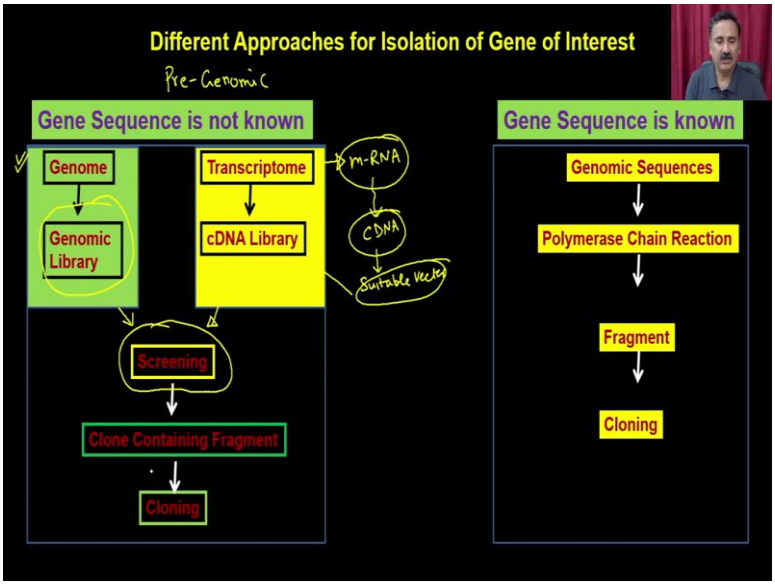
Enzyme Science and Technology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati

Module - III
Enzyme Production (Part 1: Cloning)
Lecture - 13
Identification of Enzyme Gene (Part-II)

Hello everyone. This is Dr. Vishal Trivedi from Department of Biosciences and Bio Engineering IIT, Guwahati. And, what we were discussing? We were discussing about the different properties of the enzyme in the course Enzyme Science and Technology.

(Refer Slide Time: 01:18)



So, in this context in this particular module, we are discussing about the enzyme production. And, if you recall in the previous lecture, we have discussed about the different approaches what one can go for to identify and isolate the gene responsible for a particular enzyme.

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 discuss about the how you can be able to identify the gene, if the genome is known.

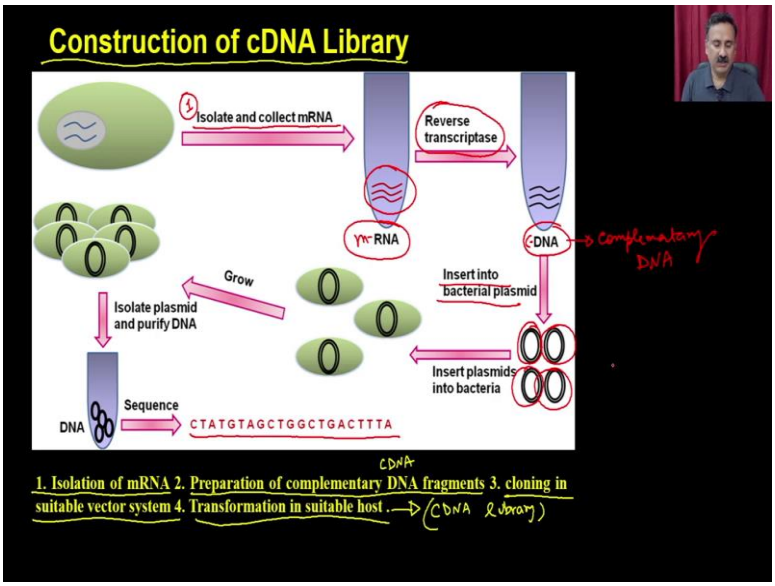
So, in that context what you have to do is you have to prepare a genomic library and genomic library is going to be a combination or the collection of the genes which are going to be cloned into the individual clone. And, it is going to represent the complete genome which means the genomic library is also going to represent the cDNA library as well ok.

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 ok, 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 this 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.

(Refer Slide Time: 03:48)



So, the construction of the genomic library, construction of the genomic library is a multi-step process where in the step 1, you are going to do the isolation of the messenger RNA. In the step 2, you are going to prepare the complementary DNA or cDNA, in short and then the step 3, you are going to clone that into a suitable vector system.

And, in the step 4, you are going to transform that into the suitable host so, that you can be able to prepare the cDNA library ok. So, cDNA library is a combine 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 RNAs right.

