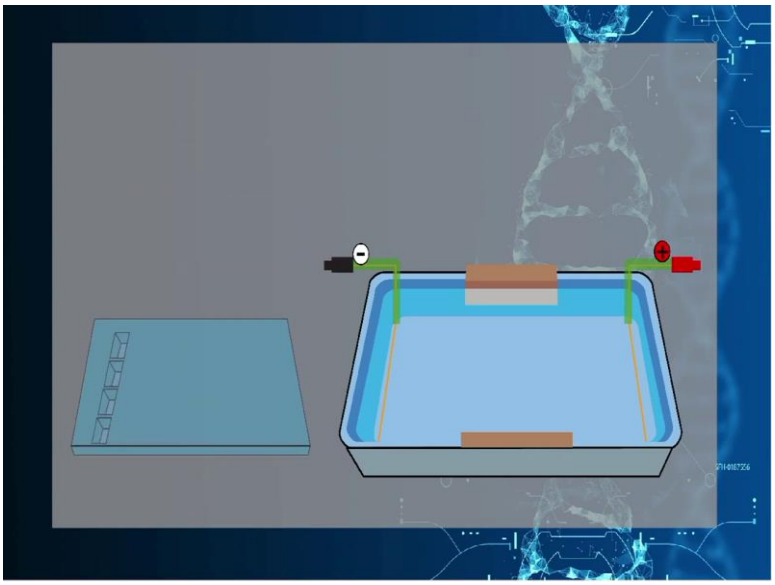

Once you got the DNA being resolved onto the agarose.

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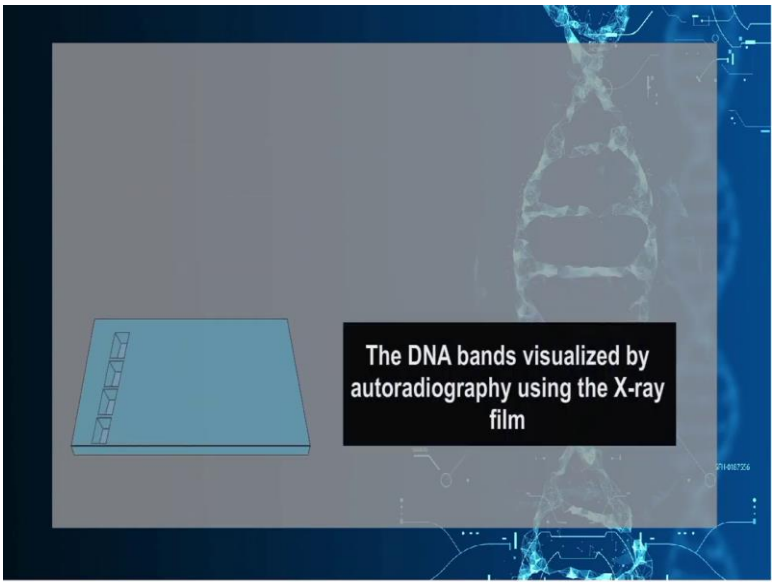

Then you have to do is you have to visualize the DNA band with the help of the auto radiography.

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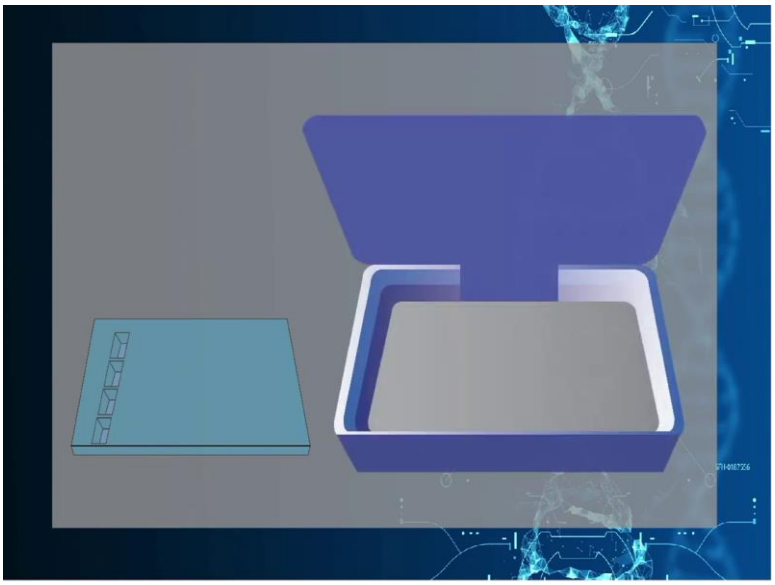

