Molecular Biology

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Module - 10

Molecular Cloning

Lecture-41 Cloning (Part 2)

Hello everyone, this is Dr. Vishal Debeli from the Department of Biosciences and Biosciences and Biosciences. So, in this context, in the previous lecture, we have discussed about the approach which is related to the condition when the gene sequence is not known, right, which means these are the conditions when the pre-genomic era, the people were only knowing the property of the enzyme, but they were not very sure about the, what is the gene sequence. And in that case, either you will have the information about the genome of that particular organisms or you will actually going to have the expression of that particular gene. So, in the previous lecture, we discussed about the, how you can be able to identify the gene if the genome is known. So, in that context, what you have to do is you have to prepare a genomic library and genomic library is going to be a combination or the collection of the genes which are going to be cloned into the individual clone and it is going to represent the complete genome, which means the genomic library is also going to represent the cDNA library as well, okay.

Whereas, if the protein or the, you know that the protein is getting expressed and you have actually some tools which are, you know, going to be tell you that, okay, there is a, you know, expression construct or you have the antibodies or something, right, and you want to screen these, you know, the expression clones, then you have to, then you have to isolate the transcriptomes. And in that case, what you have to do is you have to first isolate the messenger RNA and then from the messenger RNA, you have to prepare the cDNA lab, cDNA and that cDNA, you have to clone into the suitable vector and that is actually going to give you the cDNA library. And once you have the cDNA library, you can be able to use that for identifying the clone of your interest. So, in today's lecture, we are going to discuss about the, how you can be able to prepare the cDNA library and then how, whether it is the genomic library or whether it is cDNA library, how you are going to screen the clones for identifying the clone of your interest and how you can be able to then isolate the particular fragment so that you can be able to clone that into the expression vector and that is why you can be able to produce the protein in the large quantity.

So, the construction of the genomic library, construction of the genomic library is a multi-step process where you are, in the step one, you are going to do the isolation of the messenger RNA. In the step two, you are going to prepare the complementary DNA or

cDNA in short and then the step three, you are going to clone that into a suitable vector system and in the step four, you are going to transform that into the suitable host so that you can be able to prepare the cDNA library. So, cDNA library is a collection of the clones which are going to show you the expression status of that particular cell. So, first thing what you have to do is you have to first break open the cells, you have to isolate the messenger RNA. So, you have to isolate the messenger RNA.

The step two, so first you are going to break open the cells. You are going to get the cell lysate and from the cell lysate, you are going to isolate the messenger RNA and we are going to discuss how you are going to do the messenger RNA and once you have the messenger RNA from the cell, so these are the total messenger RNA and then you are going to use these messenger RNA and with the help of the enzyme reverse transcriptase, you will be able to synthesize the DNA which is called as cDNA. cDNA means complementary DNA, right? So complementary DNA to all the messenger RNA what you have isolated and then once you have the complementary DNA, then you can insert that into the bacterial plasmids or other suitable vectors. So that is actually going to give you the different clones and then these clones are actually going to transform into the suitable host and that is how you are going to get the cDNA library. From the cDNA library, you are actually going to isolate the clones of your interest and that is how you are going to identify the DNA sequence and then you can be able to use that for subcloning that into the expression vector and that is how you are going to use that for protein production or the enzyme production.

So before getting into the step number one where we are going to talk about the isolation of the messenger RNA, we have to first understand the structure of the messenger RNA because that is actually going to lead to the approaches what you can actually be able to use to isolate the total messenger RNA. So if you see the structure of the messenger RNA, in the structure of the messenger RNA you have a 5 prime cap, which is actually the 5 prime cap, then you have the 5 prime UTRs, then you have the coding sequence. So this is the coding sequence which is actually going to be responsible for production of the protein because this is the region what is going to be transcribed, which is going to be translated by the ribosomal machinery and then you have the 3 prime UTRs, these are the, so 5 prime UTR and the 3 prime UTRs are the regulatory region and after this you are actually going to do the post translational modification. So one of the classical post translational modification is that you are going to add the poly A tail and this poly A tail is present in all the messenger RNA, the length could be different, some of the having the 200 messenger As, some can be having the 50 and so on. The amount of the As what are going to be present on the 3 prime end of the messenger RNA is going to decide the age or the age of the messenger RNA or the durability of this messenger RNA, this means it is actually going to decide the stability of this messenger RNA in the cytosol.