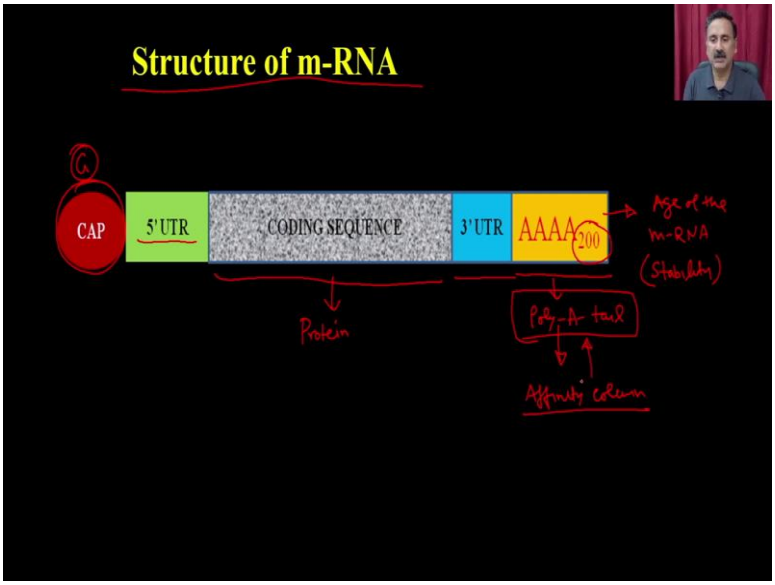
So, you have to isolate the messenger RNA and the step 2 you. So, first you are going to break open the cells, you are going to get the cell lysate and from the cell lysate you are going to isolate the messenger RNA. And, we are going to discuss how you are going to do the messenger RNA. And, once you 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 sub cloning that into the expression vector and that is why you are going to use that for protein production or the enzyme production. So, before getting into the step number 1, 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.

(Refer Slide Time: 06:16)



So, if you see the structure of the messenger RNA, in the structure of the messenger RNA you have a 5 prime cap ok 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 a protein, because this is the region which is going to be translated by the ribosomal machinery.

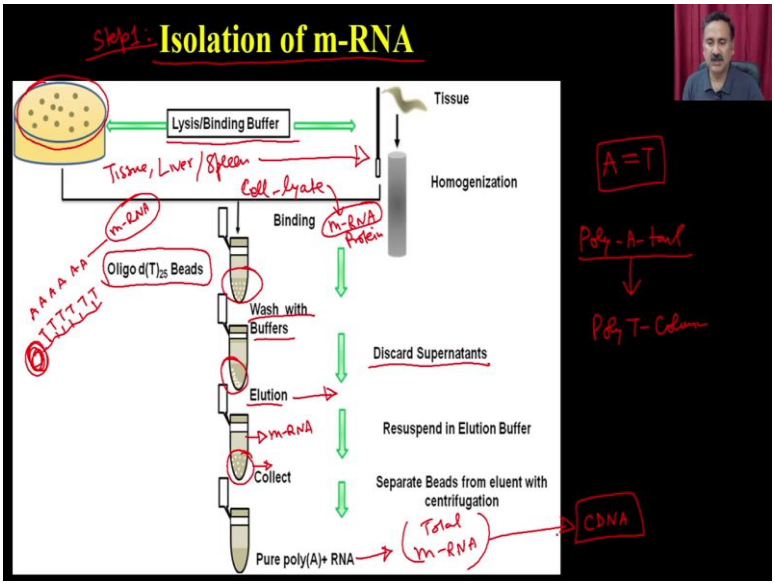
And, then you have the 3 prime UTR. So, 5 prime UTR and the 3 prime UTRs are the regulatory region. And, after this you are actually going to do the post translational modification. So, one of the classical post translational modification is that you are going

to add the poly A tail. And, this poly A tail is present in all the messenger RNA, their 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 A's 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 measured 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 a affinity column and that affinity column is actually going to have a very high affinity for a poly A tail. And, that is all you can be able to use that to isolate the all the messenger RNA what is present in the cytosol.

(Refer Slide Time: 08:00)



So, these are the different steps what you are going to follow for the isolation of the messenger RNA. So, this is the step 1 in the construction of the cDNA library ok. 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 tail has A very high affinity for a poly T columns ok, 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 and put 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 the single suspension is then going to be incubated with the lysis or the binding buffer and that is actually going to give you the lycells. Lycells means you are going to get the cell lysate.

And, the cell lysate is going to have the two things. One is its going to have the messenger RNA, it is going to have the protein and it also going to have the other RNA species. But, as we said that in the 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 amino 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 you are once the binding step is over, then you can wash the beads with the washing buffer and you can just discard the supernatant and you can collect the beads ok. And, then you are going to do the illusion.

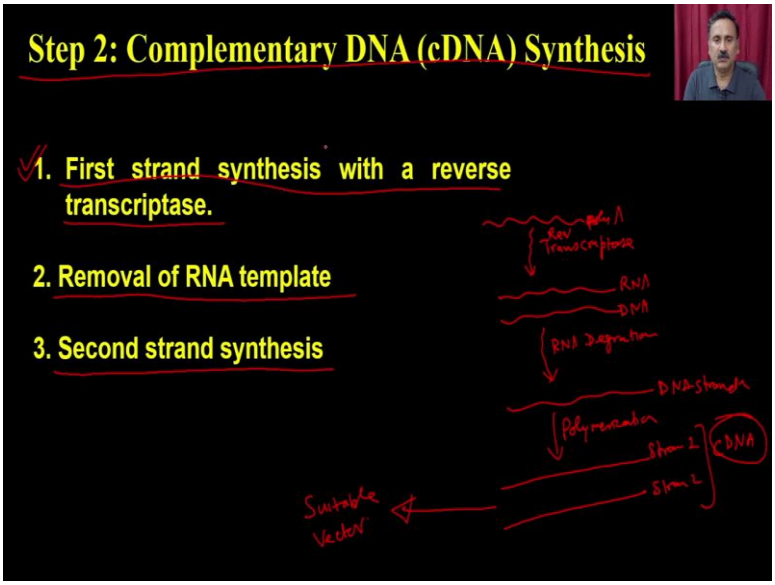
So, what you are going to do is you are going to do the illusion with the help of the either the poly A you know poly A sequences or you can change the pH and other things ok. So, once you do the illusion, you are going to have the two fractions. You are going to have the bead fractions and you are going 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 the all the messenger RNA which are actually going to have the poly A tail.

