Genetic Engineering: Theory and Application
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Module IV- Basic of Cloning (Part II)
Lecture: 13- Cloning Vectors (Part II)

Hello everybody, this is Vishal Trivedi from department of Biosciences and Bioengineering, IIT Guwahati.

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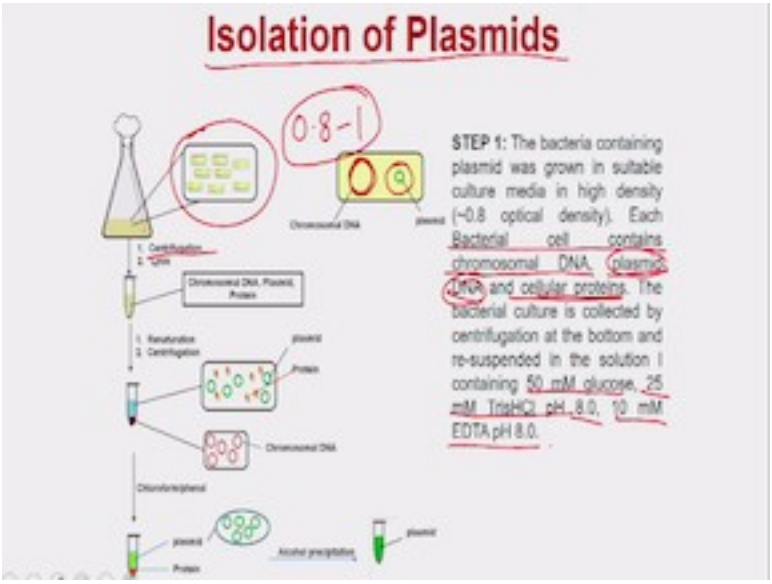

Let us discuss how to isolate the plasmids from the bacterial cell so the first step is that you have, you have to transform the plasmids into the bacterial cell. Let them to grow for overnight, so that they will have the OD of 0.8 to 1. Now if you take the 0.8 to 1 OD bacteria but in the first few steps what you have to do is you do the centrifugation and collect the bacteria of cells, ok and then, so each bacterial cell contain mostly the three components; one is the chromosomal DNA which is actually the genome of the particular equally which you are using for as a host, then it is going to contain the plasmid DNA which you have to isolate and then it also going to contain the lipids as well as the protein part.

So now you what, this purpose of your isolation is that you have to get the plasmid DNA and you have to destroy the chromosomal DNA as well as the cellular protein. So for first step is that you suspend this into a resuspension buffer which is actually the 50 mili molar glucose, 25 mili molar Tris HCL ph 8 and 10 mili molar EDTA ph

8, and that actually will resuspend the bacterial cell containing the plasmid into the isotonic condition.

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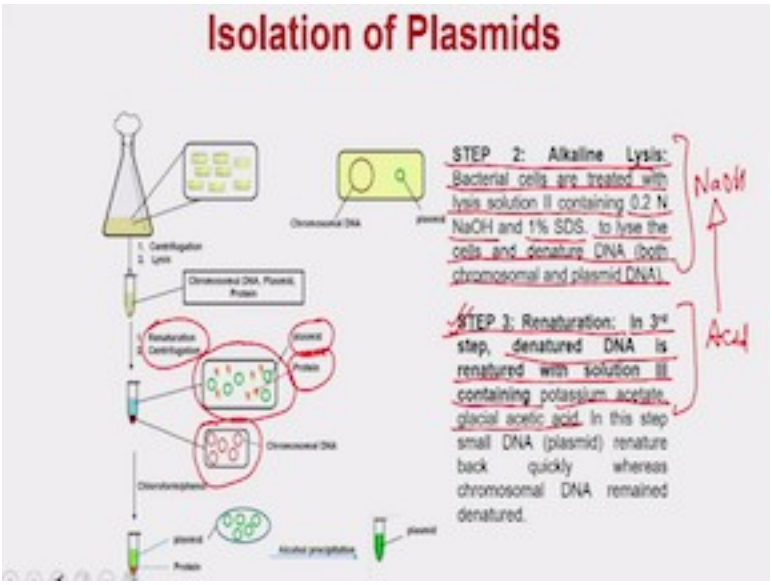

Then in the second step what you do is you lyse the bacterial cell under the strong lysis conditions so what you do is you add the resuspension buffer where the cells are responded you add the lysis buffer, solution number 2 which contains the 0.2 normal NaOH and 1 percent SDS, that actually will lyse the bacterial cell and it will going to not only that it is also going to denature the chromosomal DNA as well as the plasmid DNA.