Now this is the region which actually one can use to identify and purify the messenger RNA. So what you can do is you can actually be able to design affinity column and that

affinity column is actually going to have a very high affinity for a poly A tail and that is how you can be able to use that to isolate the all the messenger RNA what is present in the cytosol. So these are the different steps what you are going to follow for the isolation of the messenger RNA. So these are the this is the step 1 in the in the construction of the cDNA library. So first you have to isolate the messenger RNA so exploiting the structure of the messenger RNA you can actually be able to use the poly A tail and the poly A tail has a very high affinity for a poly T columns because the A is having a very high affinity for T and A is having the complementary to the T and that is why you can use a poly T column and that poly T column is actually going to give you the all the messenger RNA which having the poly A tail.

So what you are going to do is you are going to first culture the cells and then you are going to take those culture cells and you are going to do the lysis input it into the lysis buffer. If you are going to work with the tissue for example if you are going to work with the liver or spleen then in that case first you have to do the homogenization and the homogenization is actually going to break open the cells to a single cell suspension and single cell suspension is then going to be incubated with the lysis or the binding buffer and that is actually going to give you the lyses. Lyses means you are going to get the cell lysate and the cell lysate is going to have the two things one is you is going to have the messenger RNA is going to have the protein and it also going to have the other messenger other RNA species but as we said that messenger RNA is actually having the poly A tail so it is actually going to bind to the column. So what you are going to do is you are going to take the beads which are actually having the poly T linkers. So in these kind of beads what you have is you have a bead and on this bead you have a linker on which you are actually going to have the T nucleotides which are attached.

So these all these T nucleotides are actually going to have the affinity for a poly A tail what is present on the messenger RNA and that is how it is very specifically going to bind all the messenger RNA what is present in the cell lysate. So once it is once the binding step is over then you can wash the beads with a washing buffer and you can just discard the supernatant and you can collect the beads and then you are going to do the elution. So what you are going to do is you are going to do the elution with the help of the either the poly A you know poly A sequences or you can change the pH and other things. So once you do the elution you are going to you know you are going to have the two fractions you are going to have the bead fractions and you are going to have the supernatant fraction. This bead fraction is actually going to contain no messenger RNA because the messenger RNA is already been eluted.

So they are actually going to be present in the supernatant and that you can actually be able to collect into the new tube and that actually is going to give you all the messenger RNA which are actually going to have the poly A tail. So most of the messenger RNA are actually going to have the poly A tail. So now you have isolated the messenger RNA total messenger RNA from the cell and you are going to use that for synthesis of the cDNA library

or cDNA of these messenger RNA which means you are going to synthesize the complementary DNA. So in the step 2 you are going to synthesize the complementary DNA synthesis. The complementary DNA synthesis is a three step process.

In the step 1 you are going to first synthesize the reverse transcriptase. First you are going to synthesize the first strand with the help of the reverse transcriptase. Then the step 2 you are going to remove the RNA template and then in the third step you are going to synthesize the second strand which means first you have the messenger RNA poly A tail. So you are going to use that and you are going to put reverse transcriptase reactions and so you are going to put the reverse transcriptase reaction and that is how it is going to give you the two strands. So this is going to be the RNA, this is going to be the DNA.

So it is actually going to give you the DNA which is complementary to this and then you are actually going to catalyze the reaction so that it is actually RNA is going to degrade. So you are going to do the RNA degradation by many type of reactions and that is how you are going to have the RNA. It is only the DNA strand 1 and now using this DNA strand 1 you are going to put it for the polymerization reaction and the polymerization reaction is going to give you the two strands. So this is going to be the strand 1 and this is going to be the strand 2 and this is what is called as complementary DNA and this complementary DNA then can be cloned into a suitable vector and that is how you are going to get the cDNA library. So let us first discuss about how you are going to synthesize the complementary DNA.