So, most of the messengers 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.

(Refer Slide Time: 11:39)



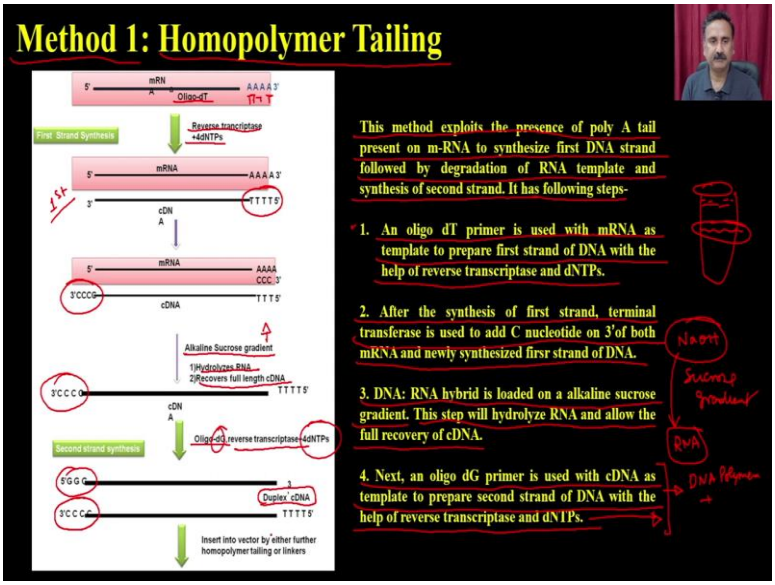
So, in the step 2, you are going to synthesize the complementary DNA synthesis. So, complementary DNA synthesis is a 3 step process. In the step 1, first you are going to synthesize the first strand with the help of the reverse transcriptase. Then, in the step 2 you are going to remove the RNA template and then in the third step, you are going to synthesize the second strand which means first you have the messenger RNA right, poly A tail right.

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 a 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 why you are going to have the RNA, that 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, what one can use to perform the cDNA synthesis.

(Refer Slide Time: 13:23)



So, in the method 1, you can use the homopolymer tailing method. I mean 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 ok.

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 ok. So, you are going to have the reverse transcriptase and you will going to add the 4 nucleotides and all the 4 nucleotides. And, this is going to work as a primer and that is

why 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 the this right 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 cDNA synthesis. And, then what you are going to do is you are going to add the then you are going to add the oligo G column right.

So, you are going to add the oligo G column, oligo dG columns and you are also going to add the reverse transcriptase and as well as or the 4 dNTPs. And, that is how what will happen is that, it is actually going to the C C C 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 a messenger RNA.

And, that you can actually be able to insert into the vector using the suitable restriction enzymes or you can actually be able to use this poly T and the poly C G 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 poly homopolymer tailing, this method exploit the presence of poly A tail present on the messenger RNA to synthesize the first strand followed by the degradation of the messenger RNA template and the synthesis of second stand. So, it has the following step. In the step 1, oligo nucleotide 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 dNTPs.

Once the first strand synthesis is over, you can actually be able to do the terminal transferase is used. Then, 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 you know terminal transferase enzyme and that is actually going to add the C C C on both the messenger RNA and as well as the cDNA right. And, then this DNA RNA hybrid is loaded onto a

alkaline sucrose gradient. So, alkaline sucrose gradient is actually going to contain the NaOH and it is going to have the sucrose gradient ok.

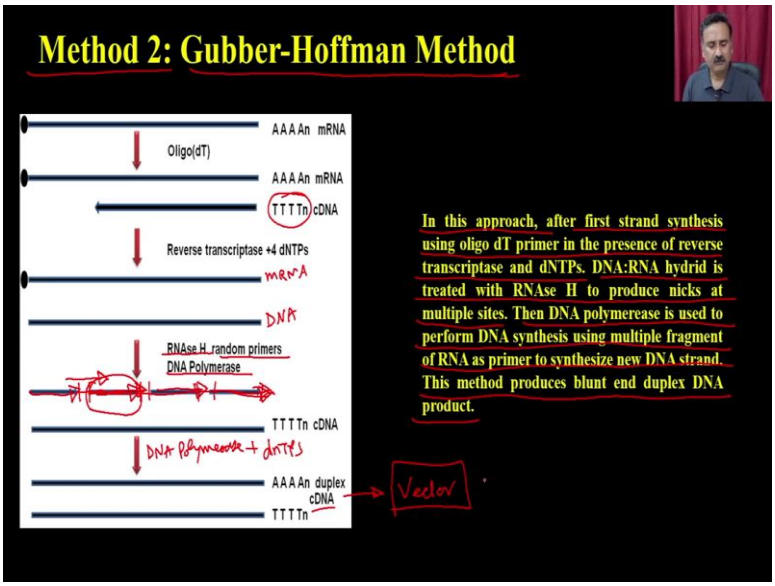
So, what will happen is when you are going to load this RNA DNA hybrid onto a alkaline sucrose gradient. So, when you load this ok and it actually contains the NaOH. So, NaOH is actually going to degrade the RNA, because it is actually going to act on to the 2 prime of hydroxyl and that is how it is 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 why it is not resist 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 a oligo dG 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, the poly G primers or you can actually be able to use the type DNA polymerase, you can use the ordinary polymerase as well and you can actually be able to use that with the dG dG primers. So, either of that can be work as and which will give you the full length the cDNA, double stranded cDNA DNA. And, that can further we inserted into the vector either by the homo polymer tailing or by the linkers.

