Genetic Engineering: Theory & Application
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Module III Basics of Cloning (Part I)
Lecture 7
Isolation of Gene Fragment (Part-I)

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Hello everybody, this is Doctor Vishal Trivedi from Department of bioscience and by engineering, IIT Guwahati and what we were discussing? We were discussing about the different properties of the host strain, which you can use to generate the chimeric organism or which you can use to genetically modified organisms, in this discussion what we have discussed from the last two module, that we have discussed about the prokaryotic as well as eukaryotic cell, we have discussed about their structures and then at the end, we have also discussed about how to separate the organelles and this is the what we have discussed in the model 1.

In the module 2 we have discuss about the different types of media, which is available for growing the prokaryotic as well as eukaryotic cell, we have also seen couple of demos how to prepare the microbiology media as well as the mammalian cell culture media and what are the precaution you should take when you prepare the microbiology media or the mammalian cell culture media and subsequently we have also discussed about the couple of salient or important biochemical reactions, what are happening in the prokaryotic as well as eukaryotic system, in that what we have discussed? We have discussed about the glycolysis, great cycle and then at the end, we have also discussed about the anaerobic oxidation.

In the same discussion we have also discuss about what is the advantage or what is the significance of each of these metabolic pathway for running the metabolism, running the life-cycle of these prokaryotic or the eukaryotic cell and then at the end, once this we have also discussed how to study the growth of these microorganisms, what are the different methods which you can use to study different microorganism or their life-cycle and then we have varied in detail, we have discussed about different stages, what a microorganism will go through to complete its life-cycle and how a particular bacteria grows in a defined culture volume.

And now what we would like to discuss, we also want to discuss that the, (if) so all these we were discussing, in context to prepare are genetically modified organism and for genetically modified organism if you recall in the lecture 1 itself, we have shown a scheme which you need to follow to generate a genetically modified organism or the organism with the improved trades by exogenously expressing the particular gene.

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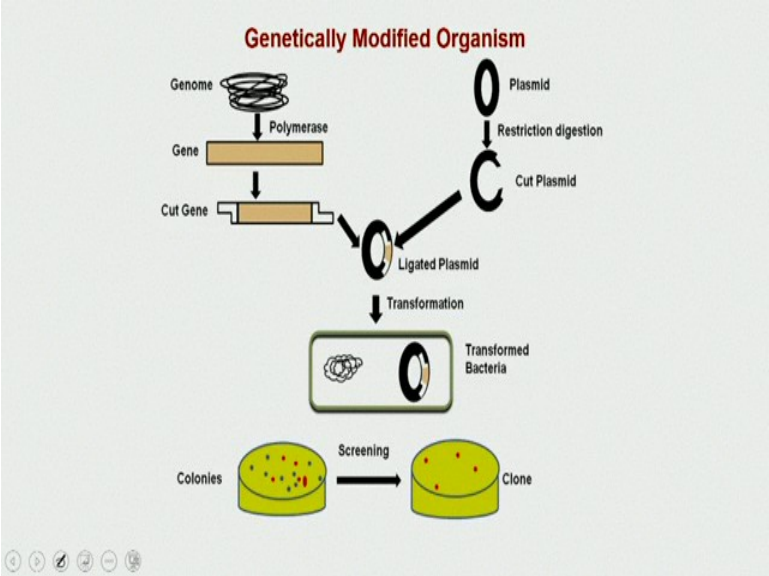

So in the scheme, what have discussed? We have discussed that once you have organism from which you want to isolate the gene, you can actually isolate a gene and then you do a restriction digestion of this gene and the similarly, you can have the transforming agents and you can do the similar kind of combination or restriction enzyme do a digestion and then you can put these two components for a lagation reaction and ultimately you can do a transformation into the host cell, which you would like to use for overexpressing or utilising this particular gene and ultimately you are going to get a genetically modified bacteria, which is going to express this particular gene, now in the todays lecture what we are going to study

is, how to isolate this particular gene and what are the different approaches one can use to isolate these different, this gene under different scenarios.

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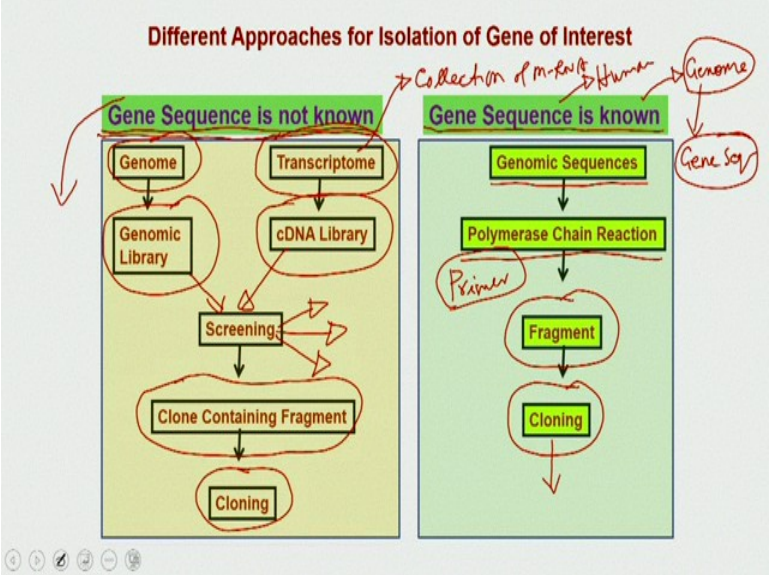

So in the biological science what you have is? You have broadly the two scenarios, so before the genomic sequences were available, the people were using a different approaches to isolate a particular gene and there are different approaches which people use when the genomic sequences are available, so you have two scenarios, in scenario number 1 what you have is, you do not know the gene sequence what you want to clone but what you know is about their character.

Okay and the other scenario what you have is that the gene sequence is known, which means you in this particular scenario, (the genomic) the gene or the genome of that particular organism is being sequenced completely, which means from this genome you know the gene sequence and there are couple of examples in this category, for example, the humans or e coli or many bacteria, mycobacterium tuberculosis and so on, so this number or this pool of information is increasing over the course of time, and that is why the people are using this approach more often nowadays compared to this approach, where the gene sequence is not known.

So this is the approach people were using when the genomic era was not started and people were using this technique to isolate the particular gene based on its particular kind of trade, so in this particular approach you have the two sources to isolate (a genome seq) to gene sequence, one you can isolate from the whole genome or the other pool is that you can use

the transcriptome, transcriptome is nothing but the collection of the messenger RNA, so these are the two sources from which you can actually be able to use, from which you can be able to extract (the genomic sequence) gene sequence of your interest, for both of these approaches, for the case of genome you have to prepare a genomic library or in the case of transcriptome you have to prepare or cDNA library.

