Genetic Engineering: Theory and Applications
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Module 3: Basics of Cloning (Part I)
Lecture 08: Isolation of Gene Fragment (Part II)

Hello everybody, this is Doctor Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And what we were discussing, we were discussing about the isolation of gene from the particular host and in that context what we were discussing, we were discussing about the preparation of genomic library as well as cDNA library. So let us summarize what we have discussed in the previous lecture. So what we have discussed, we have discussed about how to prepare the genomic library.

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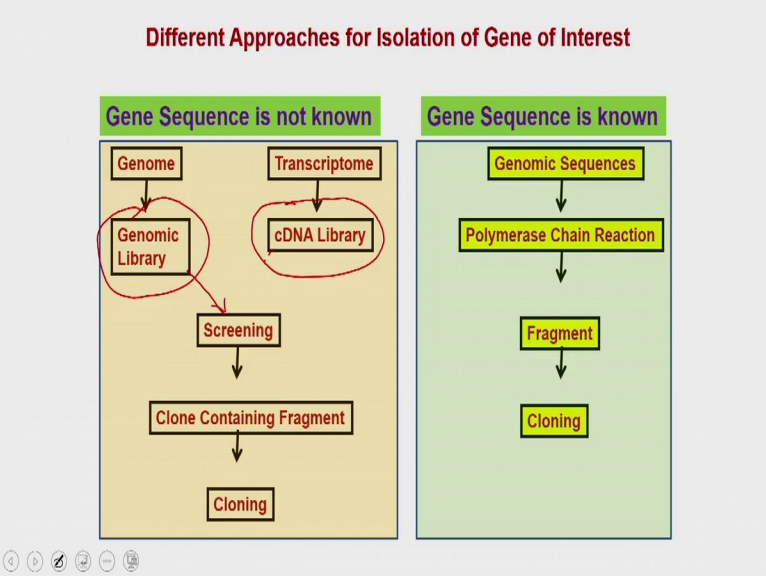

Within the genomic library we have discussed how to isolate the genomic DNA, then how to digest the genomic DNA to a suitable size fragment and then how to clone these fragments into the different vectors and what is the carrying capacity of the different vectors and so on. Similarly for the cDNA library we have prepared, we have discussed about how to isolate the RNA from the, from the mammalian cells and then how to prepare the cDNA. We have discussed different methods of preparing the cDNA.

And then ultimately we have discussed also about how to integrate this cDNA into the vectors or the carrying molecules and then at the end we have discussed about how to transform, deconstruct into the suitable host and then ultimately you are going to get either the genomic library or through the cDNA library. So now the next topic to get the gene of

interest is that you have to screen either the genomic library or to the cDNA library. So let us see how to screen the genomic library or the cDNA library.

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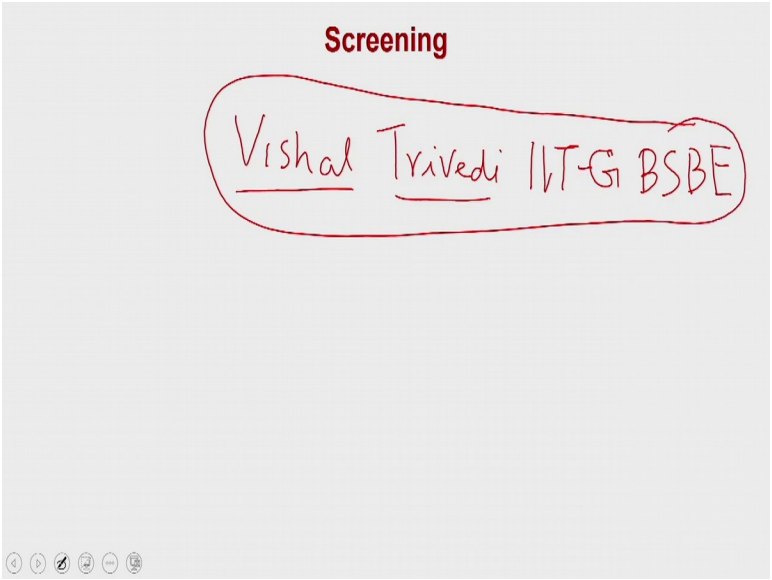

When we say about screening that means that you have the variety of molecules and among those variety of molecules, you have to choose a specific molecule. Which means the tools what you have to use is also, should be specific for that particular molecule and it should not have any cross-reactivity to the other molecules.

For example, if I have to search about myself. So my name is Vishal Trivedi. So suppose I am searching and you are searching my name on Google and if you type the, my name Vishal Trivedi and you will try to search on Google, it will tell you the different types of the names or the persons with the same name in different places, which you are going to, which are working in different, different institutions and which may be working in different countries.

So this name is not specific. This name is specific only if you put another affiliations or additional information. For example if I write Vishal Trivedi, IIT Guwahati then it becomes slightly more specific compared to the only name as Vishal Trivedi. But still it could be possibility that within the IIT Guwahati also you may find several persons with the same name.

So in that case you also have to add the department's name, for example in our case the department name is Biosciences and Bioengineering. So now if you put this as a query in the Google there is a more probability that you may find myself and you will find my profile on to the IIT Guwahati website.

