Molecular Biology

Prof. Vishal Trivedi

Department of Biosciences and Bioengineering

Indian Institute of Technology, Guwahati

Module - 10

Molecular Cloning

Lecture-45 Screening of recombinant Clones

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bionining IIT Guwahati. So, in the today's lecture, we are going to discuss about how you can be able to screen the recombinant clone. So, now, let us to 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 vector, the vectors are also or 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.

So, screening of the recombinant clone, so you can imagine that this is a vector, right, 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 a 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, okay, 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 worked 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 it would 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, okay. 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 can be used in different screening methods.

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, okay. So, it is going to give the blue color to the 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 colorless compound Xgal or it is also called as 5-bromo-4-chloro-3-endolyl-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 an initial N-terminal region like the 11241 of the protein is important for the activity of the protein. In this system, the host containing lacZ, lacZ without the initial reagent 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 synthase. The enzyme beta galactosidase oxidizes the Xgal to form the 5-bromo-4-chloro-endoxyl and galactose. The endoxyl derivative is oxidized in air to give a blue colored dibromo-dichloro 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.

So if the enzyme is active it is going to convert the Xgal 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 gene you are going to insert that it will not going to complement the remaining portion which is present in the host and as a result 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 transform cells. So this is what we have explained here. So the beta galactosidase is a protein which is actually going to be expressed for lapoprons. So what you have is you have a LAGG-xene which actually which has a missing 11 to 41 region.

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 on to the vector which actually can call as LAGG prime. So this LAGG-xene 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-dichloroindolyl. So this is the colored compound blue colored compound. So since you are cloning the gene into this particular LAGG-xene area 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 if you are going to have the two 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.

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. So these colorless colonies are the colonies where you are going to have your recombinant DNA because of simple reason that it is actually going to have the inactivation of LAGG-XE. Then the second criteria is the antibiotic sensitivity. So antibiotics are the drugs which are actually been 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. So for example there are antibiotics which are disrupting the translation steps, there are antibiotics which are disrupting the transcriptions and there are other antibiotic which are disrupting the protein synthesis.

So if you want to so if you add the antibiotic into the media it will not allow the propagation of the normal bacterial cells 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 antibiotic containing media.

In the cloning of a fragment into the plasmid, the plasmid is cut with a restriction enzyme and a fragment 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 the antibiotic containing solid media. These 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, gloramphenicol and these are the gene product from the antibiotic resistance genes. So it will actually going to be beta-lactamase, neomycin, phosphorotransferase, ribosomal protection proteins and the chloramphenicol acetyltransferase.

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 phosphorotransferase 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 cat genes and the cat gene is going to acetylate the chloramphenicol to acetylchloramphenicol and that also is going to interfere with the action of the chloramphenicol. 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 circularized. Whereas once you have the cut vector it does not have the you know its circular DNA. 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 the ampicillin.

So it will actually allow the bacteria to grow whereas when you do the transformation of this cut vector since the cut vector you 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 and form the colonies. Then the third approach is the insertional inactivations. 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 genetic system.

For example, in an insertional inactivation of the lacG right. So lacC is a part of the lac 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 bametron site and if you use this bametron 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 okay 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. Then we have the instructional inactivation of the antibiotic resistance genes and that we in this example I have taken from the vector which is called as PBR-322. So in the PBR-322 it has the two antibiotic resistance genes. It has the ampicillin resistance gene and it has the tetracycline resistance gene. 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 okay.

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 a SCA utilizing the SCA as a restriction site what will happen is that it is actually going to disrupt the ampicillin resistance okay. 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 into the bacteria and we are going to get the colonies. Now this transform bacteria what you will do is just make a replica plate of this plate and then you grow this first with the tetracycline okay. So when you grow them with the tetracycline both of these clones are actually going to grow okay 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 colony 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 okay. 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 colony which contains the recombinant DNA. 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 of lysis of the. The CI gene

encodes for a CI repressor and which is responsible for the formation of the lysogens okay. In the presence of the functional CI the plaque containing unlicensed host cells and has a terbit appearance whereas in the absence of it, it will be clear. This feature can be used to screen the clone to detect the functional CI or the absence of CI okay.