Once you prepared genomic library or the cDNA library than you have to screen these libraries for looking for this particular gene sequence, so there are different way in which the gene sequence could be, your particular gene sequence could be screened, that we are going, anyway going to discuss in a subsequent lecture, once you got the, after the screening, you are going to get the particular clone which will contain your fragment and then this fragment you can clone into a cloning vector or into a expression vector and check whether that particular gene is expressing your desired that product or not.

And in the other scenario, when the gene sequence is available, which means the genomic sequences are available, what you can do is you can take those genomic sequence information and then you perform a polymerase chain reaction with the help of the site specific primers, which means you can use the forward primer as well as the reverse primer and that you can use along with the reaction mixture and along with the different types of enzyme what we using the polymerase chain reactions and by these you are going to get the PCR product and once you get the PCR product, then you can use this PCR product and put it into the cloning vector or the over expression vector and then you can use subsequently these clones for downstream applications.

So let us see under what scenario, you can use the genomic library and under what scenario, you can use that cDNA libraries, so that you could be able to choose the particular subset, so that your screen as well as your protocol should be very, very streamlined.

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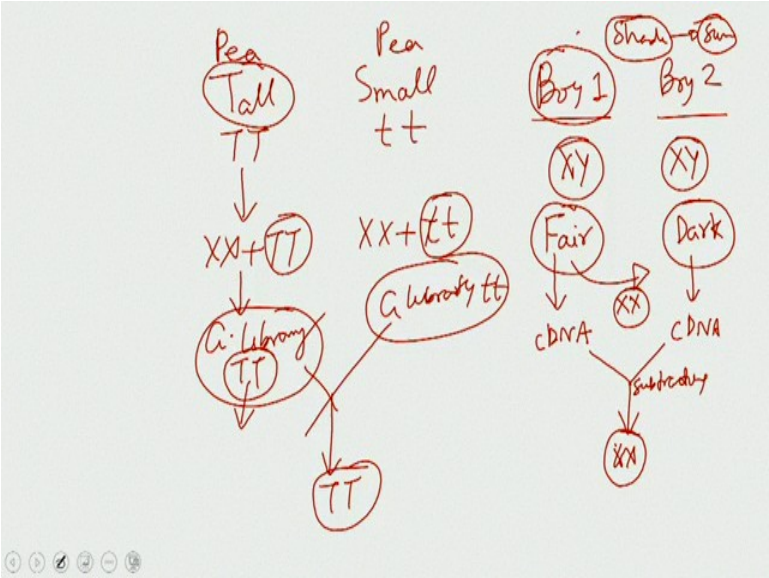

Now let us imagine that you have the two plant, one is tall and one is small, so you have the two varieties of pea plant, one is tall which is consider to be TT and you have two small and these are called small t small t, now if these are pea plants in both the cases what you can do actually and you, suppose you want to know what are the genes are responsible for making these pea plant as tall compared to these pea plant.

So what you can do, and what is difference in this is that, it contains the genome, which is actually the XX chromosome plus this particular tall is actually containing a additional gene, which is the TT, whereas in this case, it has everything identical as it was there in the tall plant, but what it has additionally is the small T and small T these two genes, so the only difference between the small as well as that tall (gene) plant is that the difference of these genes, so what you can do is you can make under, so if you have these kind of questions where you can actually looking for the phenotypic features in a particular plant and those are phenotypic features which are genetically induced.

For example the tall versus small, then you can use the genomic library, so what you can do is, you can prepare a genomic library in this case and support this is a genomic library for TT and this is a genomic library for small t small t, now what you have to do is, you have to look for this particular gene into this library and that actually will be absent from this library, so what you have to do is, you have to do a subtractive analysis and that actually will give you a gene which is TT and that is a gene which is responsible for tallness in the pea plant.

On the other hand, under what scenario, you will use the cDNA library or the cDNA approach, suppose you are actually having the two boys, the boy 1 and the boy 2, so these two are human beings, so their genomic is identical, whereas you can see in this case there genomic are different, one is having the TT as a gene, whereas all other genes are identical, but the tall one is having the TT gene, whereas the smaller one is having small t small t gene.

Whereas in this case, you are actually going to have the identical genome XY, because both are boy, but this boy is fair in color and this boy is dark in color or you can imagine that you are actually studying how the color of skin is being changed if a boy is going from a shade to Sun, so in those cases what will happen is the, and you might have noticed that if a person is goes from shade to Sun it color get change from fair to slightly dark and how that happen is because that this guy is expressing some of the additional genes, like for example XX which are responsible for giving a dark color to the skin.

So in this case is what you are going to do is, you are going to generate the cDNA library from these two individuals and then you will do exactly the same, you are going to do a subtractive analysis and that will give you the only one gene, which is being modified or the couple of genes which are being modified, when this boy is moving from the shade to the Sun.

So in totality what we are trying to explain is that if you have the questions where the genomes is getting edited or where the genome is getting modified you can use the genomic library approach to identify the gene, whereas if you have the conditions where the expression of proteins are changing, which means the expression of the transcriptome is changing or the pool of transcriptome is changing, then you can use the cDNA library, because the cDNA library approach actually corresponds to the expressions status of itself.

Whereas the genomic library corresponds to the genomic content of a particular cell, so you know that a genomic contain does not very, if you change the environmental condition or if you change the many of the parameters, whereas all these phenotypic changes what you see under these conditions is mostly been done simply by an expression of the additional genes.

so if your question is about looking for a gene which is getting activated when you are moving from the shade to Sun, then you cannot get that question or you cannot get that the answer to that question simply by looking at the genome, because the genome of a particular individual will remain constant, whether it go from the Sun to shade or shade to Sun, but

under these conditions, the only thing will change is the expression of additional proteins or the suppression of the, some of the proteins.

So, when if you do the subtractive analyses, which means if you do a cDNA library preparation under the two conditions, when a person is in the shade or when the person is in the Sun, these are the conditions will tell you the corresponding genes responsible for a phenotypic change, so let us discuss these things in detail, how to prepare the genomic library? How to prepare the cDNA library? How to screen them to get your gene of your choice and then at the end we are also going to discuss about the polymerase chain reactions and how you can use that also in the modern world to extract the gene of your interest from the genome and you can use that for subsequent cloning applications.