Similarly when we would like to screen the clones for a desired property or for a desired clone of containing a desired gene you have to also use a specific analytical tool or analytical techniques to screen the clones. In the biological systems you have the only two molecules which are having the some kind of ordered arrangement.

For example if you take the DNA, the DNA is, can be used to screen the clones because the DNA is having a systematic ordering of nucleotides and that the arrangement of the nucleotide within the DNA molecule is very, very specific for a particular set of gene, a particular set of genes.

Similarly if you go with the proteins, the proteins also could let you to have specific searches for a particular clone. So in that regard, for screening of different clones of the genomic or the cDNA library we have the three different analytical tools which can be used.

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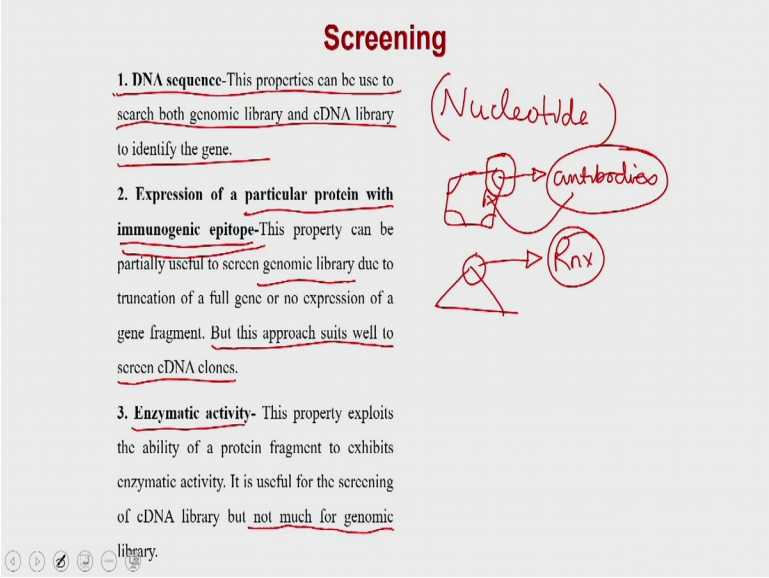

So what are the different tools? We have the DNA sequences. So DNA is a sequence which is made up of the nucleotides. So nucleotides are, and these, the arrangement of nucleotides in a particular stretch of DNA is very, very specific especially if you are talking about the nucleotide sequence or nucleotide sequence of a small stretch of DNA. So that is very, very specific.

Similarly the protein is also having the immunogenic epitope. For example if you have a protein, what is meant by the immunogenic epitope is that if you have a protein which is of three dimensional conformation, what will happen is the some portion of the protein is going

to react for the, with the immune system and these regions are going to give you the antibodies and so, and which is going to be very, very specific for this particular protein.

These antibodies may not react with the other proteins because they are very specific for this particular epitope. So this property can be also used to screen genomic library or the cDNA library. The third is that you have an enzyme and this enzyme has a specific reaction which it is catalyzing. And that reaction can be used also to screen genomic library or cDNA library. So at the end what we have concluded?

We have the three analytical tools, either the DNA sequences or the DNA sequences of the clone or the antibodies which you can use or over-express or which you can develop against these proteins which are present inside these clones and the enzymatic activity which will be coming out from the enzyme which is present inside the clones.

As you can see in these kinds of classifications, the use of antibody approach is more suitable for screening the cDNA clones whereas the approach of enzymatic activity is more appropriate for the genomic library.

So this means that the, while you can use any of the analytical tools, you can, you can still be having a better results if you use the antibodies for screening the cDNA clones, cDNA libraries or the enzymatic activity for the genomic library whereas the DNA sequence is a generic approach. It can be used for screening of both, the genomic library as well as the cDNA library to identify the gene. So let us start with the approach where the DNA sequences could be used as a analytical tool to screen the genomic or the cDNA library.

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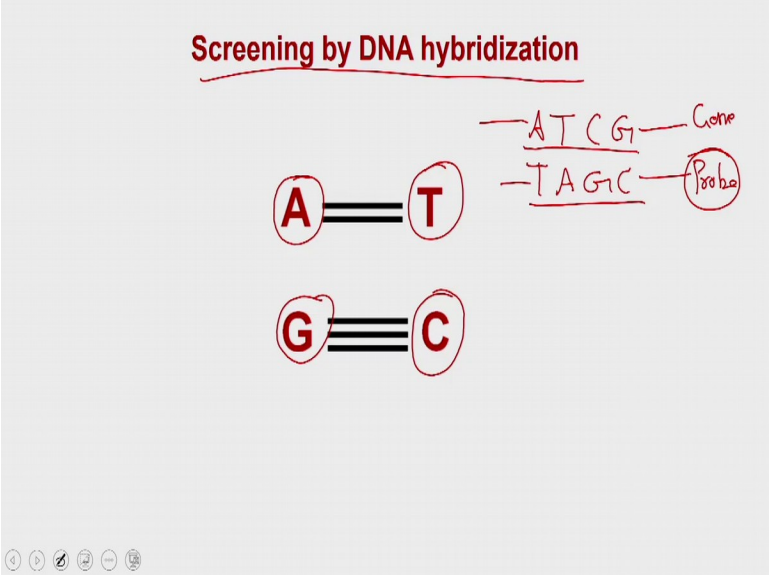

So the basis of screening by the DNA hybridization depends on the property that, within the DNA you have the specific base pairing between the nucleotides. For example adinine is always making a pair with the thymine and guanine is always making a pair with cytosine so adinine is making a pair with thymine with the 2-Hydrogen bonding whereas the guanine is making the hydrogen bonding with the cytosine with the 3-Hydrogen bonding.