(Refer Slide Time: 18:26)



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 oligo dT 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 end duplex DNA. So, in this first step is same as the homopolymer tailing that you are going to add the poly T primers.

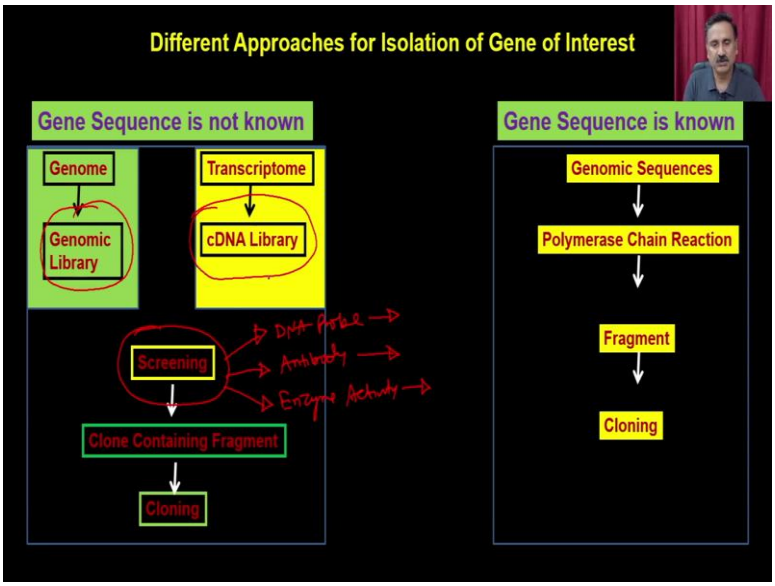
And, that is actually and in the with the help of the reverse transcriptase, it is actually going to give you the messenger RNA as well as double stranded RNA. So, it is going to give you the messenger RNA and as well as the first strand of the DNA. And, now what you are going to do is you are going 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 the 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 ok and the same is true for this one also ok. 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 adapter proteins.

(Refer Slide Time: 20:47)



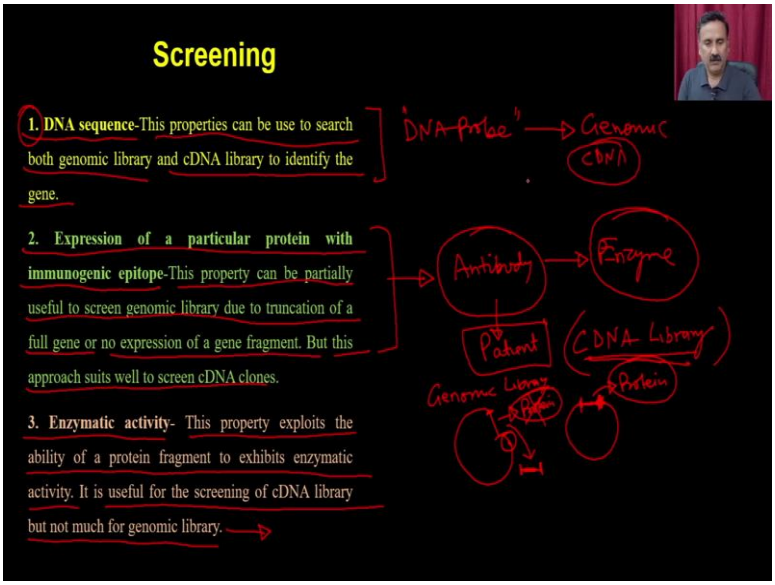
So, this is all about that how you are actually going to prepare the genomic library or the cDNA library. And, once you prepare the genomic library and cDNA library, you are going to get the number of clones right. And, then the next task is that you are actually going to do the screening of these clone 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 ok.

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 method can be used.

So, if you have any enzyme activity, you can actually be able to use that also to identify the clone of your interest. So, let us discuss about the screening of the genomic library or the cDNA library and what are the different approaches you can use.

(Refer Slide Time: 22:19)



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 our interest. Then, we have the approach number 2, 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 well suits to the cDNA library. So, if you have a antibody which recognizes the with the protein of interest or the enzyme of your interest right.

For example, if you know that in a particular pathological conditions, this particular antibody is being produced in the patient right. So, you want to 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 ok. So, in the expression clones, the clones are actually going to start; suppose this is the clone and this is the gene what you have inserted right. This is the messenger RNA the cDNA, it is actually going to produce the protein ok 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 libraries sometime may have the truncated proteins right. 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 that time you can be able to use.