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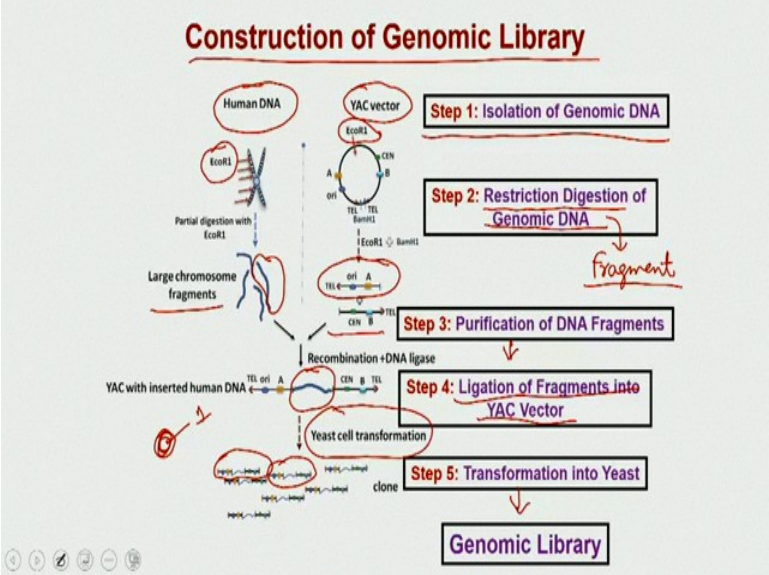

So let us start with the genomic library, so the protocol of the construction of genomic library is where I have taken an example of human genome, so what we have to do is, you have to first isolate, so in the step one, you have to first isolate the genomic DNA from the particular human being for which you are interested to prepare the genomic library, (once you gen) once you have prepared the genomic DNA, then you know that the human, the genomic DNA or the genome is arranged in the form of chromosome.

Then the next step is that you are going to digests the genome with a suitable restriction enzyme which in this case we are going to, we have given that example with the help of ecoR1 which is an restriction enzyme and we are going to discuss about the restriction enzyme in a subsequent lecture, so in the step two you are going to create the fragments of

the genomic DNA, which can be done by simply restriction digesting the DNA with a suitable restriction enzyme.

Once you got (the) these fragments, so after this, you are going to get the fragments of genome and once you got these fragments you have to purify these fragments, using the suitable techniques, so that you have the fragment of a suitable size, so which means you cannot take a fragment which is very large or you cannot take a fragment which is very small, because the reason is that a large fragment is going to correspond for multiple genes, whereas a small fragment may not correspond to even for a single gene and you are looking for a suitable size fragment, so that it should correspond to at least one gene.

Once you got the purification fragment, then you are going to put these fragment into a yeast artificial vector or yeast artificial chromosome vector, so for this what you are going to do is, you are going to take a YAC vector, then you also going to digest that with the ecoR1 and now your YAC vector is ready for the ligation reactions, then what you do is take these fragments from the genomic DNA and put it along with the YAC vector and put it for the ligation reaction, this particular fragments, all these fragments are going to get inserted into the vector and then you will do the transformation.

So in the last step you are going to do the transformation into the yeast cell and then what will happen is that different vector are going to carry the different gene and ultimately what you are going to get? You are going to get the clones which will contain the single gene or the multiple genes, which means it depends on the size of the fragment and these colonies or these clones can be considered or it is going to represent the whole genome and that is why this technique is called as the genomic library, which means it is exactly the same as the library which is present in our Institute.

So our Institute has a library, which represent the different types of books which are present in the collections, similarly in genomic library is going to have the collection of the genes which are constituting the genome and all these genes are going to be present in different clones which you will get by doing these reactions at the end, so after the step five you are going to get different clones, which are constituting the genomic library, so let us discuss each and every step in details so that you will be able to understand the process as well as you will be able to perform, if you can get the necessary infrastructures.

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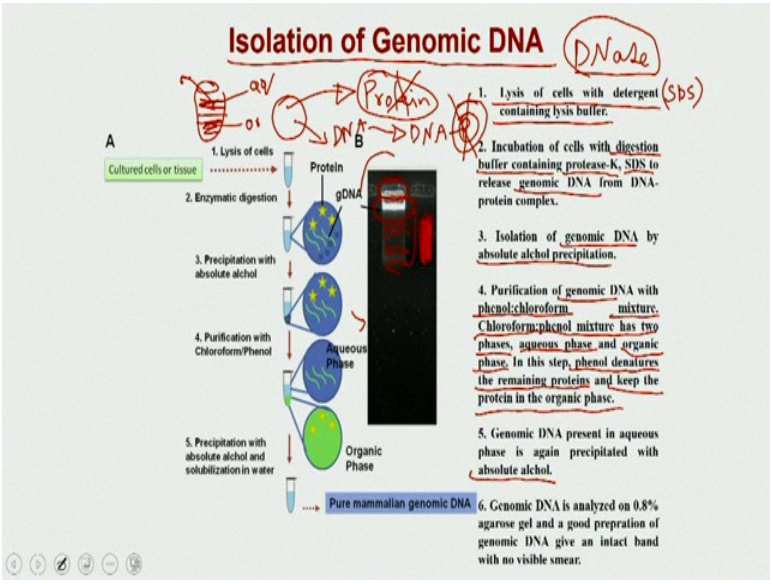

So in the step one, what you are supposed to do is? You have to do a isolation of genomic DNA, if you remember in our previous lectures we have also discussed about the isolation of different organic, so if this is what we are doing for from a mammalian cell, for example if you are trying to generate a genomic library for humans, it is a good idea first to isolate the nucleus instead of lysing the cells, so what you can do is you lyse the cells and then you isolate the nucleus because that actually will make the genomic preps more purified and more the quality of DNA what you are going to get is going to be better.

So once you isolate the nucleus, then you break open the nucleus and get the genomic DNA, so under this protocol what you are going to do is, in the step one you will lyse the cells with the detergent, for example, you can use the SDS and you can use a lysis buffer, the lysis buffer is actually the buffer, which contains the trice EDTA and the detergent which is called as, which is in this case, for example, you can use the SDS.

So that actually is going to lyse the cells and will release the DNA okay, now apart from DNA it also going to release many things, for example, it is going to release the other biomolecules such as protein and you know that DNA may not be present alone, DNA may be present in the form of the DNA protein complex, so what you are going to get (a) by lysing the cell is that, you are going to get the DNA which you need actually for making a genomic library, you are also going to get the protein, then you are going to get the DNA which is in complex with the protein, apart from that you are also going to get the lipids and carbohydrates and other biomolecules.

So the first tasks would be to remove the protein which is actually either present alone or which is also present along with the DNA, so that what you will do is, you will do that by putting these into a digestion buffer and this digestion buffer contains the proteinase-K which is actually a protease, the SDS and, so job of the SDS is to release the genomic DNA and the proteinase-K is going to degrade the protein whether it is the isolated protein means the individual protein or protein which is bound to the DNA and that actually will release the genomic DNA or the, it will free the genomic DNA from all types of the complexes.

Subsequently what you will do is once you got the genomic DNA, then you will do the alcohol precipitation and then if you add the alcohol to this prep, you are going to get the genomic preps, but this is genomic what you are going to get is not purified, you have to again do a several rounds of purification, so that you are going to get the high-quality genomic DNA and that you will do by mixing the genomic DNA with the phenol chloroform mixture, once you add phenol chloroform and mixture, what will happen is that the chloroform phenol mixture has two phases, you are going to have an aqueous phase and a organic phase.