And this is very, very specific because of the several reasons which we are not going to discuss here. But as we know that this is a specific base pairing and you can use a particular DNA stretch to isolate a clone which is containing the complimentary DNA into the bacterial cell or the clone.

Which means, for example if you have this as a DNA which you are looking for, then for screening this molecule or for screening this particular gene containing the clone what you have to do is you have to synthesize a small stretch of DNA containing the complimentary sequence of this and that actually will let you to screen. So this is actually, is called as the probe.

The molecule which you are going to use to screen the gene and this is the gene sequence and you can use this particular probe with this particular sequence to screen this gene within the clone. Let us see how you can do this in a more, more elaborated way.

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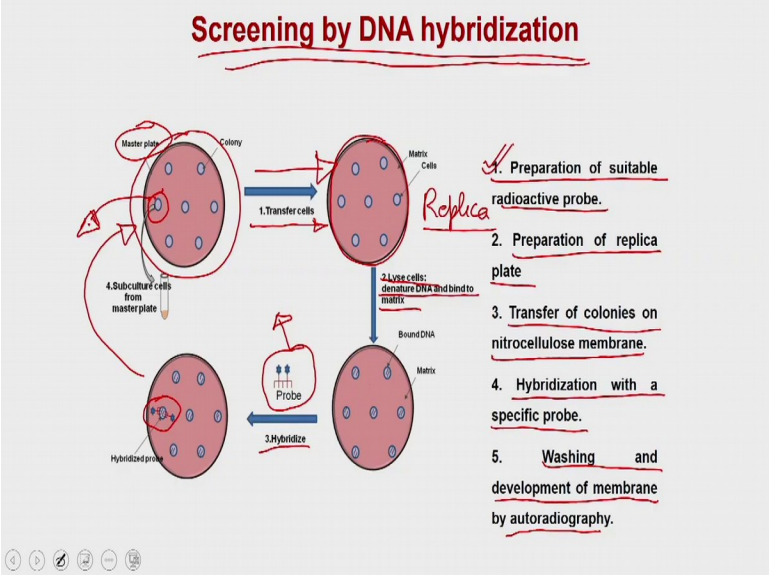

So the screening of DNA hybridization is a multi-step process where, first you have to do is you have to take the genomic library. So genomic library could be, or the cDNA library, so either of, whether you have, you will take genomic library or the cDNA library it is going to have the different colonies on to the plate. And this original plate which contains the genomic or cDNA library is labeled as the master plate or the original plate.

Then what you have to do is take this master plate and prepare a, transfer the cells from, so what you have to do is you have to take the membrane and then transfer the, some of the cells from the master plate to a another plate and this plate is called as the replica plate which means it is actually going to be a replica of the arrangement of colonies on to the membrane.

Then you, once you got the clones on to the membrane then you will lyse the DNA, and you lyse the bacteria. You will denature the DNA and then you will allow them, you will allow this DNA to bind to the membrane and there are many steps which you have to do and then what you do is you will prepare the probe and you will allow them to incubate with the DNA which is present inside the cells. And then you will do the hybridization. After hybridization, the probe will go and bind to a specific clone.

Once you got this specific clone on to the replica plate then what you will do is you will go to the master plate and extract the cells which you are, which are containing this particular clone and then you will take this clone and grow and reconfirm that this contains the gene of your interest simply either by sequencing or you can do further downstream screening as well with the another kind of analytical or analytical tools.

So for this screening of DNA hybridization is a multi-step process. What you have to do is, first you have to prepare a suitable radioactive probe which is actually this one. So you have to first prepare a radioactive prepare. Then you have to prepare the replica plate which is actually, already we have discussed that from the master plate you will prepare a replica plate. Then you will transfer the colonies on to the nitrocellulose membrane.

Once the colonies are been transferred on to the nitrocellulose membrane then you will do the procedure and let the DNA is coming out from the cell. And then you will put the radioactive probe and do the hybridization and once the hybridization is over then you will do the washing and you will develop the membrane with the autoradiography and with that you will going to know that where your clone of interest is present.

And then you can extract the original clone from the master plate and you can use that for extracting the plasmid and then downstream you can, you can use another type of, or additional analytical tool to know the presence of particular gene inside that particular clone.

So let us see what are the different steps and we will discuss these steps in more detail. So the first thing what we are going to discuss, how to prepare the radioactive probe for screening the clones by the DNA hybridization.