So we have multiple approaches one what one can use to perform the cDNA synthesis. So in the method 1 you can use the homopolymer tailing method. In a homopolymer tailing method what you have is you have this is the messenger RNA what you have and what you can do is you can just put the oligo-dt primers. So what will happen is when you put the oligo-dt primers they will come and sit next to the poly A tail. So they will sit on the poly A tail and then you can actually be able to use the reverse transcriptase and the reverse transcriptase is actually going to synthesize the first strand.

So you are going to have the reverse transcriptase and you will going to add the four nucleotides and all the four nucleotides and this is going to work as a primer and that is how you are going to have the synthesis of the first strand. So this is the first strand synthesis. Once this is done then you can actually be able to get this and then you are going to perform the alkaline sucrose gradient. When you do the alkaline sucrose gradient it is actually going to hydrolyze the RNA and it is actually going to give you the first strand the c-densin synthesis and then what you are going to do is you are going to add oligo-g column. So you are going to add the oligo-dg columns and you are also going to add the reverse transcriptase and as well as all the four dNTPs and that is how what is happen is that it is actually going to all these all the GG is actually going to bind to the CCC on which is present on the first strand and that is how it is actually going to start the synthesis of the second strand and that is how you are going to get the duplex cDNA from the messenger RNA and that you can actually be able to insert into the vector by using the suitable restriction

enzymes or you can actually be able to use this polyT and the polyC sequences as well.

So you have two choices here either you can use the linkers or you can use the restriction enzymes. So in the homopolymer tailing this method exploits the presence of polyA tail present on the messenger RNA to synthesize the first strand followed by the degradation of the messenger RNA template and the second synthesis of second strand. So it has the following step. In the step one oligonucleotide dt primer is used with messenger RNA as a template to prepare the first strand of DNA with the help of the reverse transcriptase and the dnt piece. Once the first strand synthesis is over you can actually be able to do the terminal transferase is used then the terminal transferase is used to add the c nucleotide on the 3 prime of both the messenger RNA and as well as the newly synthesized strand of the DNA.

So after this you are going to add the you are going to run the terminal transferase enzyme and that is actually going to add the ccc on both the messenger RNA and as well as the cDNA. And then this DNA RNA hybrid is loaded onto alkaline sucrose gradient. So alkaline sucrose gradient is actually going to contain the NaOH and it is going to have the sucrose gradient. So what will happen is when you are going to load this RNA DNA hybrid onto alkaline sucrose gradient. So when you load this and it actually contains the NaOH.

So NaOH is actually going to degrade the RNA because it is actually going to add on act onto the 2 prime of hydroxyl and that is how it is actually going to form a cyclized product and that is how it is actually going to degrade the RNA. Whereas DNA does not contain the 2 prime hydroxyl and that is how it is not susceptible for the alkaline lysis. So once you do the alkaline lysis and you are going to load this complex onto the sucrose gradient the RNA is going to be degraded and the DNA the first strand of the DNA is actually going to be can be isolated after the gradient. So this step will hydrolyze the RNA and it will allow the full recovery of the cDNA. Once you got the first strand of the cDNA then you actually can use oligoDg primer and you can use the cDNA as a template to prepare the second strand of DNA with the help of the reverse transcriptase and the dNTPs.

At this stage the you know the second step synthesis you have the choice you can use the reverse transcriptase and polyD polyG primers or you can actually be able to use the tag DNA polymerase you can use the poly DNA polymerase as well and you can actually be able to use that with the dG primers. So either of that can be work as and which will give you the full length cDNA double standard cDNA DNA and that can further be inserted into the vector either by the homopolymer training or by the linkers. Then you have the step 2 the method 2, method 2 is called as the Gubber Hoffman method. So Gubber Hoffman method in this approach first strand synthesis using oligoD primer in the presence of reverse transcriptase and then dNTPs then DNA RNA hybrid is treated with the RNase H to produce the nick at the multiple site. Then the DNA polymerase is used to perform the DNA synthesis using the multiple fragments of RNA as a primer to synthesize the new DNA strand.