So in, what will happen is the phenol which is present in the phenol chloroform mixture is going to denatured whatever the remaining proteins present and that is how and it will keep the protein in the organic phase, whereas the aqueous phase is going to have the DNA part, so once you do that, what you are going to get in your append off is two layers, one which is that aqueous layer and the top you are going to get the, on the top you are going to get the aqueous layer and at the bottom you are going to get the organic phase.

What you can do is you can just remove this aqueous phase from the top and store it in a different append off and then you can again precipitate this DNA using the absolute alcohol and now you got the genomic DNA, isolated from the cell, but you have to analyse these on a agarose gel or a agarose electrophoresis, so that you can check whether the quality of a genomic DNA what you purified is good enough to prepare a genomic library or not because the genomic library preparation is a long process and (it) you have to ensure that the genomic library, genomic DNA what you purified is of very, very high-quality.

How to do that? When you run these genomic DNA on a particular agarose gel, what you will get is? You will see a intact band of genomic DNA and the other characteristic feature of the genomic DNA is that it runs very close to the well, so if you load is into the well, normally the genomic DNA are very, very heavy, if it genomic DNA and it is not fragmented, it is not

that than it will grow, it will run very close to your well, the other thing is, you also have look for, if it has any kind of trailing signal.

For example, in this case you will see a very faint band, a faint signal what you see at the bottom, if it is very, very large or if it is very intense that actually signifies that the genomic DNA what you have purified is got fragmented into different fragments and all these fragments, which means your genomic DNA may show a fragment like this and if that happens going to show you a smear like this from the well, which means you are going to, you are not actually purifying the intact genome, you are actually missing some fragment of some part of the genome in this process.

So if that happens, you have to purify the genomic DNA, you have to increase the amount of the stringency in your process because the every cell has the DNase and these DNase are actually going to degrade the DNA, if you do not do the purification with a lot of precautions and if you do not do the isolation on by following the subsequent steps meticulously, sometime it also happens that when you purify the genomic DNA, you may be not doing the pipetting properly and because of that, also sometimes the genomic DNA get broken down into multiple pieces and that also appears like a smear on the gel, so now we have purified the genomic DNA from the cell.

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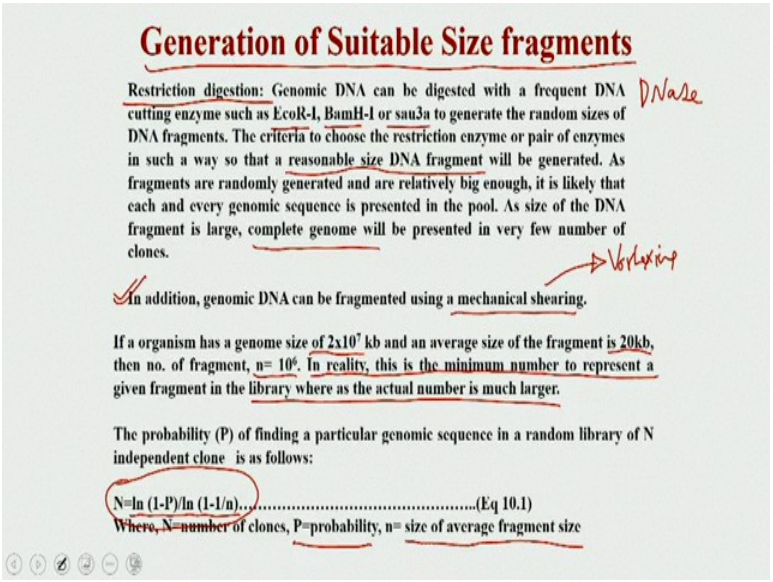

And now we will go, move onto the next step, so in the next step, we have to do a generation of suitable size fragments, which you are going to do with the restriction digestions, so restriction enzymes are the enzymes which actually degrades the DNA in a very, very precise

manner, so restriction enzymes cuts at their specific size, so they are not non-specific as like DNase, because DNase degrades the DNA on random places.

So for this purpose what you do is, you take an enzyme which actually cuts the genomic DNA very frequently. Although we do not know the genomics sequence of that particular organism, but as an experience it is found that if you use the ecoR1, BamH-1 or sau3a, most of these are actually the cutting side of these enzymes is very, very frequent and they are very, very dominant or abundant into the any genome of any organisms.

The idea of using of any of these enzymes either individually or in combination is that you want to have the fragment of suitable size, why it is important to have a suitable size? Because as I said, if it is a fragment of very, very big size you are going to have the multiple genes into that, if you have a fragment of very, very small size it is going to be a fragment which will not going to represent even a single gene, it may have a fragment of a particular gene.

So you need a suitable size fragments, so that it will at least express, at least one gene or maximum one gene or the flanking sequence of the other things, this, so if you digest the genomic DNA with restriction enzymes either alone or in combinations, most likely you will generate a suitable size DNA fragment and as the (so) genomic is very big. The number of fragments what you are going to get and subsequently the number of clones what you are going to get is going to be very, very large, if the fragments size is very big, so there is a formula for which you have to use to ascertain that the genomic library is representing your fragment optimally.

The other way of generating the suitable size fragment is that you can do a mechanical sharing, for example, you can use the vertex, so you can do a vertexing, so if you isolate the genomic DNA and if you do the vertexing it will actually going to also share the DNA and it will going to generate the fragments.

Now as I said, you have to generate a minimum number of fragments so that it will represent a particular genome, now, for example if you are working with an organism which has a genome size of 2 to power, 2 into 10 to power 7 KB and the average size of the fragment is what you are putting into this is 20 KB, which means the number of fragment what you are going to generate is 10 to power 6, so in reality this is a minimum number of, minimum

number to represent in the given fragment in the library, whereas the actual number is going to be much larger.

Now if you see, if you want to calculate what is the probability that you will found your gene or your fragment into a individual independent clone, then you can use these particular formula which is that the number of clones what you need to represent your gene fragment into individual clone is lean 1 minus P divided by lean 1 minus divided by N, where the P is actually the probability factor, which means the probability through by which you are going to have a independent clone containing the your gene fragment and N is the size of the average fragment which you are going to use to generated the genomic library.

So you can use this particular formula to are certain yourself that you are going to have the 100 percent probability that my particular gene fragment is going to be represent in that particular genomic library, now we have generated this suitable size fragment, now this fragment in the third step, this fragment has to be inserted into a particular vector, but before getting into that I would like to show you the genome sizes of different organisms.