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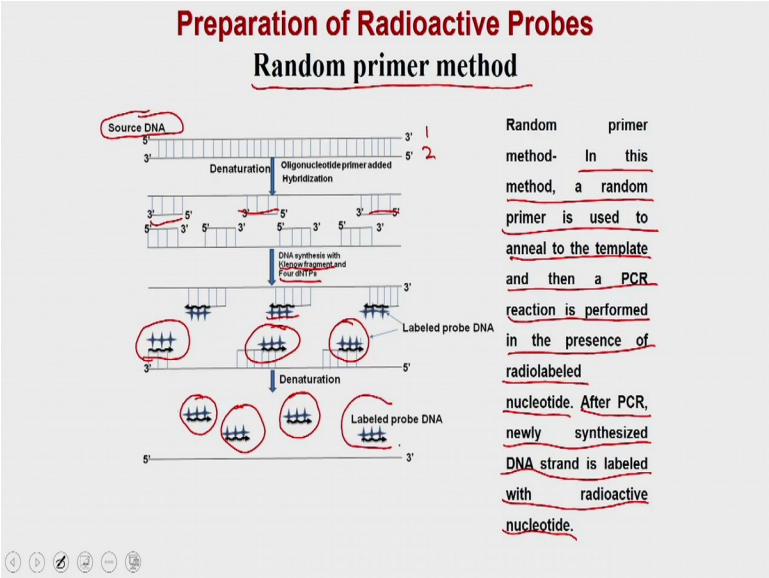

So there are two methods of preparing the radioactive probe. The first method is called as the random primer method. So random primer method is a method, in this a random primer is used to anneal to the template and then a PCR reaction is performed in the presence of radio-labeled nucleotide.

So normally what happen is when you do the PCR you are normally using the non-radioactive nucleotides, whereas in this case what you are going to do is the, the gene which you want to use or the stretch of DNA you want to use as a probe, what you will do is you will put a random primer and you will do a PCR in the presence of radio-labeled nucleotide.

After the PCR is over you are going to have the newly synthesized DNA strand but in this strand the nucleotide which are going to be incorporated are not going to be normal nucleotides, they are going to be the radioactive nucleotides and that is how the probe that you are going to develop is going to have the radioactive, radioactivity into that.

So what you have to do is, first you will use, first you will take the source DNA from which you would like to make the probe, then what you do is you do the denaturation, which means you are going to separate the strand 1 and strand 2. And then you allow your random primer to be added, the random primer is going to be attached to the multiple places and then you will add the Klenow fragment and you will add the, all the nucleotides and you can add the radioactive nucleotide into the mixture.

What will happen is that Klenow fragment will take this particular primer and it will synthesize the stretch and because of that, it will incorporate the radio-labeled nucleotides instead of normal nucleotide and ultimately you can denature the, denature the synthesized material.

And once you denature, this molecule will come out from the template and this is going to be your radio-labeled probe which you can purify from the reaction mixture and that can be used to screen the genomic or the cDNA library using the DNA hybridization approach.

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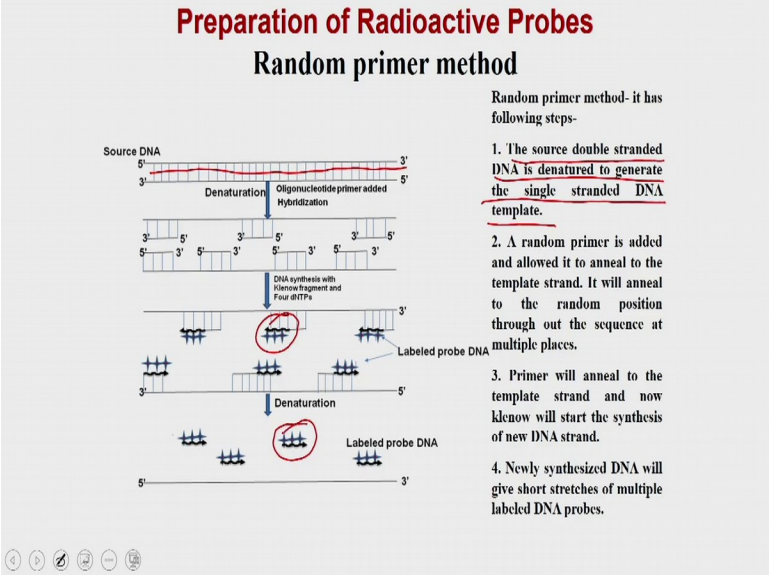

So this is what is given in the templates, the source double stranded DNA is denatured to generate the single stranded DNA as a template. So in the first step you will denature so that all the hydrogen bonding between the two strands are going to be broken down. That is going to give you the templates. And then the random primer is going to be attached and it will anneal to the strands.

And then you will add the Klenow as well as the nucleotide to synthesize the DNA and in this process the Klenow fragment is going to be attached the radioactive nucleotide instead of the normal nucleotide and that is how you are going to produce the radio-labeled nucleotides.