This method produces the blunt and duplex DNA. So in this first step is same as the homopolymer telling that you are going to add the quality primers and that is actually and in the with the help of the reverse transcriptase it is actually going to give you the messenger RNA and as well as double standard RNA. So it is going to give you the messenger RNA and as well as the first strand of the DNA and now what you are going to do is you are going to add the RNase H and you are going to add the random primers and DNA polymerase. So once you add the RNase H it is actually going to chew the RNA at multiple places. This means it is actually going to add the primers at multiple places.

So it is going to leave some amount of RNA and it is going to keep some nicks. So because of that this sequence is actually going to be used. In the second step what you are going to do is you are going to use these sequences for with the help of the DNA polymerase. So when you add the DNA polymerase to this along with the dNTPs plus dNTPs what will happen is that it is actually going to use this as a primer and that is how it is actually going to start synthesis. And you know that when the DNA polymerase will run it is actually going to remove this particular sequence and it is going to synthesize its own sequence and that is how it is actually going to synthesize the new DNA strand.

So there will be no RNA present. And the same is true for this one also. And that is how you are going to get the duplex cDNA and this duplex cDNA then can further be ligated or inserted into the vector either by the with the help of the linkers or the adaptor proteins. So this is all about that how you are actually going to prepare the genomic library or the cDNA library. And once you prepare the genomic library and cDNA library you are going to get the number of clones. And then the next task is that you are actually going to do the screening of these clone with the help of the different types of analytical tools.

These tools can vary and depends on the what kind of diagnostic probe you have. So for example if you have a gene fragment right if you have a fragment of the DNA which is known that it is actually going to give you the that particular enzyme or suppose you have a antibody or suppose you have some kind of activity which is actually be associated with the unidentified enzyme X then all these things can be used for screening. So in the screening you have three options either you can use as DNA probe and you can actually be able to use that for screening the clones or you can actually have the antibodies. You have antibody which is recognizing a particular enzyme or you can actually have the enzyme activity. So either of these three methods can be used.

So if you have the enzyme activity you can actually be able to use that also to identify the clone of your interest. So let's discuss about the screening of the genomic library or the cDNA library and what are the different approaches you can use. So as I said you know you can have the two three choices either you can use the DNA sequence. So this property can be used to search both the genomic library and as well as the cDNA library to identify the gene or the clone of your interest. Then we have the approach number two the expression

of a particular protein with the immunogenic epitope site.

So this property can be partially useful to screen genomic library due to the truncation of a full gene or no expression of a gene fragment but this approach will suit to the cDNA library. So if you have antibodies which recognizes the with the protein of interest or the enzyme of your interest. For example if you know that in a particular pathological conditions this particular antibody is being produced in the patient. So you want to identify if you want to identify the enzyme what you can do is you can take this antibody and you can prepare a genomic library or the cDNA library and then you can use that as a probe to identify the clone. In this approach the cDNA library is more suitable because cDNA library means you are actually going to have the expression clones.

So in the expression clones the clones are actually going to start suppose this is a clone and this is the gene what you have inserted this is the messenger RNA the cDNA it is actually going to produce the protein and this protein is then can be detected with the help of the antibody. Whereas in the case of genomic library the problem is that genomic library sometime may have the truncated proteins. So sometime it may have half protein and the half gene may be of the other. So in that case it may actually give you the protein and that time you can be able to use but if it does not if it only gives you a truncated protein for example if it only gives you a half protein and that half protein does not contain the antigenic site then in that case it is actually not going to work. So 100% if you have the antibody which is going to use as a tool to recognize the enzyme you can be able to use the cDNA library.

But for the other case like the genomic library the DNA is more suitable. Then we have the enzymatic activity for example if you are trying to explore enzyme which is associated with a particular activity but you do not know the gene in that approach you can actually be able to use the enzymatic activity. So this property exploits the ability of a protein fragment to exhibit enzymatic activity it is useful for the screening of cDNA library but it is not useful for the genomic library because of the simple reason that the genomic library may or may not be complete. So the gene fragments are not complete they may give you the truncated proteins and those truncated proteins may or may not give you the activity.