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 okay recombinant DNA okay. So this is what it is shown here right. So CI is a temperature sensitive repressor so if you change the temperature the CI repressor is going to be expressed and it is actually going to shuttle the virus from the lytic to the lysogenic phase okay. 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 okay.

And when you present the non-functional CI repressor it will actually going to shift the protein shift the cycle towards the lytic phase okay. 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. 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 plagmit containing the missing gene and the transomemant 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 requirements in this approach okay. So what is the complementation of the mutation is that the host is mutated for a crucial gene okay. 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 okay.

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 oxotroph 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 provide that product into the media okay and as a result it is actually going to give you the growth of this mutant or this mutated host in the presence of this particular product in media.

For example if this gene is responsible for the synthesis of uracil okay 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 the 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. Now let's 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 transform 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's 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 transform 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 but the host strain transform with the recombinant clone has non-functional gene product and it 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 mono phosphate 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 we have is we have the gene of URA3 which actually provides a pro 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 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 insertional inactivation of this particular gene. Now if you have done the insertional 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 under URA3 minus minus cells. So this is actually going to give tell you that if you got the colonies this means the OMP decarboxylase is inactive. Then these are the methods are more popular in the prokaryotic system. Let's talk about now how you can be

able to screen the clones into the mammalian system.

So screening of the transfected mammalian cells. First method is the reporter gene assay. So in the reporter gene assay system a chimeric construct is produced 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 agents. Afterwards the cells are being 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 UV festivals. So these are the reporter gene construct what you can use for screening the mammalian clothes like the CAT gene, LAGZ, luciferase, 4a and the GFP. And the gene product are CATG chloramphenicolosyltransferase then LAGZ beta galactosidase is for luciferase and 4a is for alkaline phosphatase and GFP is the green fluorescent protein.

And the reaction what you are going to see for catalyzing is that when you have the chloramphenicolosyltransferase it is going to run like chloramphenicol to acetyl chloramphenicol and so on. 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, 14S spiralis. The enzyme utilizes the duluciferin as a substrate to form the axial luciferin. In the presence of ATP magnesium luciferin is getting converted into the luciferin adenylate involving pyrophosphate cleavage and the transfer of AMP into the luciferin and the luciferin adenylate undergoes oxidative decarboxylation to form the oxyl luciferin and simultaneously there will be emission of light.

The reporter gene construct containing luciferase is transfected into mammalian cell. The cells are washed with PBS and lysed with a lysate buffer take the lysate into the luminomotor cubate and luciferin substrate is injected to start the 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. So what you are going to do is you are going to first take the look expressing vector, you are going to clone your recombinant DNA into this right and then you are going to do the transfection. So once you got the transfection you are going to have the eukaryotic cell which has this recombinant DNA which contains the look gene in front of the promoter and then you are 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 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 lyse with a lyse buffer, take the lysate into the luminometer cubate or plate and you add the luciferase substrate and it is injected to start the reaction and measured immediately in a luminometer. 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 to cells containing recombinant protein, fluorocellently tag 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 cells. Flow cytometer analysis the cell based on the shape, size and fluorescence a non fluorescent cell is giving separate peak as compared to the fluorescent label 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 transfect 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 cells, these are the GFP expressing recombinant DNA containing cells and that is how you can actually be able to collect 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. 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 the integrity or integrity of the how you can be able to verify the clone.

So, you because that is very important right. So, you can actually be able to verify the clone by several method. 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 LB agar plate. Now, the next question is how you can be able to verify the clone because 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. 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 that is how these fragments are going to be 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's method. So, the first method what is been discovered is the Dideoxy chain termination or the Sanger's method.