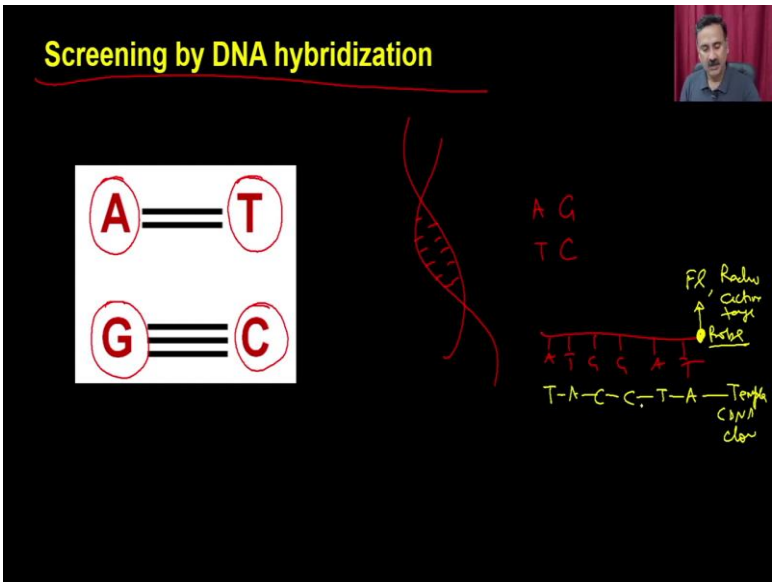
But, if it does not, if it only gives you a truncated protein; for example, if it only gives you a half protein and that half protein does not contain the interchanging site, then in that case it is actually going not going to work. So, 100 percent if you have the antibody which is going to use as a 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 a enzyme which is associated with a particular activity, but you do not know the gene. In that approach, the you can actually we will 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 right. So, the gene fragments if 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.

(Refer Slide Time: 25:42)



So, DNA if you see the structure of a DNA, DNA has the double helical structure right where you have the nucleotides, what is present inside the helix right. And, these nucleotides have the very peculiar the peculiar base pairing right. You always know that the adenine is always making a pair with thymine and whereas, the guanine in is always making a pair with cytosine.

So, because they are very strict and they are also only making a pair right, 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 ok which I want to screen, then what I will do is I will prepare a probe.

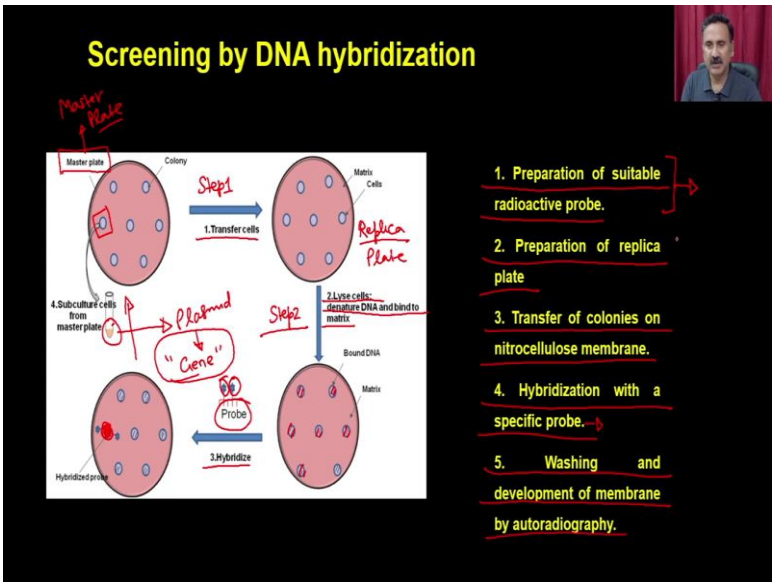
Like for example, I have prepared a probe like this ok. So, this is the probe I have prepared right. So, because this is the sequence, I know that it is actually going to bind to that particular gene which is responsible for the production of this particular design. So, now, when it is actually going 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 ok 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 a 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 tags ok. 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.

(Refer Slide Time: 28:00)



Now, how you are going to do this? You are 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 ok.

So, imagine that these are the clones you have. So, then first in the step 1, 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 on to nitro

cellulose membrane. And, that is how it is actually going to give you a impression of the clones onto the nitro cellulose membrane. Now, in the step 2, you are going to do the lysis of these cells.

So, once you lyse 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 right. So, they will be get lysed and that is how the DNA will come out right. So, DNA will come out from these cells. But they will not going to washed away because they will go and bind to this nitro cellulose membrane.

Now, what you are going to do is in the step 3, 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 ok.