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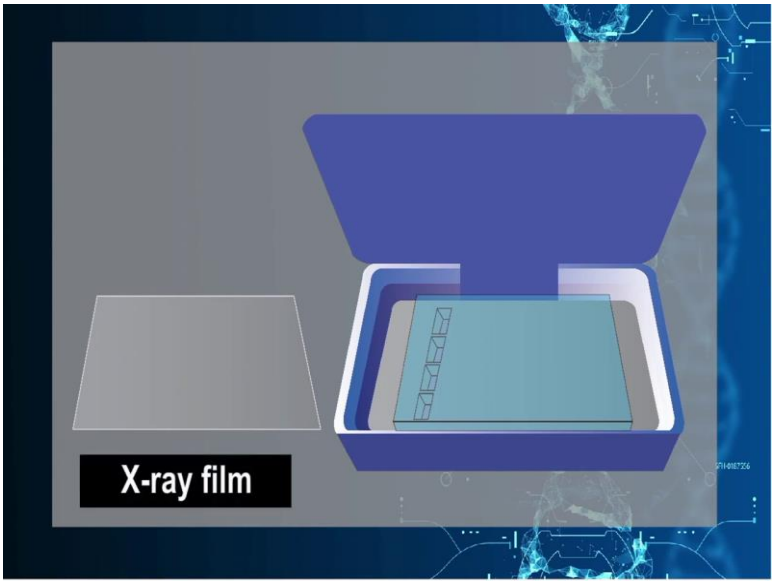

So, what you have to do is you have to take this gel you have to take the agarose gel and put it into the gel cassette put the X-ray films.

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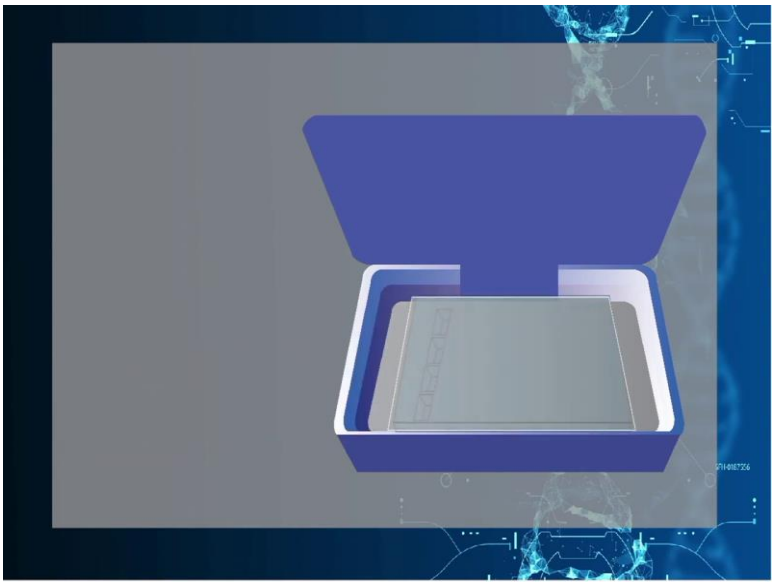

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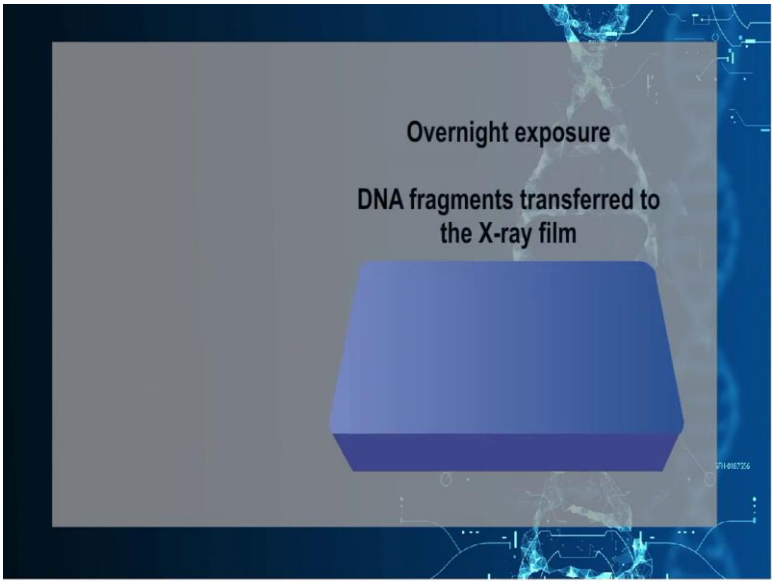

And then you close it and let it be exposed for overnight or 72 hours.