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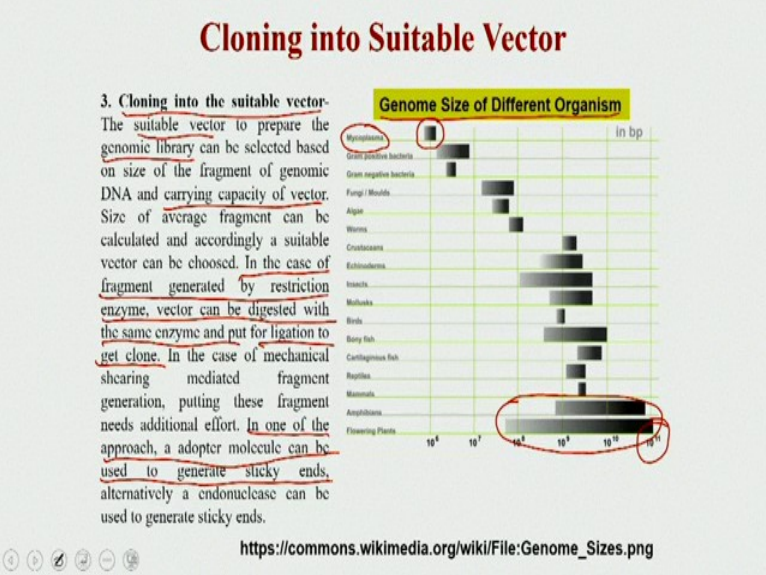

Now, once you want to clone these into a suitable vector, what you have to remember is that the genome sizes of different organisms vary from very, very small to a very, very large, for example in the case of flowering plants it can go up to 10 to power 11 base pair, whereas in the case of mycoplasma which is actually the smallest organism, it could be even 10 to power 6 base pair, so and all other organisms are in between these two extremes, so what you have to remember or what you have to consider is that the number of fragments what you are

going to generate from these genome are going to generate a suitable fragment size and that fragment size will be very, very large if the genome is very, very big, it is very, very small, if the genome is also small.

So depending on the fragment size which you are going to generate from these genome, you have to choose a suitable vector for generating a genomic library, which means there are vectors which can take up, so there are vectors which can take up the DNA of different lengths and every vector has a carrying capacity of the particular size of fragment, and accordingly, you have to use the vector which can be used to generate or prepare the genomic library.

In the case of the fragment generated by restriction enzymes, vector can be digested with the same enzyme and put it for ligation to get the clone, whereas in the case of mechanical sharing, for example if you do a mechanical sharing it is not going to generate the fragment which are going to have the course events, so in those cases what you have to do is, the subsequent cloning is going to be very, very tedious and difficult, so in those cases you have to be little more, you might have to put more effort is in cloning those fragments and one of the approach in that case is that you can put an adapter molecule and to generate the sticky ends and then you can use those sticky ends to insert that particular fragment into the particular vector which also will going to be digested with enzyme for which the adapter molecule you have used.

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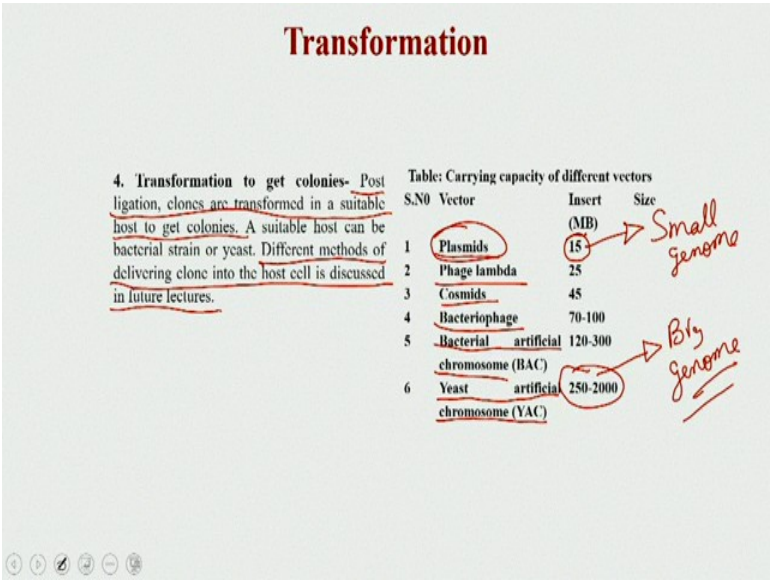

Now let see, so these are the different vector which you can use, so plasmids is the bacterial plasmids, then you have the phage lambda, you have cosmids, you have the bacteriophage, you have the bacterial artificial chromosome or BAC and then you have the yeast artificial chromosome or YAC, every of these or all these carrying vectors have the different carrying capacity, for example in the case of plasmids you have the caring capacity of 15 MB.

Whereas in the case of yeast artificial chromosome you have the carrying capacity of 250 to 2000 MB, which means (the) this is good for the organism of small genome, such as the bacterial genome or the some or the other infectious organism, whereas the yeast artificial chromosome is for the organism which contains the big genome or the genome size is very big, for example, the human, so you have to keep these under consideration, when you will generate the particular size fragment and then use that for subsequent ligation reactions.

Now, once you have done the ligation reactions you are going to get the clone and then what you will do is, you will take those close and transform into a suitable host, for example if you have used the plasmid, then you will transform that into the bacteria and once the bacteria will take up that particular plasmid it is going to give you the colonies and each colonies are going to represent the single clone.

So once you do the transformation, the clones are going to be transformed into the host to get the bacterial colonies, a suitable host can be either bacterial strain or the yeast, we are anyway going to discuss the different methods of delivering clones into the host cell in our subsequent lecture and so by following these 4 or 5 steps, you could be able to generate the genomic library subsequent to these you have to screen the genomic library to get your gene of your interest, that we are going to discuss in the subsequent lecture.

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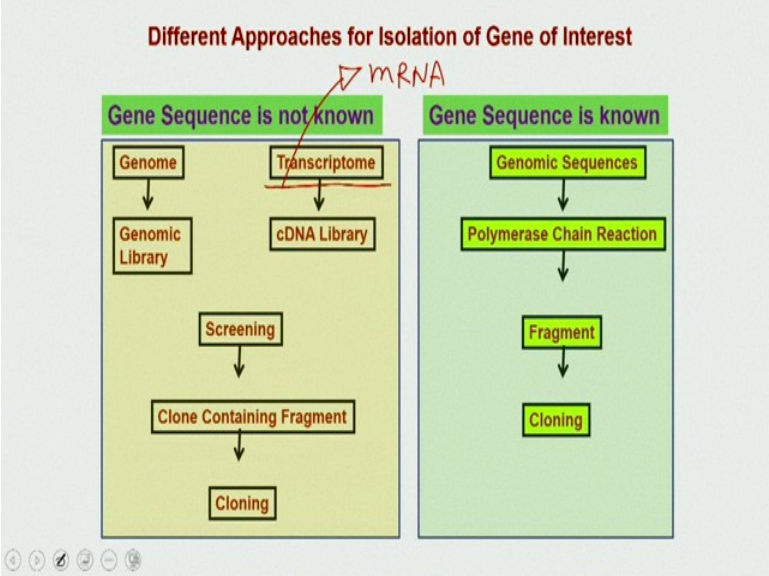

Now let us move onto the next approach and the next approach is called as the cDNA library preparation, now, so as I said cDNA library, you can use only if you are working with the transcriptome of a particular cell, which means you are actually going to or you want to see the change in the messenger RNA pool of that particular organism or that particular cell type, we have already discussed couple of examples, under what conditions you can be able to use the cDNA library to identify the gene of your interest.