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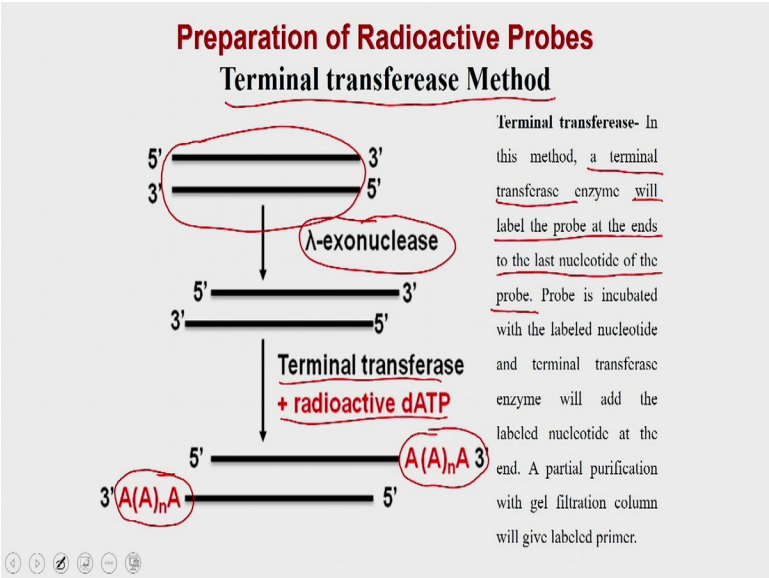

The other method is called as the terminal transferase method. So the terminal transferase method is using a enzyme which is called as the terminal transferase or, and that will label the probe at the end of the last nucleotide of the probe, which means if you have the probe DNA ready, what you can do is you can add the lambda exonuclease and that actually is going to create the nicks into this and then what you do is, you add the terminal transferase along with the radioactive ATP.

Once you do the terminal transferase and add the radioactive ATP, what will happen is it is going to attach the radio-labeled ATP or on the both strands of the probe and that is how the radio-labeled probe is going to be ready by this method.

Irrespective of whether you prepare the probe by the random primer method or the terminal transferase method, this probe can be used for downstream screening purposes. Apart from these two methods which we have discussed there are many other methods which also can be used to prepare or screen, to prepare the radio-labeled probes. Now once the probe is ready you can use the probe to screen.

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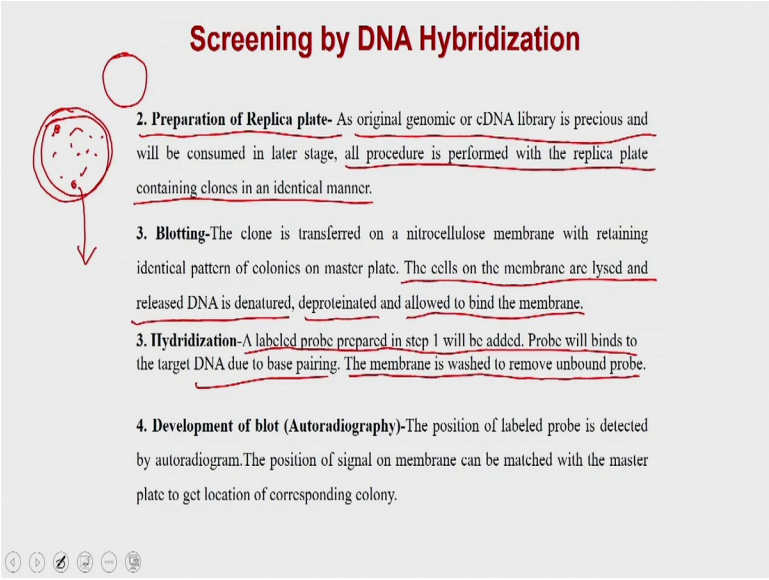

For that what you have to do is you have to prepare the replica plate as the original genomic or cDNA library is very precious because it can be used on multiple occasions. What you have to do is you have to keep your master plate conserved and because you don't want to destroy the genomic or the cDNA library.

If you remember in our previous lecture as well, what we have discussed, we have discussed that the preparation of genomic library or the cDNA library is a very, very tedious and a

lengthy procedure. That is why once you prepare a genomic library of a particular host; you do not want to destroy the genomic library.

Whereas in the screening of genomic library or the cDNA library by DNA hybridization methods is going to destroy the library. So that is why you have to prepare the replica plate so that once you do the procedure on the replica plate, you will destroy whatever is being transferred on to the replica plate. But at least you are going to have your original plate and from that you will not only, only extract the clone what you are looking for but also you can use that library on several occasions.

So that is why it is important that you prepare a replica plate and what you do is, if you want to prepare a replica plate is that you take your master plate and suppose it has the colonies like this, what you have to do is you take a nitrocellulose membrane filter and then what you do is you put the filter on top of this, your library and then you take out this and by doing so, the some of the bacterial colonies are going to stick to your replica plate.

To make the identification easy because, at the end you have to superimpose your replica plate to your master plate to know which clone you are looking for. You are also supposed to put the reference point. For example in this case I can make three reference points and in such a way so that it would not be having any kind of symmetry. And in the absence of those kind of things, what will happen is you can match these spots and that will allow you to identify or superimpose your replica plate to the master plate.