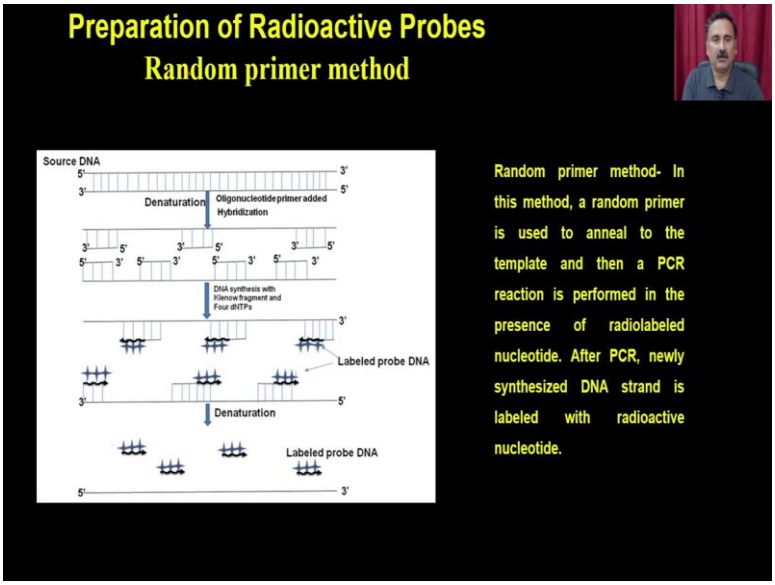
And, then what you can do is you can just take out this gene of interest and you can just grow them into the media. And, that is how you can be able to isolate the plasmid or you can actually isolate the recombinant DNA. And, from this plasmid you can be able to isolate the gene of your interest and that is why you can use this gene for further downstream applications.

So, it has the following steps ok. The step 1, 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 nitro cellulose membrane. Then, you hybridize that with a specific probe which means a 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 probe it is actually what on which colony it is binding and that is why you can actually go back to the master plate and it can

actually be identified. 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.

(Refer Slide Time: 31:31)

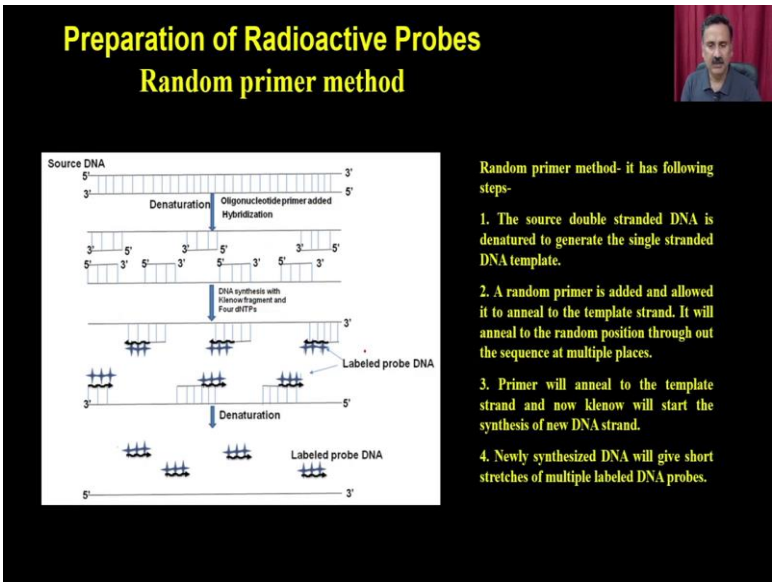


So, in the preparation of the radioactive probes, you can use the random primer methods. So, in the random primer methods, in this method random primer is used to anneal to the template and then a PCR reaction is performed in the presence of the radio labeled nucleotide. After the PCR, the newly synthesized DNA strand is labeled with a radioactive nucleotide.

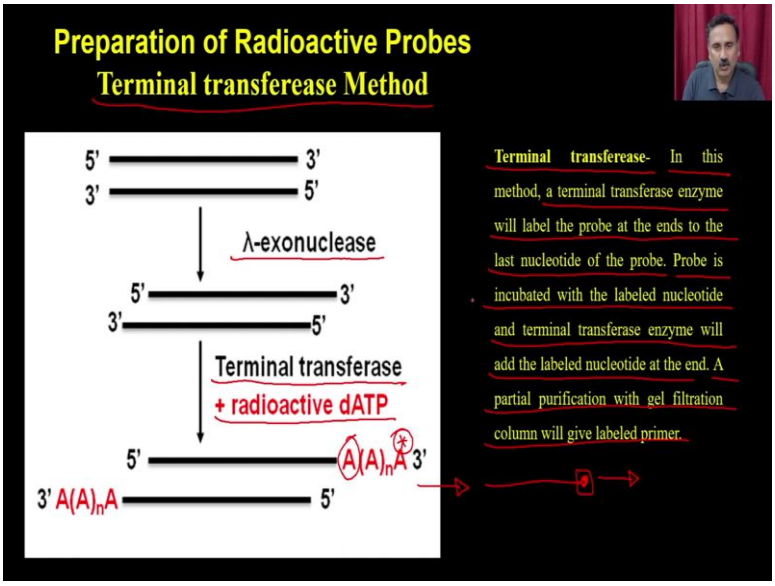
So, what you are going to do is suppose this is the template which for which you want to synthesize the probe. So, what you are going to do is you are going to add the oligo nucleotide primers and you will do the hybridizations. So, it will actually go and hybridize.

And, then what you are going to do is you are going to do a DNA synthesis with the help of the Klenow fragments and the 4 dNTPs which 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.

(Refer Slide Time: 32:36)



(Refer Slide Time: 32:41)



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.