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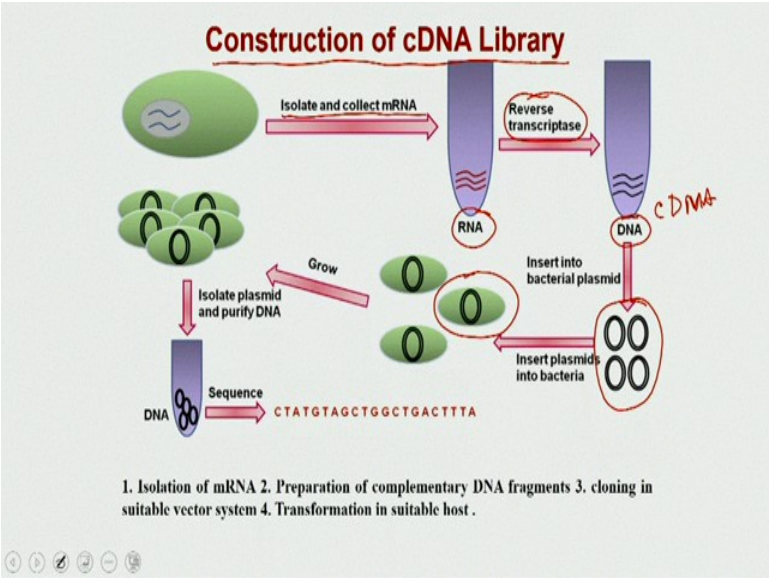

In a particular cDNA, so what are the different steps you have for generating the cDNA library? As I said in a particular given conditions what you are going to do, your first step is that you isolate and collect the messenger RNA, so messenger RNA are the RNA which

actually represent the transcriptome of a particular cell or particular organism, in the subsequent step from the RNA you are going to generate the DNA, the RNA and the DNA what you generated is called as the complimentary DNA or the cDNA and that is why this library is called as the cDNA library.

So from the RNA you will generate the DNA with the help of the enzyme called reverse transcriptase, then once you got the DNA, double standard DNA, then you will insert these DNA into a plasmid or a suitable carrying capacity that and once you got this then you are going to do the transformation, that transformation will give you the clones and this is actually your genomic library, from her you can actually do the screening and then you can be able to get the gene of your interest.

So let us discuss how to isolate and collect the messenger RNA, but before we will discuss about the approaches what you use to collect the messenger RNA from the cell, let us discuss what is the general structure of a messenger RNA?

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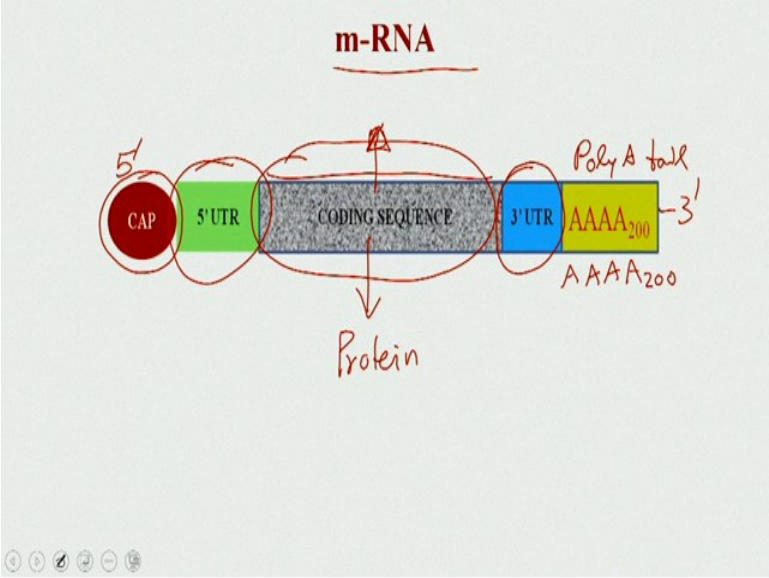

So our general structure of messenger RNA that it has the 4 or 5 definite regions which are important for the functioning of a messenger RNA, so what you have? You have the 5 prime cap, this cap is important for protecting the messenger RNA from any kind of degradation and this cap is also providing the binding side for the initiator messenger RNA or initiator tRNA, then you have the 5 prime UTR region, which is actually the region for responsible for guiding the or ribosomal assembly and then this is the coding sequence, which means this is

the sequence which is going to be responsible for generating the protein and this is the only sequence which is going to give you the, so this will be getting generated from the DNA.

,Subsequent of that you have the 3 prime UTR that also has a specific functions in the messenger RNA or in the translation process and about to this, you have a poly A tail, this poly A tail is actually a series of A which are present on the 3 prime end and these numbers could be of 200, it could be 400, it could be any number and this number of poly A tail actually decides the age of this particular messenger RNA in the cell.

So what we can do is because we do not have to purify a single messenger RNA because otherwise you can use a coding sequence or a sequence which is complementary to the coding sequence and that will give you the particular type of messenger RNA, but that is not what we want? What we want to prepare is, we want to prepare, we want to isolate the messenger RNA pool of that particular cell under a specific conditions, so under those conditions you want to use a generic sequence or generic sequence, so that you can use that as a probe to isolate all the messenger RNA, which is present in that particular cell.