So let us start the first method. The first method is where you are going to use the DNA sequence or DNA probe for screening the clones and that can be used both for genomic library and as well as for the cDNA library. So DNA if you see the structure of a DNA, DNA has the double helical structure where you have the nucleotides what is present inside the helix and these nucleotides have the very peculiar base pairing. You always know that the adenine is always making a pair with thymine and whereas the guanine is always making a pair with cytosine. So because they are very strict and they are also only making a pair you can actually be able to use that as a sequence. So wherever you have the A you are going to have the T on the template wherever you have the G on the probe it is actually going to have the C on the template.

So suppose I have a template DNA or if I have the genomic sequence which I want to screen then what I will do is I will prepare a probe like for example I have prepared a probe like this. So this is the probe I have prepared. Because this is the sequence I know that it is actually going to bind to that particular gene which is responsible for the production of this particular gene. So now when it is actually going to recognize it is actually going to recognize a protein or the DNA. What will what DNA sequence it is going to identify? It is actually going to identify a DNA sequence of this.

So wherever it will find a DNA sequence with this it is actually go and bind and that is how you can be able to identify this template DNA or this cDNA clone or the clone DNA with the help of the probe. So this is going to be the probe this is going to be this and the probe will where the probe is binding for that you have to put some kind of you know the tag actually. So you can actually be able to put the fluorescent tag or you can actually be able to put the radioactive probe or radioactive tag. So if you add the radioactive probe which has this sequence it will go and bind to all the DNA sequences or the gene sequences where you have the this particular sequence present. Now how you are going to do this? You are going to use this with the help of the from cDNA library.

So this is suppose this is the cDNA this is the library whether it is the genomic library or cDNA library. So you are going to have the main plate or the plate where you are going to have all the clones and that plate is called as the master plate which means this is the original plate where the your clones are present. So imagine that these are the clones you have. So then first in the step one what you are going to do is you are going to first transfer the master plate and you are going to prepare a replica plate. So that you can actually be able to work with all the clones without destroying the master plate without destroying the original clones.

So you are first going to prepare a replica plate. So you are going to just insert invert this onto another plate and that is how it is actually going to give you the replica plate or you can actually be able to transfer that onto a nitrocellulose membrane and that is how it is actually going to give you a impression of the clones onto the nitrocellulose membrane. Now in the step three the step two you are going to do the lysis of these cells. So once you lysed the cells it is actually going to denature the DNA and it will actually going to bind the matrix which means all the clones are all these are you know cells. So they will be get lysed and that is how the DNA will come out. So DNA will come out from these cells but they will not going to washed away because they will go and bind to this nitrocellulose membrane.

Now what you are going to do is in the step three you are going to add the DNA probe which actually has the tag. So either it can have the radioactive tag or is a fluorescent tag. So once the tag is there it will actually go and bind to its specific you know for example if it is binding to this particular clone and then what you can do is you can just take this replica plate or you can take this replica membrane and then you can actually compare that with

the help of the master plate and you will know that this is the clone what is response or which is the where the gene of my interest is present. And then what you can do is you can just take out this gene of interest and you can just grow them into the media and that is how you can be able to isolate the plasmid or you can actually isolate the recombinant DNA and from this plasmid you can be able to isolate the gene of your interest and that is how you can use this gene for further downstream applications.

So it has the following steps okay. The step one you are going to prepare a suitable radioactive probe you can prepare also the fluorescent probe then you are going to prepare a replica plate. So this is what the replica plate then you are going to transfer of the colonies on the nitrocellulose membrane then you hybridize that with a specific probe which means the radioactive probe and then you are going to wash and development of the membrane by the audio radiography and that is actually going to tell you on which colony it is binding and that is how you can actually go back to the master plate and you can actually identify. Now how you are going to prepare the radioactive probes so you can actually be able to use the multiple methods of preparing the radioactive probes. So in the preparation of the radioactive probes you can use the random primer methods. So in the random primer methods in this method random primer is used to anneal to the template and then a PCR reaction is performed in the presence of the radiolabeled nucleotide after the PCR the newly synthesized DNA strand is labeled with the radioactive nucleotides.