Once your replica plate is ready, then what you have to do is you have to lyse the cells which are been transferred on to the membrane and the, you have to release the denatured DNA. You have to remove the proteins and then the DNA is allowed to bind to the membrane. So the nitrocellulose membrane what you use is having a very high affinity for the DNA molecules.

And because of that once the bacteria releases the DNA from their cell, it actually gets stored there itself or it gets blotted on to the membrane itself. And in this process you denature the protein so that the protein part is going to be get away. Then what you do is you will prepare, you will incubate this probe or this membrane with the radio-labeled probe which we have prepared either by the random primer method or the terminal transferase method.

And then you let it be, it probe to bind the target DNA due to the base pairing and then you will remove the, you will wash the membrane to remove the unbound probe and you will also

wash it with the high stringent buffers so that you will reduce the non-specific interactions and at the end, what you will do is you will develop the probe with the auto radiography.

So auto radiography means you will use the X-ray films and put the X-ray film on top of the, on top of the membrane and what will happen is the membranes are having the radioactivity. So that will actually, they will produce the beta particles and these beta particles are going to interact with the film and once they will interact with the film they are going to give you the signal. And that signal can be used to tell that this is the location of the clone.

And then what you can do is you can take that location and put it into the master plate to extract the clone of your interest which contains the gene. So that is what is written. The position of the signal on the membrane can be matched with the master plate to get the location of the corresponding colony.

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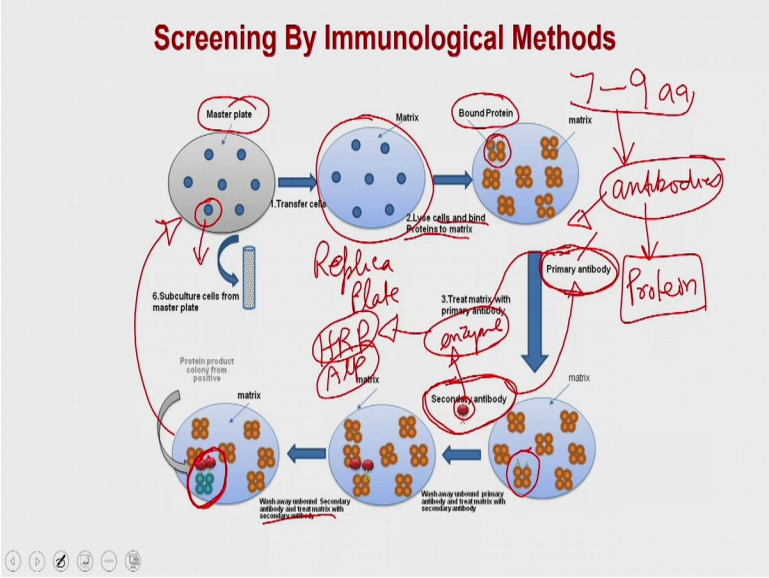

Now let us move on to the next method. And the next method is called as the screening of immunological methods. And this is actually based on the, based on the fact that every protein what we use is having the stretch of the amino acids which are actually immunogenic, which means these stretch of amino acids are going to produce the antibodies and these stretch of amino acid which is present on the protein is called as the epitope.

Normally you have an epitope of 7 to 9 amino acids and that actually is giving you the antibodies and the antibodies which they give is specific for the particular protein. So that antibodies can be used as analytical tool in this case. There are several similarities between

the screening of the genomic library or the cDNA library by the immunological method or to the DNA hybridization method.

So the first step is the same that you are going to start with the master plate. You will prepare your replica plate. So you will prepare the replica plate, so once the replica plate is over then what you will do is you will, you will lyse the cells and allow the protein to bind to the membrane. So the nitrocellulose membrane what you will use is having very high affinity for the protein.

So you can imagine that the cell got lysed and all the protein are now been bind to the protein within that vicinity and now you have the bound protein. Now this bound protein is, you are going to incubate with the primary antibody. So the antibody what you are going to develop against your gene of interest or the protein of interest is called as the primary antibody.

So what will happen is the primary antibody will go and bind to the protein which are being coming out from a particular clone or the set of clones and then what you do is you will add the secondary antibodies.

So the secondary antibodies does not have any affinity for your protein. Instead the secondary antibodies are having the affinity for the primary antibodies, which means the secondary antibodies are going to bind your primary antibodies. And why it is called as secondary antibodies? Because you are adding it to the second time whereas the secondary antibodies are also been coupled to a particular type of enzyme and this enzyme can be used to catalyze the reaction, because at the end you have to develop.

So once you add the secondary antibodies then you will wash the membrane to remove the secondary as well as the primary. This washing is going to be very harsh, so that you will remove the non-specific binding. And then what will happen is depending on this enzyme you can use specific substrate to develop, develop the membrane.

And the enzyme which are very, very popular are the HRP, horseradish peroxidase or the alkaline phosphatase; so based on whether you are using the horseradish peroxidase or the alkaline phosphatase you can use their corresponding substrates and then these substrates are actually been processed by the enzyme which is been present on to the secondary antibodies and then they will be precipitate these substrates on the site of these clones.