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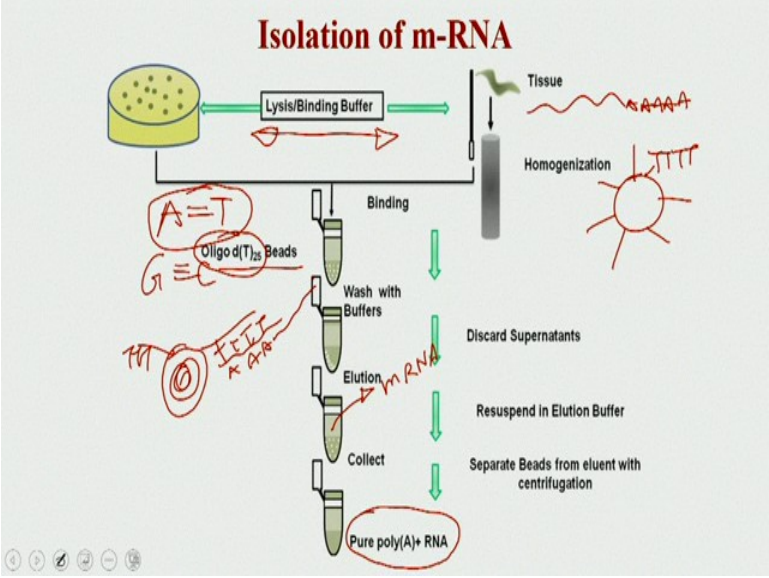

For that what you are supposed to do is, first you isolate the cells. Okay, then you put under the lysis buffer and that lysis buffer is going to lyes the cells and relief your messenger RNA, so imagine that you have isolated the messenger RNA, which actually contains a poly A tail on the other hand, then what you do is you take the agarose beads or the sugar beads and all these sugar beads are going to have poly T sequence and you know that as per the

complimentary theory, the A is always making base pair with the T, whereas the G is making base pair with the C.

Which means A is making double bond with the T and G is making triple bond with C, so exploiting this complimentary or this affinity of this A for the T, what you can do is you can take agarose beads which are tagged or which are having the poly T on the surface, which means they are going to have the poly T on their surface, so what will happen is, these beads are going to bind your RNA, which is present in the supernatants, so you can imagine that it is going to be like this, like this.

So one messenger RNA will buy into 1 tail, then the second will buy into the other one like that, so what will happen is at the end the pool, the messenger RNA pool, which is present in your cell isate is going to bind the poly T tails, which is present on these beads and these beads, and then what you do is, you watch these beads with a washing buffer and then you do the evolution, once you do the evolution, it is going to break the interaction between the poly T versus to A and that actually will release the RNA into the supernatants and that supernatants you can use or supernatants you can take to collect the messenger RNA, so ultimately what you are going to get? You are going to get the pool of messenger RNA and with the, in the append of and the beads you can discard.

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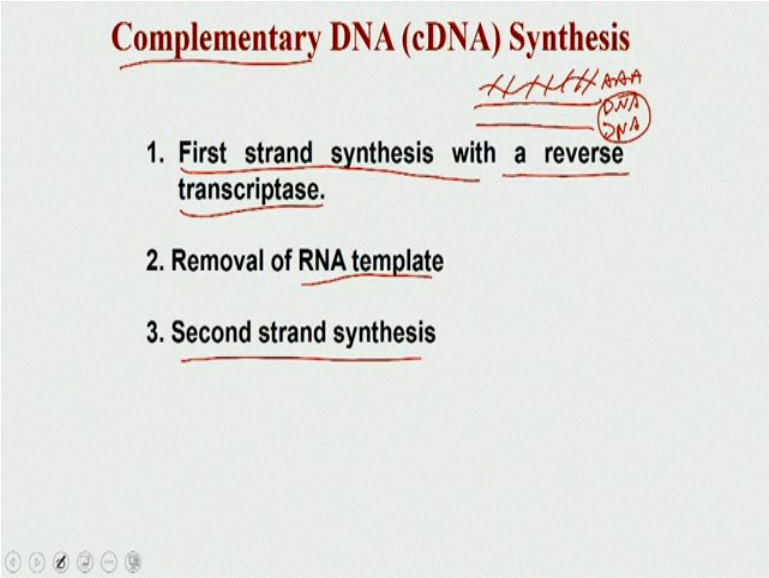

Now you got the messenger RNA in the second step you have to generate the cDNA or the complimentary DNA, which means you got the message RNA, now you have to generate the DNA and this you have to do in three different steps, in the first step you will do a first strand

synthesis of the DNA which is the reaction, which is going to be catalyzed by the enzyme called reverse transcriptase and then what you will do is, you will remove the RNA template which means you are going to degrade the RNA from this and then you will induce the second strand synthesis and that is how you are going to get the double-stranded DNA for subsequent cloning reactions.

So there are many methods which anybody can use to do a cDNA synthesis, there are many popular methods which you can use, so we are not going to discuss all these methods because all these methods are exploiting one or other different types of approaches, what we are going to discuss our couple of very popular methods, so one of the most popular method in this is that where you are going to use the, homopolymer tails or this method is called as the homopolymer tailing.

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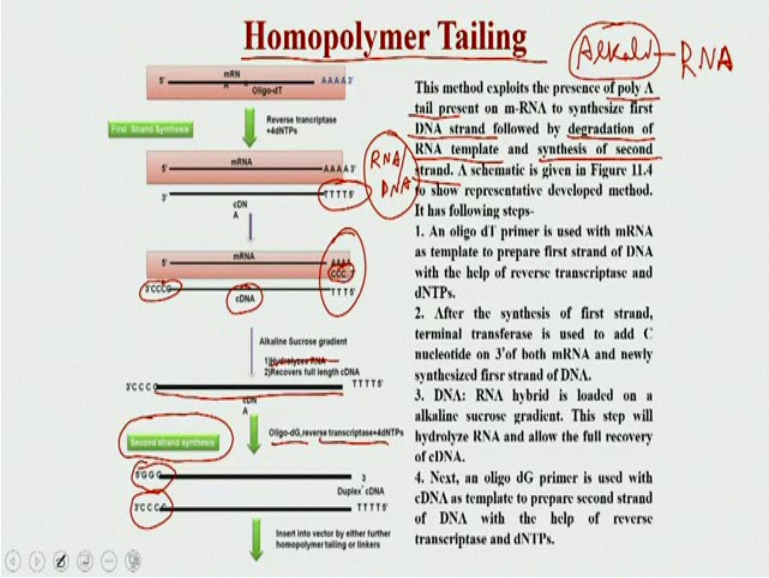

So in the homopolymer tailing, what you do is this method actually exploits the presence of poly A tail on the three prime of the messenger RNA, so what you do is first you used this poly A tail on the messenger RNA to synthesize the first strand which you do by the reverse transcriptase and then you induce the degradation of RNA template and the synthesis of second strand okay, so how to do this?

In the first step what you do is you put a poly T primer. Okay, so once you put the poly T primer the reverse transcriptase is going to use this as a template to synthesize the one strand of DNA okay and then you are going to get the RNA, DNA hybrid, now what you have to do is, you have to degrade the RNA okay, so once you got this RNA, DNA hybrid which is

actually like these, you are also going to synthesize the or you are going to add the terminals C, which means you are going to put the, you are going to put the C and you are going to put an enzyme called the terminal transferase, once you do that it actually going to put the CC on the three prime end of both the strands.

In this case, it is going to put on this side and in this case, it is going to put on this side, so in this case what you are going to have is, you are going to have the CCC on the DNA strand because you have used the terminal transferase, so that the terminal transferase is going to put the CCC on the DNA, on the three prime end, now what you do a is this DNA, RNA hybrid you will put it on to the alkaline sucrose gradient and you know that the alkaline solution is degrade the RNA and it does not do anything to do DNA, which means if you have the RNA, DNA hybrid and you put it into the alkaline solution, that actually is going to induce the hydrolysis of RNA molecule, but it is not going to affect the DNA molecule.