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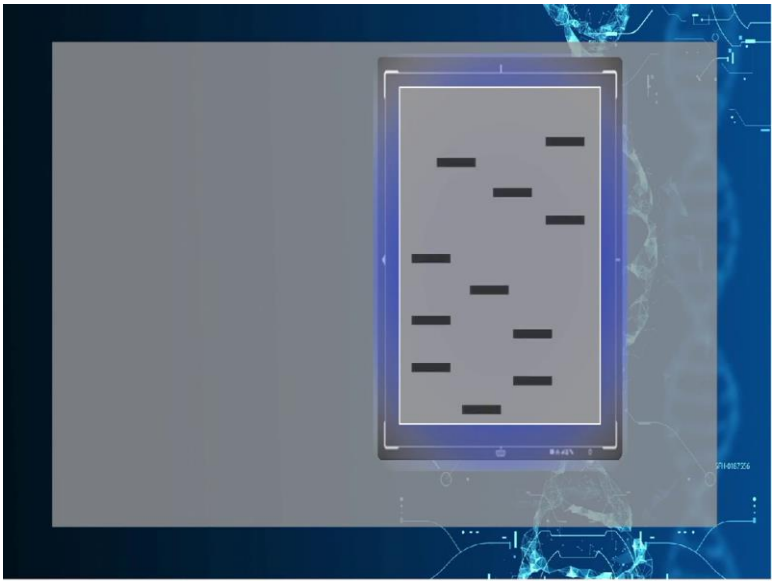

During that period the radioactivity what is present on the gel is going to export the X-ray film and that is how you are going to get the bands of the DNA.

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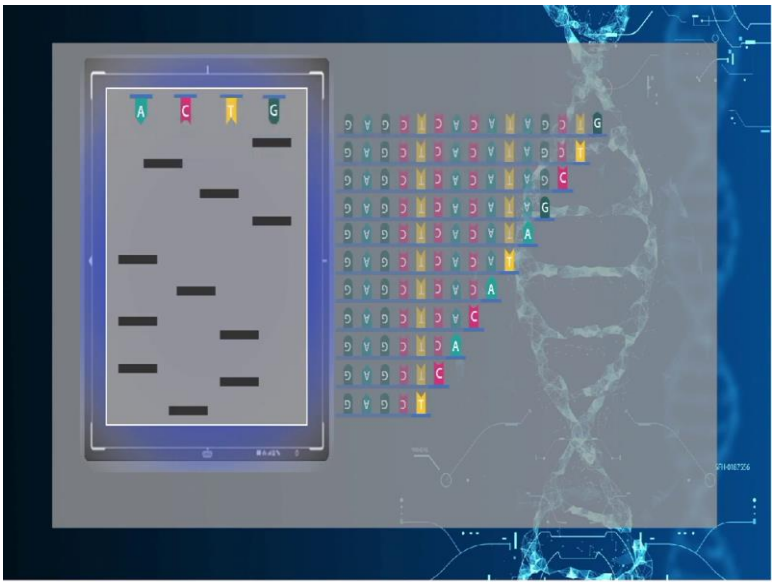

Now, what as we discussed before you have to read it in the reverse orientation which means you have to read it for this sequence, then this sequence, then this sequence, then this sequence, then this sequence and this sequence and that is how you are going to get the sequences from each band and what you have is this is yours DNA sequence. So, what you have to do is you have to take this sequence and then these sequences and that is actually is going to be the DNA sequence.

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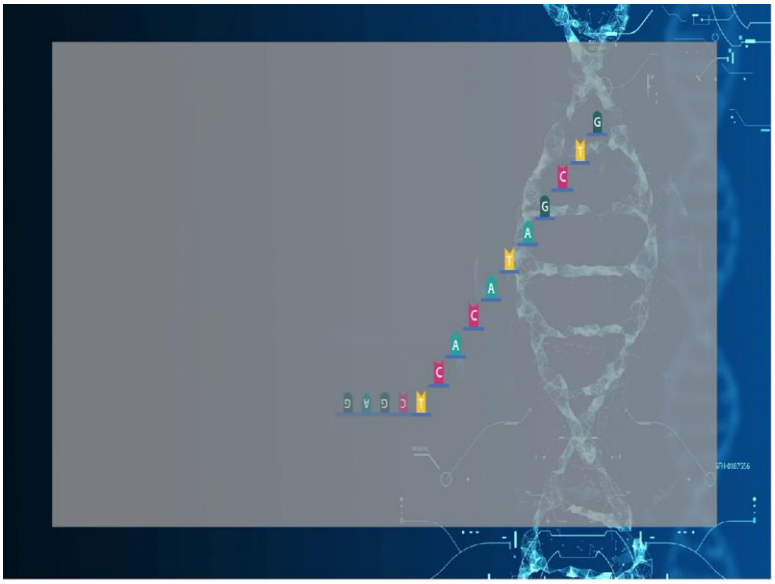

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What you are going to get from the Sanger sequence. So, this is all about the Sanger sequencing method what we have discussed.