If you remember we have discussed in the past that DNA is very very sensitive for alkaline conditions, so once you add the alkaline condition, alkali to the bacterial cell and as well as the SDS, SDS is going to lyse the bacterial cell and alkali what you have added is going to denature chromosomal DNA as well as the plasmid DNA.

In the step three what you do is you renature these biological molecules. So in the step three you will do the renaturation as well as the centification. So in the step three what you do is you will add the denaturation buffer or you add the solution III which actually contains the acetate, potassium acetate, glacial acetic acid. So what will happen is because these contains NaOH which is actually the alkali and is contain the acid which is actually the potassium acetate as well as the glacial acetic acid.

The acid is going to neutralize the alkali and because of that it actually going to renature the DNA but the problem is that the chromosomal DNA is going to be very very big compare to the plasmid so the time you are providing for renaturation is only very short, may be like half a minute or one minute. So in that short period of time the renaturation will only going to occur for the plasmid DNA not for the chromosomal DNA.

So because of that the chromosomal DNA is going to be denatured or is going to be remain as denatured and then when you (4:32) what will happen is the chromosomal DNA will present into the precipitate it whereas the top portion is going to contain two component; one is the plasmid the other one is known as the protein which is also still present. So after this your next task is to remove the protein part so that you are going to have the say plasmids.

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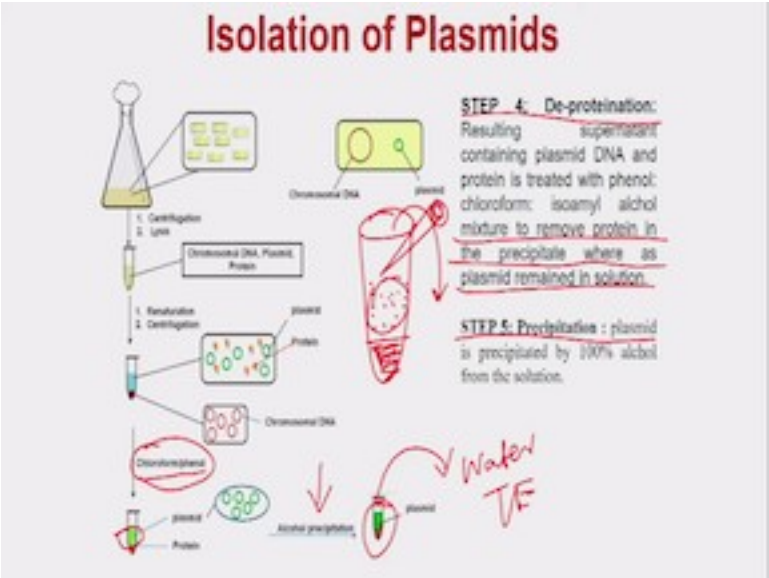

So in the step four you will do the de-proteination and how do you do the de-proteination is that you are going to treat the solution with the chloroform phenol mixture, the chloroform phenyl mixture when you treat then what will happen is that will remove the protein in the form of precipitate.

So this actually is going to precipitate the protein where as your plasmid is going to be present in the aqueous phase which is present in to the top layer. So when you will do that actually into the append of what will happen is you are going to have the protein at the bottom where as the plasmid is going to be present in the solution. Now what

you have to do is put your tip or put your pipette and suck this particular liquid into the next vessel and then you have the plasmid into the solution.