Now once you know that Ok, this is my site of, this is my specific clones you can actually go back to the master plate and extract this particular clone and use it for downstream applications. You can take out this clone, you can sequence the clone to know that whether it contains that particular enzyme, particular gene or not. And you can do protein production also and to know whether this is producing that particular protein or not.

Now the question comes, why we are using the primary antibodies and why we, why it is not, just like that we use the primary antibodies labeled with the enzymes. So the reason why we use the two antibodies instead of one antibody is that once you use the primary and secondary antibodies you actually amplify the initial sequence.

So the amount of protein what is present inside this clone is very, very low. They may be in the nano gram range and that is why you are actually developing a primary antibody which is very, very specific. So with that actually, it goes and binds.

But with that small amount of antibody which are actually binding to your antigen inside this colony, it is not going to give you any signal if you develop that with the enzyme which is bound to the primary antibody. Because of that what you do is you go and use the secondary antibody. So the secondary antibody is specific only for the antibodies which you have used.

So that is why the secondary antibody will go and bind to the primary antibody and as per our rough estimate it is found that every primary antibody is being bound by three secondary antibodies. So, because of that, if you, if you calculate the signal get amplified several folds because the enzyme is attached to all those three secondary antibodies and it will give you a very, very large signal compared to, only by using the primary antibodies.

Now once you got these clones what you have to do is, you got the clone either by the immunological method or the DNA hybridization method you have to use these clones and you have to do a downstream applications, you can do further analytical tools to know whether the gene what we are looking for is right or not.

So that is why whether you use the DNA hybridization method or the immunological method you have to repeat this procedure on multiple occasion so that you will be sure and confident that the clone what you are purifying is actually the specific clone and the reliable clone. Now let us move on to the third method.

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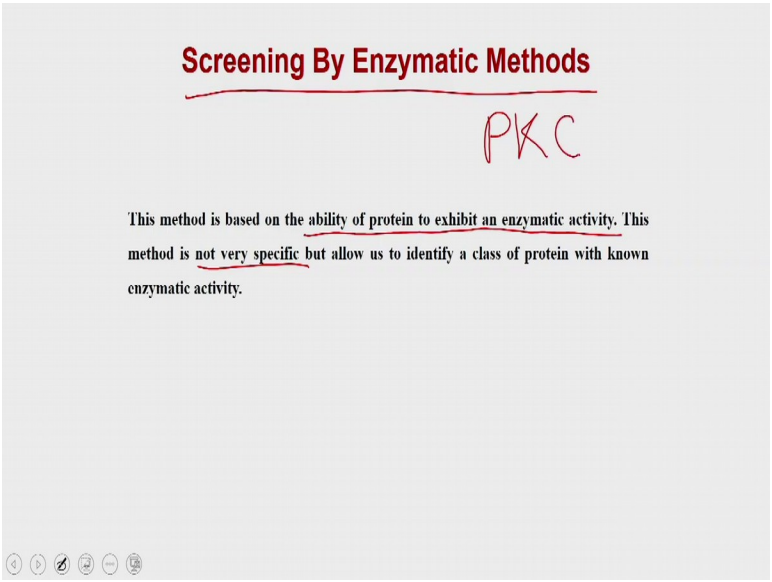

And the third method is called the enzymatic method. And this method is simply based on the ability of the protein to exhibit enzymatic activity. One of the major drawback of this approach is that it is not very, very specific.

For example if you are looking for kinase and suppose you are looking for kinase called PKC which is called Protein Kinase C but if you do an activity for kinase you will also going to, the other enzymes also going to catalyze the reactions which are falling in the kinase category.

For example if you do a reaction for PKC, the P 30 Map K and all other kind of classes of enzymes or kinases are going to also catalyze the reaction. That is why with this approach the background is going to be very, very high. And you may have the large number of false positives.

We have so far, what we have discussed; we have discussed about the hybridization techniques. We have discussed about the immunological techniques. And we have also discussed about the enzymatic method to screen the genomic or cDNA library so that you can be able to get the clones which contains your specific gene.

But once you got these clones, you have to get your gene and then you also have to do the some more confirmatory test before you can use them for downstream applications. So at the end of these screening of these, screening of these genomic library or the cDNA library, what you are going to get?

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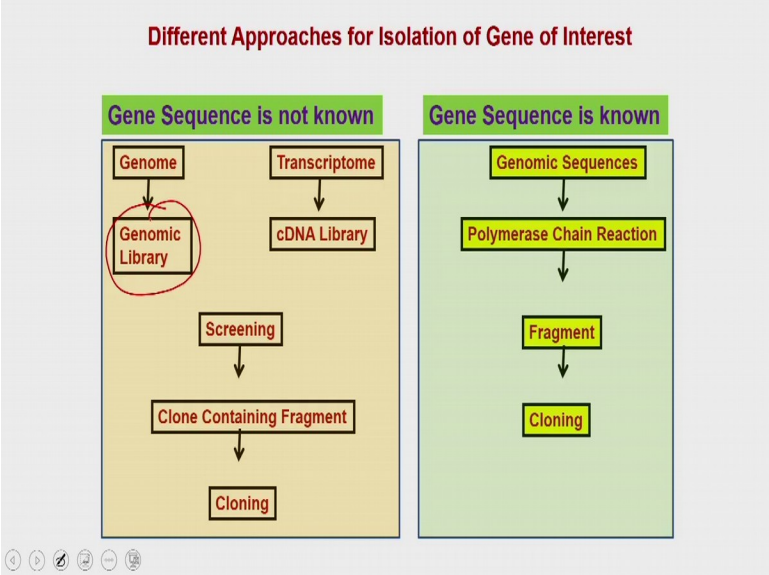

You are going to get a set of clones which are containing your fragments.