So what you are going to do is suppose this is the template for which you want to synthesize the probe so what you are going to do is you are going to add oligonucleotide primers and you will do the hybridization so it will actually go and hybridize and then what you are going to do is you are going to do a DNA synthesis with the help of the clinofragments and the 4 dNTPs so it will actually going to synthesize the strands and that is how it is actually going to incorporate the radioactive nucleotides and that is how it is actually going to produce the labeled probe and that labeled probe you can actually be able to purify with the help of the gel filtration chromatography and that can be used for further downstream applications. Then we also have the terminal transferase method so we also have the terminal transferase method so in the terminal transferase method you are going to use an enzyme which is called as terminal transferase so in this method a terminal transferase enzyme will label the probe at the end of the last nucleotide of the probe. Probe is incubated with the labeled nucleotide and the terminal transferase enzyme and will add the labeled nucleotide at the end. A partial purification of the gel filtration column will give you the labeled probe. So for example this is the gene so what you are going to do is first you are going to rotate it with the alpha endonuclease and that is actually going to cause the nicks and then you are going to have the terminal transferase and as well as the radioactive DATPs for example if I want to you know radio labeled DATP so what will happen is it is actually going to add the A's on one end and that is how it is actually going to incorporate the radio activity in both the strands of this particular gene and that is how I am going to get the radioactive probe with one end of the probe as radioactive and that I can further purify with the help of the gel filtration protocol.

And now once the probe is ready I can use that for the screening purpose. So for the screening purpose I have to prepare the replica plate so as the original genomic DNA or cDNA library is precious and will be consuming in the later stage all procedure is performed with the replica plate containing the colonies in a identical manner. First you are going to transfer so the clone is transferred on to a nitrocellulose membrane with retaining the identical pattern of the colonies on the master plate. The cells on the membranes are lysed and the released DNA is denatured, deprotonated and allowed to bind to the membrane. So this step is very crucial and it actually going to decide what will be the what will be the success of your screening because if you could be able to do this successfully then you what you are going to do is you are going to actually lyse the cells and the DNA is actually going to immobilized to the site of that particular clone.

Then you are going to do the hybridization so available probe prepared in step one will actually going to be added the probe will add to the target DNA due to the base pairing the membrane is washed to remove the unbound and then you are going to do the development of a durodogram. So this position of the durodogram is detected by the autoradogram the position of the signal on the membrane can be washed with the master plate to get the location. For example if this is the plate and this is my master plate so I will actually super impose both of them and then I will know that okay this is the clone which actually is giving the signal or the durodogram. So then I can actually be able to isolate this and I can isolate the gene of interest. Now the second step second method so second method is the screening by the immunological method.

So in the immunological method you are going to use the antibody as the probe. So antibodies can be tagged with the enzyme or you can actually be able to add the fluorescent. So antibody can be tagged to the fluorescent dye or it can be attacked to the enzyme such as HRP or alkaline phosphatase. So in this case what you are going to do is step one is same you are actually going to prepare first master plate and then you are going to prepare the replica plate and from the replica plate you are going to transfer the cells and onto the membrane and that is how you are going to prepare the netocellulose membrane and then you are going to light the cells and allow the protein to bind to their site. And once that is done then you are going to add the primary antibodies and once you add the primary antibody the primary antibody is actually will go and bind to the sites wherever you have the antigen of interest.

You treat the matrix with the primary antibody and the primary antibody is actually going to you know bind the proteins what is present within the cell and that is how it is actually going to give you and then what you are going to do is you are going to add the secondary antibodies. So secondary antibody is actually going to be tagged with the enzyme or the fluorescence right so that secondary antibody will go and bind to the primary antibody and wherever it will bind it is actually going to give you a signal. So looking at the signal right for example this is the clone which is giving you the signal you can go back to the original

plate so original plate is saying that this is the clone from where I am getting the signal. So in that case you actually can use that and you can isolate that clone and you can actually be able to grow that into the large quantities and that is how you can actually be able to isolate the plasmids and from the plasmid you can be able to isolate the gene and that gene you can actually be able to use for the over expression purpose. So these are the two methods which are very very popular either the immunological method where you are going to use the antibody as a probe or the DNA probes.

Apart from that you can also use the screening by the enzymatic method. So this method is based on the ability of a protein to exhibit an enzymatic activity. This method is not very specific but it allow us to identify a class of protein with the known enzymatic activity. So in this case also you are going to have the same steps what you are going to do is you are going to first prepare the master plate right.