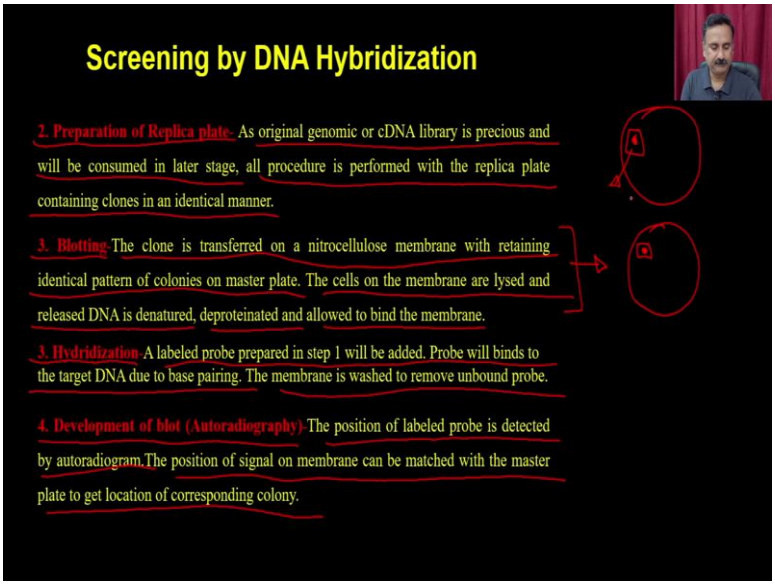
Probe is incubated with the labeled nucleotide and the terminal transferase enzyme and will add the labeled nucleotide at the end. A partial purification with the gel filtration column will give you the labeled probe. So, for example, this is the gene ok. So, what

you are going to do is first you are going to treat it with the alpha endonuclese 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 dATP for example, if you want if I want to you know radio labeled dATP right. So, what will happen is it is actually going to add the A's on one end ok. And, that is how it is actually going to incorporate, the radio radioactivity 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 chromatography. And, now once the probe is ready, I can use that for the screening purpose.

(Refer Slide Time: 33:58)



So, for the screening purpose, I have to prepare the replica plate. So, as a 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 onto a nitro cellulose membrane with retaining the identical pattern of the colonies on the master plate.

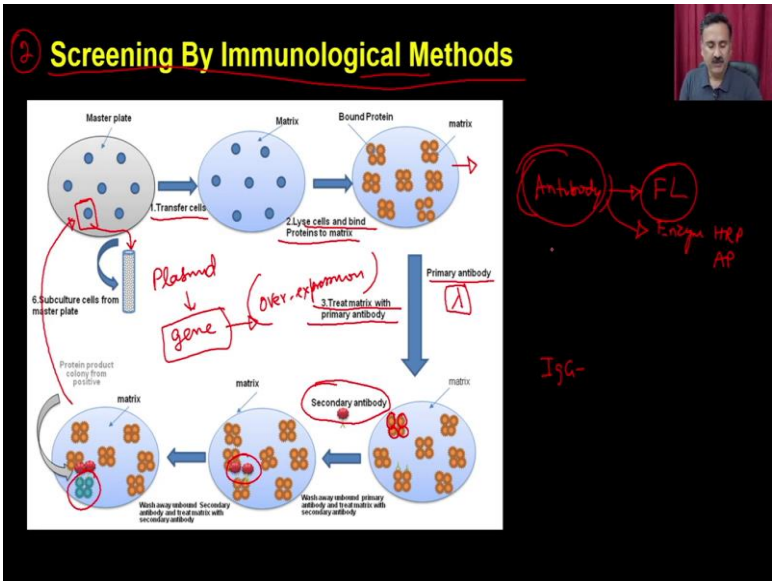
The cells on the membranes are lysed and the released DNA is denatured, deprotonated and allowed to bind to the membrane. So, this step is very crucial and it actually going to decide what will be the success of your screening. Because, if you could be able to do

this successfully, then 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, a labeled probe prepared in step 1 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 blot autoradiogram.

So, this position of the radio leveled is detected by the autoradiogram. 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 right and this is my master plate. So, I will actually superimposed both of them and then I will know that ok, this is the clone which actually is giving the signal or the radio radiogram. So, then I can actually be able to isolate this and I can isolate the gene of interest.

(Refer Slide Time: 35:37)



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 right. 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 tagged to the enzyme such as HRP or alkaline phosphates.

So, in this case what you are going to do is step 1 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 nitrocellulose membrane.

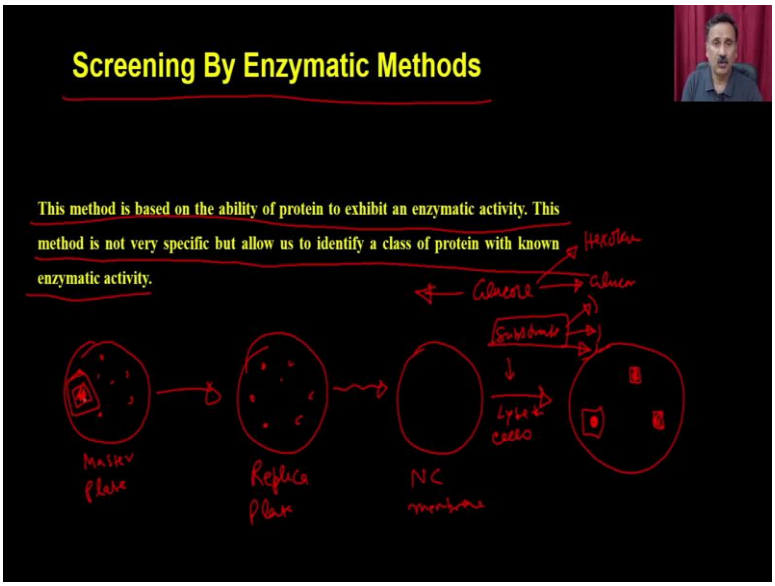
And, then you are going to lyse the cells and allow the protein to bind to that site. And, once that is done, then you are going to add the primary antibodies. And, once you add the primary antibody, the primary antibody is actually will go and bind to the sites wherever you have the antigen of interest.