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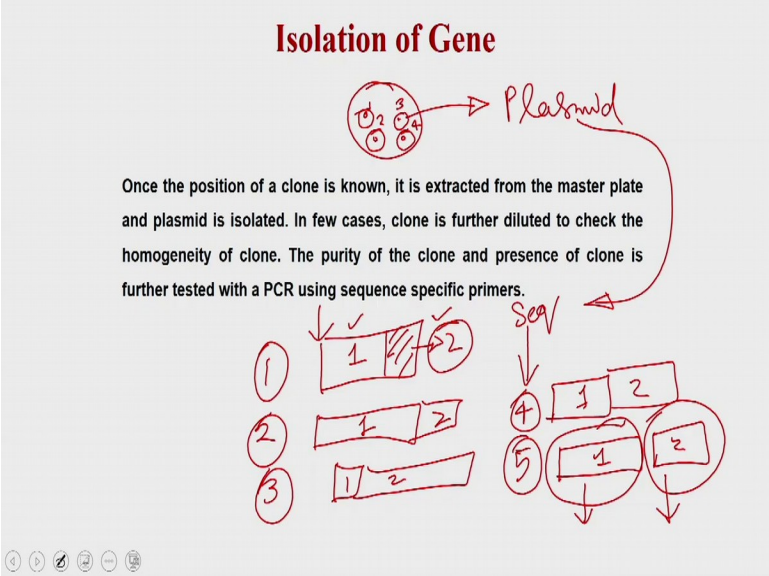

So it is always important to remember that you are not going to get the single clone. You are going to get multiple clones. And as I said in the past also when we were discussing about the genomic library that if you do the screening of genomic library the success of having a preparation, success of getting a right clone depends on the number of clones what you have prepared in the genomic library or the number of clones which are representing your genomic content as well as how that particular content is being distributed.

So that actually will decide whether you will be able to screen a specific gene or not. Let me give you an example. For example, you have done the screening and these are the three or four clones which are got highlighted. And you did it multiple times and every time what you do, when you do, you are getting these four clones are being highlighted and they are giving you the positives. Or they are giving you the positive signals which means all these four are containing your gene.

Now what you are going to do is you are going to go to the master plate and extract these particular clones and extract the plasmids. Now you take out the plasmids and do a sequencing. Now once you do the sequencing you will know whether this plasmid contains, what genomic sequence this plasmid contains. So it could be a possibility that suppose this is the clone 1, this is the clone 2, this is 3 and this is 4. Now for clone number 1, you may have a gene 1 which is half and you may have another gene which is gene 2, Ok.

So you have a small fragment of 1 and a small fragment of gene 2. So which means this gene got broken down from here and then you also have a some more DNA from the gene number 2.

Now for the clone number 2, what will happen is that you have this. You have the full 1 and you have the small portion of 2, Ok. Now for the clone number 3, you may have this small 1, small 2, and for clone number 4, you may have both of these 1 and 2, Ok. So in these cases what will happen is it is very, very difficult to decide whether the gene of your interest is 1 or whether the gene of interest is 2.

And that is why, when I was, when we were discussing about the preparation of genomic library it is very important that you may have a specific clone which will be like this, either 1 or either 2 so that once these clones are going to highlight, you will say this is my gene of interest or this is my gene of interest. So if that is not the case and you have the overlapping gene and you have multiple genes within the same clone, it is difficult to say whether the, your gene of interest is gene number 1 or gene number 2.

But even then, once you do the sequencing of these clones, probably you will be able to distinguish which is the gene which is corresponding to your protein. Because in some of the cases you may have the full gene and the other one is the partial gene and that clone may be giving you higher signal.

And because all these clones may not be giving you the similar kind of signal, some clone may be giving the more signal, some may be giving the less signal, so because of that if you do the sequencing of these clones then you will know that which clone is containing the full gene, full length gene of my gene of interest and that could be your gene fragment which you are looking for. So with this, we would like to conclude our lecture here.

So with this, we would like to conclude our lecture here and in this lecture what we have discussed? We have discussed about how to screen the genomic library or the cDNA library and we have also discussed in detail how to, what are the different approaches can be used to screen the genomic or the cDNA library and we have discussed about the DNA hybridization method, immunological method as well as the enzymatic method.

And in totality in combination of all the three, you will be able to screen the gene of your interest for the downstream applications and in the subsequent lecture we are going to discuss about the alternate approach where the genomic sequences are known, which means we are going to discuss about the polymerase chain reactions and how you can use the polymerase chain reaction to get the gene of your interest or the fragment of your interest and how you can use, how you can clone that into the vector of your interest. So with this I would like to conclude our lecture here, thank you.