So at the end of this sanctifications you are going to get the first strand of the cDNA, now what you do is you put the second strand synthesis with the help of a poly G primer, so what will happen is, this poly G primer is going to bind to the poly C tail, which you have added in the previous step and utilizing these poly G tail, poly G primer is going to synthesize the second strand with the help of the enzyme called reverse transcriptase.

Now your gene is ready or the duplex DNA is ready, that you can use into the subsequent cloning reactions, which means you can digest this particular DNA and you can put it into the cloning vectors or alternate option is that you put the adapter molecules to this and you put it into the cloning vectors.

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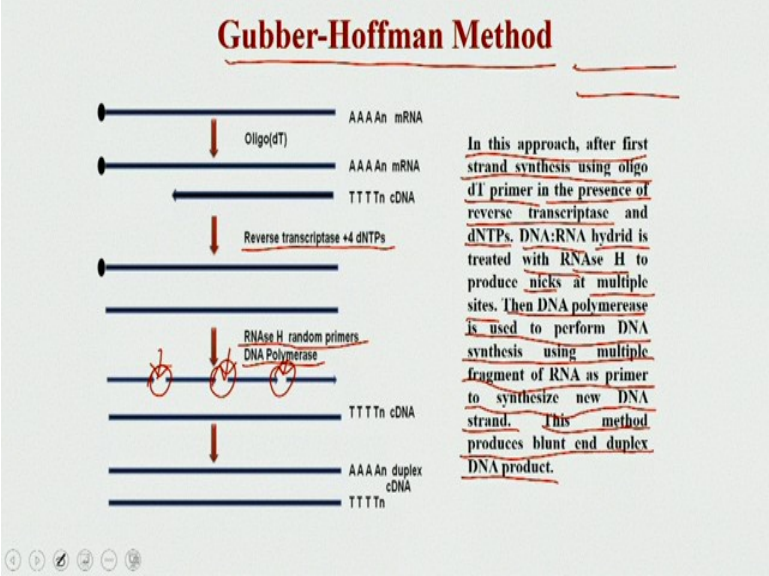

Now the next approach is call as the Gubber-Hoffmann method or Gubber-Hoffmann method, in the Gubber-Hoffmann method, it actually synthesizes the first stand using the poly T tail, so and with the help of reverse transcriptase and then in the subsequent step what it do is, instead of putting into the alkaline sucrose gradient or alkaline sucrose solutions, what you do is you treat the solution with the RNase H and then you use RNase H, so what happen is RNase H actually degrades the RNA in multiple places and that is how it actually generates the primers or that the remaining RNA molecules behaves like a primer and then you add the DNA polymerase.

So what will happen is (this primer) these RNA molecules are working as the primer and the DNA polymerase uses this RNA fragments and to synthesize the second strand, so that is what is written here, in this approach after first strand synthesis using the oligo DT primers in the presence of reverse transcriptase and DNTPs, the DNA, RNA hybrid okay, the DNA, RNA hybrid is treated with RNase H, so RNase H is a very, very specific RNase which actually detects and degrades the RNA when it is present along with the DNA, so in that what happened is it produces the nicks at the multiple sites, that is what you see here right, it produces the nicks at the multiple sites and the remaining RNA which you can use as a primer for the DNA polymerase.

So in the subsequent step what you do is, then you use the DNA polymerase to perform the second strand synthesis using the multiple fragment of RNA as a primer to synthesize the new strand and at the end what you are going to get this method is going to generate the blunt

end DNA, which means you are going to get DNA with no cohesive ends, it is going to be a blunt end, which means this DNA if you use this method, you have to use this, you have to use either the restriction digestion or you have to use the adapter molecules or you have to use the linker molecules to put this DNA into the cloning vectors to prepare the cDNA library.

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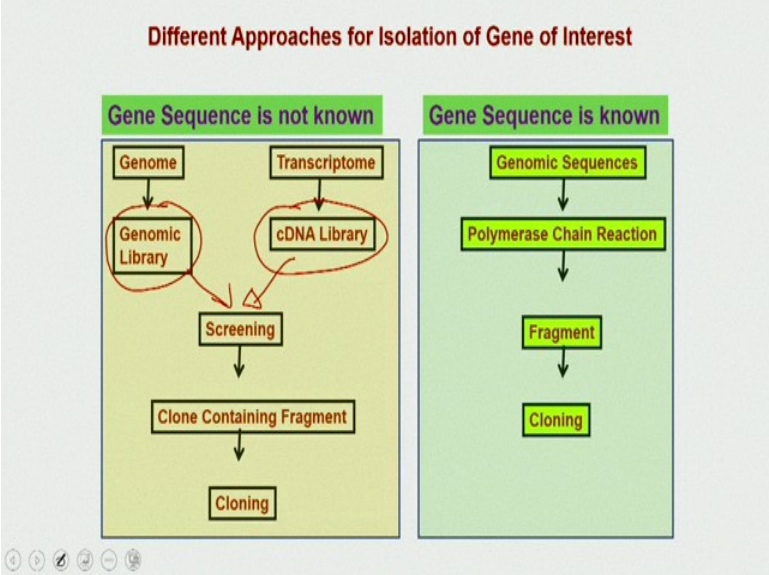

Now the next step is cloning of these cDNA into the suitable vector, so in the subsequent step what you are going to do? You are going to do exactly the same what we have discussed before, as we discussed for the genomic library preparation, once your cDNA library, once your cDNA is prepared then you will digests those or you will use your adapter molecule or you use linker molecule and insert these fragment into the suitable vector depending on the size of these fragments and then you will transform those into the suitable host either the bacteria or the yeast and that actually is going to give you the cDNA library, which is going to represent the transcriptome of that particular organism in that particular conditions.

Which means if you use cDNA library, you can actually be able to study the expression status of that particular cell, whereas if you use the genomic library, you are going to use the changes in the genome of that particular organism under a different, under the subsequent, under these specific treatments, now subsequent to this weather you have prepared a genomic library or whether you have prepared the cDNA library, all these libraries have to be screened using a particular type of criteria to isolate your particular gene and this is what we are going to discuss in our subsequent lecture.

So what we have discussed so far in this lecture is that we have discussed under what are the different approaches are available for you to isolate a particular gene sequence, you have the two different approaches, you have the approach when the gene sequence is not available or you have the approach when the gene sequence is known and under these we have also discussed about how to prepare a genomic library or how to prepare a cDNA library and now in the subsequent lecture we are going to study how to screen the genomic library or cDNA library to isolate your specific gene sequence. Thank you.