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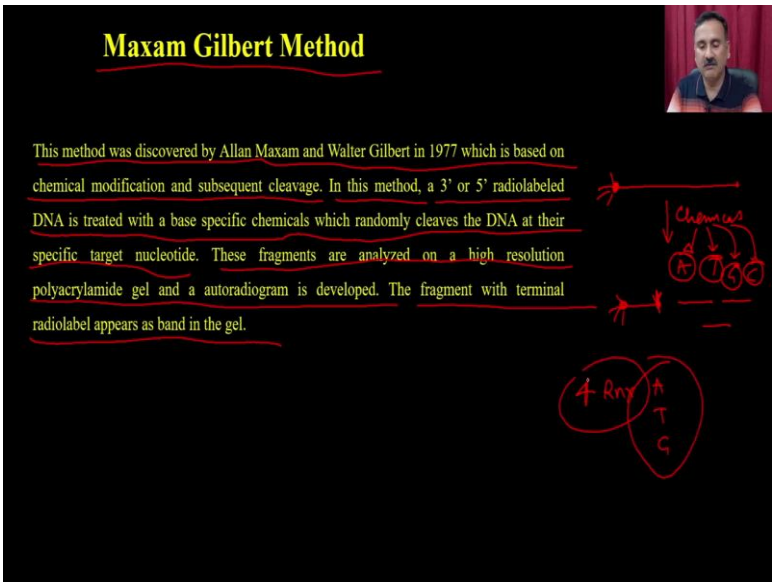

Now, the second method is the Maxam Gilbert method. So, this method was discovered by the Maxam and the Gilbert in 1977 which is based on the chemical modification and the subsequent cleavage. In this method a 3 prime or 5 prime radio labeled DNA is treated with a base specific chemical which randomly cleaves the DNA at their specific target nucleotide.

These fragments are analyzed on a high resolution polyacrylamide gel and autoradiogram is developed the fragment with the terminal radio labeled appear as a band in the gel. Which means what it is going to do is it is going to take a DNA fragment; it is going to label on one side with the radioactivity and then it is actually going to treat with the chemicals these chemicals are specific.

So, they will either going to target the A nucleotide, they are either going to target T nucleotide, the G nucleotide or the C nucleotide. And as a result, what you are going to get you are going to be small fragments of this DNA where the terminal nucleotide you know that. So, on this side you already have radioactivity, right.

But the place where it is actually going to be cleaved is either A or T or G or C. This means here also you are going to run the four reactions the A reactions, T reaction, G reaction and C reaction and that is how you are going to analyze these reactions and that is how they will going to give you the pattern of the DNA sequence.

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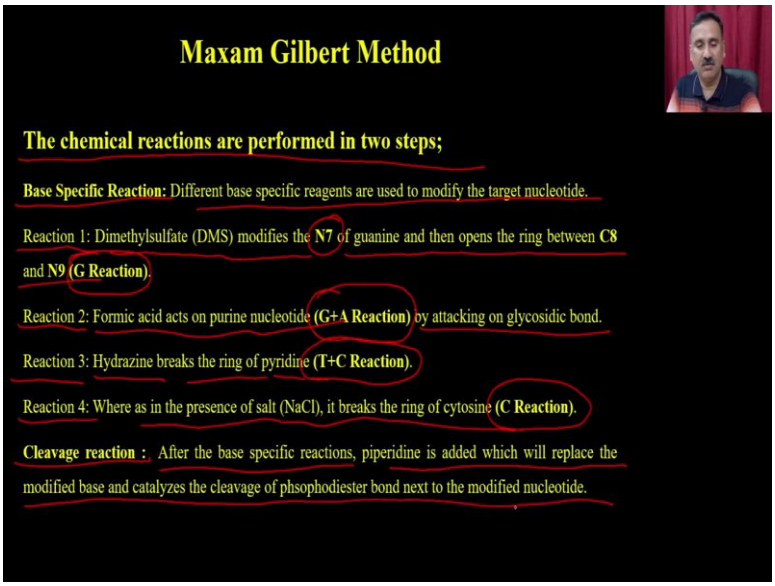

So, the chemical reactions are performed in two step. The base specific reaction and the cleavage reactions, ok. So, in the base specific reaction different base specific reagents are used to modify the target nucleotide. Reaction 1 where you are going to use the dimethyl sulfate DMS and that is going to modify the N7 of the guanine and then open the ring between C8 and C9. So, that is called as G reaction. Then you have a reaction 2 which is you are going to add the formic acid act as a purine nucleotide.