So this is the master plate. So this is the master plate from the master plate you are going to prepare the replica plate like this okay and this is going to be your replica plate and replica plate from replica plate you are going to first transfer that onto a nitrocellulose membrane. So you are going to prepare that onto the NC membrane and on this NC membrane you are going to add the substrate for your enzyme. So you are going to lyse the cells right you are going to lyse the cells and you are going to add the substrate. So once you add the substrate it is actually going to give you the signal okay. This signal you can actually be able to compare which is there on the replica plate to the master plate and that is why you will say that okay this is the clone of my interest and that is why it is actually going to give you.

The only issue with the screening by the enzymatic method is that it may not be unique because in some cases you might have to see that multiple clones are actually going to give you the activity because the substrate is a very very you know non-specific probe because substrate can be used by the multiple enzymes and that is how it may actually give misguide you in terms of the getting the clones. For example if I use the glucose as a substrate right so glucose can be used by the hexokinase glucose can be used by the glucokinase glucose can be used in any other reactions also. So in that case you may get the clone of your interest but it is not very specific and you may actually be able to use or you might have to use the other screening method to further verify the clones. So this is all about how you can be able to screen the genomic library and as well as the cDNA library and once you actually have screened and you say that okay this is the clone I have to isolate you can actually have to perform the multiple steps to isolate this particular clone also. So how you are going to isolate the gene so once the position of a clone is known it is extracted from the master plate and the plasmid is isolated in few cases the clone is further diluted to check the homogeneity of the clone the purity of the clone and the presence of clone is further tested with the PCR using the size sequence specific primers.

So what happen is that you what you are going to do is you are going to first isolate the

clone okay and then you are actually going to dilute that into 1 is to 10 or 1 is to 100 dilutions okay and then again you are going to plate that onto a plate okay so in that case you are going to get all the colonies okay. Ideally if this clone is pure which means it only has a single gene it actually will all these clones are identical to each other. So again if you repeat the probing reactions like either you use the DNA probe or antibody probe or the enzymatic method all these clones should actually give you the signal which means all these clones should give you the signal because if it could also happen that some of the clones actually will give you the signal okay. In those cases what happen is in that case the this particular clone is not pure it may have actually the multiple clones which are coming together or when you are isolating the clone you actually got the cross contamination from the neighboring clones also. So in that case you have to first do the you know this is the primary screening through which you got this clone then you might have to do the secondary screening by further diluting these clones and doing the same reaction again and again and you have to repeat that until you are actually getting all the clones which are actually going to give you the signal.

So that is what you have to do if you want to isolate the gene. So once you got this you can actually be able to isolate the plasmid and that plasmid is actually going to contain the gene of your interest so that you can actually be able to use the gene and then you can actually be able to use the PCR and you can actually we get the amplified gene and that amplified gene you can put into the expression clones or expression vector and that is why you can be able to use that for protein production. So this is what we have discussed. So we have discussed about the approach one where which is very very common or very very much popular into the pre genomic era when the genomic sequence were not known and you were only knowing that there is an antibody which has been found in the patient or there is a genomic sequence or the DNA fragments is found and something like that. So in those cases you have to use the genomic library so you have to take the genome you have to prepare the genomic library or you might have to isolate the messenger RNA and you have to prepare the cDNA library and then once you are you prepare the genomic library or the cDNA library you can use that for screening the clone of your interest with the help of either the DNA probe or the antibodies or the enzymatic method. Once utilizing these three screening tools you can be able to screen the clone of your interest and once you got the clone of interest you can just isolate the plasmid you can prepare the you can isolate the gene of your interest you can use the PCR to amplify the gene of your interest and then you can actually clone it into the suitable expression vector and that is how you are going to get that particular gene responsible for the production of the enzyme into a expression vector and that is how you can be able to use that for enzyme production.

So with this I would like to conclude my lecture here in our subsequent lecture we are going to discuss about the second approach where the gene sequence is known and that is very common and very popular nowadays for the enzyme production. So with this I would like to conclude my lecture here. Thank you. you