This method is originally been 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 complementary 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 three normal nucleotides and a 2 prime, 3 prime di-epsi nucleotide triphosphate. This means it is actually you are going to take the single-stranded 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 d d n t p's.

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 d d n p p's. In some cases you are going to take the ATP, in some cases you will take the GTP, in some cases you will take the CTP and in the other case you will take the TTP. This means for every DNA sequence you are going to run the four reactions and in four reactions you will take either the d d d d t p plus you will take the all other remaining three nucleotides. So, that is how you are going to you know make the four different types of reactions.

When the DNA polymerase utilizes di-deoxy nucleotide as a nucleotide it gets incorporated into growing chain, but the chain elongation stops at the d d n p's due to the absence of the 3 prime hydroxyl group. In the typical sequencing reaction you are going to run four different d t m p's are taken into the four separate reaction and analyze onto a high resolution polyacrylamide gel electrophoresis. The ratio of NTP and DTP is adjusted so the chain termination occur at the each position of the base in the template. 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 and you are going to have the terminal sequence then you add the primers and when you add the primer and you are going to have the two

options either you go with the Sanger's protocol or you go with the labeling 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 d t p's and the clino fragments and you are going to you know 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 d t t a t p and the remaining three NTP's. In the second reactions so this is the reaction number one in the second reaction you are going which is called as T reactions you are going to add d d d t t p but and the rest three nucleotides which are normal nucleotides. Then in the reaction number three you are going to add the or which is called as G reaction which is called as d d g t p and the three g d NTP's and in the fourth reactions you are going to take the C reactions which is called as d d c t p plus the four three remaining NTP's and after every reactions you add you are going to put them for chase which means you allow the DNA to be synthesized. So, in the step one your primer is added and annealed to the three prime of the DNA template the radio labeled ATP is been added to label the primers then the step three the polymerase reaction is divided into the four reactions and in the step four DNA synthesis continue until terminated by the incorporation of the specific d d NTP's either the A, T, G or C and in the step five a chase of polymerase reaction is performed in the presence of high concentration of NTP's to extend all non terminated sequences into the high molecular weight DNA.

This high molecular weight DNA will not enter into the sequencing reaction. In the labeling or the chain termination protocol this is the labeling and the chain termination protocol in the step one a primer is added and annealed to the three prime of the DNA template then the step two a limited amount of NTP's are added along with the one of the radio labeled nucleotide to label the DNA throughout the DNA throughout the length. Then the step three the polymerase reaction is divided into the four 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 dideoxy NTP's. Synthesis is terminated at the specific d NTP's either A, G, C or T to give 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 and so all these fragment has to be analyzed onto a high resolution SDS page and when you analyze them you are going to get this kind of fragments. So you are going to divide that into four reactions A reactions, T reactions, 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 and for G reaction you got this spot here and for the C reaction you got the fragment here. Now what you have to do is you have to run in a reverse orientation like this. You have to walk and you interpret this you are going to walk like this and that is how you are going to get first sub A then AT then ATT then ATT A. 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.

Now the second method is the Maxim-Gilbert method. So this method was discovered by the Maxim 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 G nucleotide or the C nucleotide and as a result what you are going to get is you are going to get the small fragments of this DNA where the terminal DNA nucleotide you know that so on this side you already have a radioactivity.

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 4 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. So, the chemical reactions are performed in two step the base specific reaction and the cleavage reactions. 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 where in the presence of salt it breaks the ring of the cytosine and that is called as C reactions. Once you are done with this 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 phosphatidyl ester bond next to the modified nucleotide. This means at the end what you are going to get you are going to get a pattern like this 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, but 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 and if you also have the same band in the G plus A reaction this means you are not going to read this.

It 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 will going to go with the from the lower band to higher band. 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. So, if you have got the two bands which are present both in the T plus C and C then you are going to read that as C other than T. 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 the DNA. So, so far 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 in the where in 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. Thank you.