You treat the matrix with the primary antibody and the primary antibody is actually going to you know bind and 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 very which are very very popular either the immunological method where you are going to use the antibody as a probe or the DNA probes.

(Refer Slide Time: 37:59)



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 will 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, right this ok. And, this is going to be your replica plate and replica plate from replica plate, you are going to first transfer that onto a nitro cellulose 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 ok. 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 ok, this is the clone of my interest and that is why it is actually going to give you.

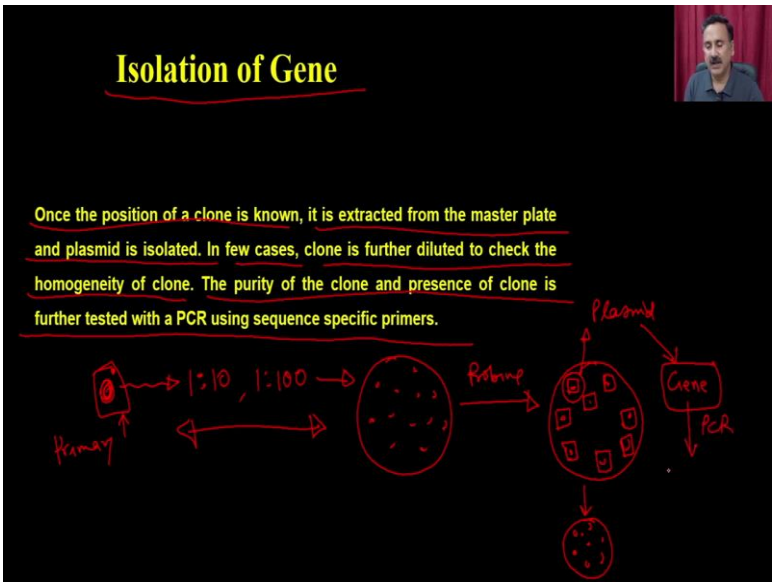
The only issue with the screening by the enzymatic method is that it may not be unique, because in some cases you might have to see that the multiple clones are actually going to give you the activity. Because, the substrate a substrate is a very very you know non-

specific probe, because substrate can be can be used by the multiple enzymes and that is how it may actually 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 the you may get the clone of your interest. But, the 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 ok, this is the clone I have to isolate; you can actually have to perform the multiple steps to isolate this particular clone also.

(Refer Slide Time: 40:31)



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 sites sequence specific primers.

So, what happen is that what you are going to do is you are going to first isolate the clone ok and then you are actually going to dilute that into 1 is to 10 or 1 is to 100

dilutions ok. And, then again you are going to plate that onto a plate ok. So, in that case you are going to get all the colonies ok.

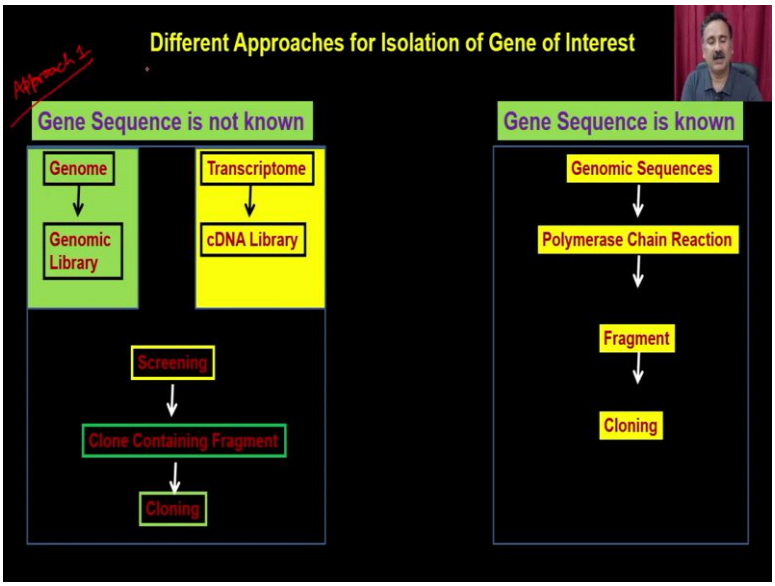
Ideally, if this clone is pure which means it only has a single gene, it actually will all these clones are identical to each other. So, again if you repeat the probing reactions like either you use the DNA probe or antibody probe or the enzymatic method. All these clones should actually give you the 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 ok.

In those cases, what happen is in that case 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 be 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.

(Refer Slide Time: 42:51)



So, this is what we have discussed. So, we have discussed about the approach 1, where which is very very common or very much popular into the pre-genomic era when the genomic sequence were not known. And, you were only knowing that the there is an antibody which is being found in the and patient or there is a genomic sequence or the DNA fragment is found in 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 your 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 the 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 to for the enzyme production.

So, with this I would like to conclude my lecture here.

Thank you.