So, it is actually act on purine nucleotide. So, it is actually going to be called as G plus A reaction by attacking on the glycosidic bond. Then reaction 3 it is going you are going to use the hydrazine and that is going to break the ring of the pyrimidine. So, it is going to be called as T plus C reaction because it is not specific for the only C or T it is actually going to attack on the pyrimidine basis. Then in the reaction 4 wherein the presence of salt it breaks the ring of the cytosine and that is called as C reactions.

Once you are done with these base specific reactions then you are going to have the cleavage reaction. So, after the base specific reaction the piperidine added which will replace the modified bases and catalyze the cleavage of the phosphodiester bond next to the modified nucleotide.

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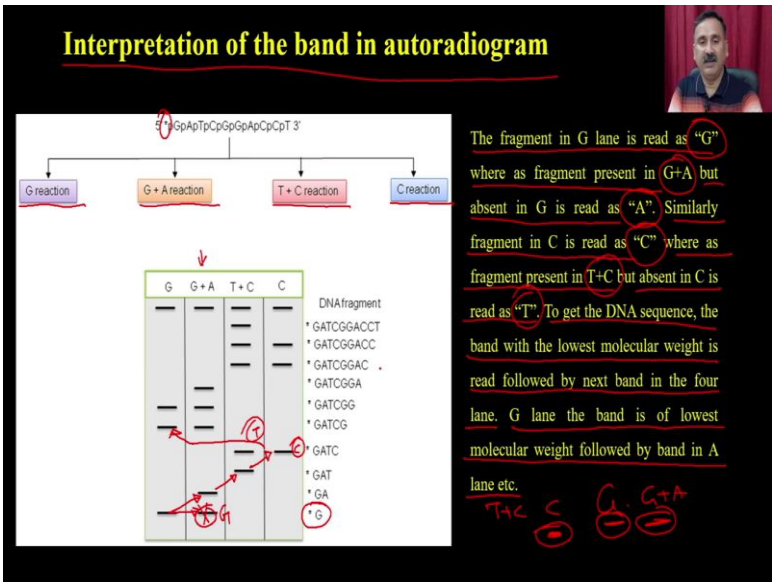

This means at the end what you are going to get you are going to get a pattern like this ok, because here also you have added a radioactivity on to the 5 prime end. So, you are going to have the G reaction, G plus A reaction, T plus C reaction and the C reactions. The fragment in the G lane is read as G whereas, the fragment present in G plus A, but absent in G is read as A.

Similarly, the fragment in C is read as C whereas, the fragment present in T plus C, but absent in C is read as T. To get the DNA sequence the band with the lowest molecular weight is read followed by the next band in the 4 lane. For example, this means you are going to start from here and you are going to read like this, ok.

But there is a; there is a issue. G lane the band is of lowest molecular weight followed by the band in the A lane which means if you have the G, ok. And if you also have the same band in the G plus A reaction this means you are not going to read this, ok. This means this is also going to be considered as G reaction. This means from here you are going to read this.

And then from here you are going to read this. From here you are going to see all these two bands are of the same level. This means it is going to be read as C then from here you are going to read this as G and so on. So, you will actually go with the you are going to go with the from the lower band to higher band, ok.

So, for the G between the G and G plus A you are going to if you have the bond in the same line you are going to read them as G. Similarly, if you have the T plus C band and C band you are going to read them as C, ok. So, if you have got the two bands which are which are present both in the T plus C and C then you are going to read that as C than T, ok.

So, same is true for the A plus G and G plus G reactions. So, this is the way you can actually be able to sequence the cloned DNA and that is how you can be able to verify that DNA. So, for what we have discussed? We have discussed about how you can be able to utilize the different types of tools or as well as the features what are present into the vector and recombinant DNA and those are the which you can use for the screening of the recombinant DNA.

And at the end we have also discussed about the DNA sequencing reactions. So, that you can be able to sequence the cloned DNA and that is how you can be able to verify the clone. So, with this I would like to; I would like to conclude my lecture here.

Thank you.