Now into the step 5 you will add the alcohol and once you add the 100 percent alcohol the plasmid is going to be precipitated in the form of the white colour pellet and that you can resuspend with the help of the water or the TE which is called Tris-EDTA solution and that's how you are going to get the plasmids.

So the way the theoretically I have shown you the plasmid isolation the similarly the plasmid isolation has also when you perform the plasmid isolation in your laboratory or in your institution you are going to face many technical difficulties.

To overcome these particular thing what we have done is we have also demonstrated you with a small clip from my lab where my student has shown or demonstrated how to isolate the plasmid from the transformed bacterial cell and what are the different type of precaution you should take while you are isolating the plasmid from the bacterial cell.

Demonstration by Sooram Banesh

In this video we will be demonstrating how to isolate plasmid. You see manual method and how to extract the plasmid DNA using phenol chloroform extraction method or isopropanol method. Both the methods will be demonstrating in this video and also we will show how to finalize the result like what are the different results you will get of the plasmid, analyzing the gel.

Hello everyone in this video we will show you how to isolate plasmid DNA using alkaline lysis method. For preparation of plasmid DNA we need resuspension buffer, lysis buffer and a neutralization buffer. In addition to that we need isopropanol, RNase A and ethanol. Resuspension buffer contains 25 mili molar Tris ph 8 and 10 mili molar EDTA and then we have to add RNase A in the final concentration of 100 microgram lysis buffer. Lysis buffer contains 0.2 normal Sodium Hydroxide and 1 percentage SDS. Neutralization buffer contains 3 molar Pottasium Acetate ph 6.0.

For isolation of plasmid DNA we need at least overnight grown culture with (())(9:13) 3.0. So this is already cultured one, we have to harvest the cells by centrifugation.

These cells we have to centrifuge 11000 rpm at least one minute to get the cells precipitated. Now we got the cell pellet. We can proceed for alkaline lysis to isolate plasmid DNA

In first step we are going to add resuspension buffer, which contain RNase A, mix thoroughly until all the cells suspended in resuspension solution. After the cells got suspended completely, now we have to lyse the cells using strong alkaline condition that is 0.2 normal Sodium Hydroxide and also 1 percentage Sodium dodecyl sulfate. Now we have to gently flip the tube in order to lyse the cells completely.

We can keep in this condition for upto 5 minutes but not more than 5 minutes, which will degrade the plasmid DNA and also genomic DNA will come out and it will interfere with the (())(13:13).

In next step we have to neutralize the Sodium Hydroxide using neutralization buffer to prevent any further degradation. After adding neutralization buffer you can see there is a white precipitate; that means all the proteins precipitated by the neutralization buffer. You can flip the tube 2 - 3 times, completely precipitate all the remaining proteins.

Now the solution contains, solution part contains our plasmid DNA and all the precipitate contains genomic DNA and also the proteins from bacteria. Now we have to centrifuge this lysed for 10 minutes at 11000 G. The precipitate got settled, now we try to transfer the white clear supernate to another tube.

This clear supernated contains plasmid DNA. Now we have to precipitate this plasmid DNA with the isopropanol followed by washing with the 70 percent ethanol. We can see white precipitate in the solution. Now we have to centrifuge it, collect the white precipitate and wash with the 70 percent ethanol.

After precipitating plasmid DNA with the isopropanol we will get the pellet of plasmid DNA. Now we have to wash the pellet. We wash this pellet with the 70 percent ethanol. Again centrifuge the pellet. Now we got the pellet, we have to air dry the pellet and dissolve it in deionize with water or the buffering. We keep leave at room temperature till the ethanol get evaporated. Next we will add (19:05).

To easy the process of manual alkaline lysis method there are several kits available from commercial vendors. The basic difference between alkaline lysis method and the kit based method is, kit based method contain silica bases columns where lysis lysed which containing plasmid DNA bites through these beads and after washing whatever the unwanted components are left they will elute out and we will elute the plasmid DNA in TE buffer or water.

The composition of the lysis buffer is same as previous method and also neutralization buffer, every buffer contain same component but in commercial kits we have one extra wash buffer, which will remove any unwanted contamination and give pure DNA.

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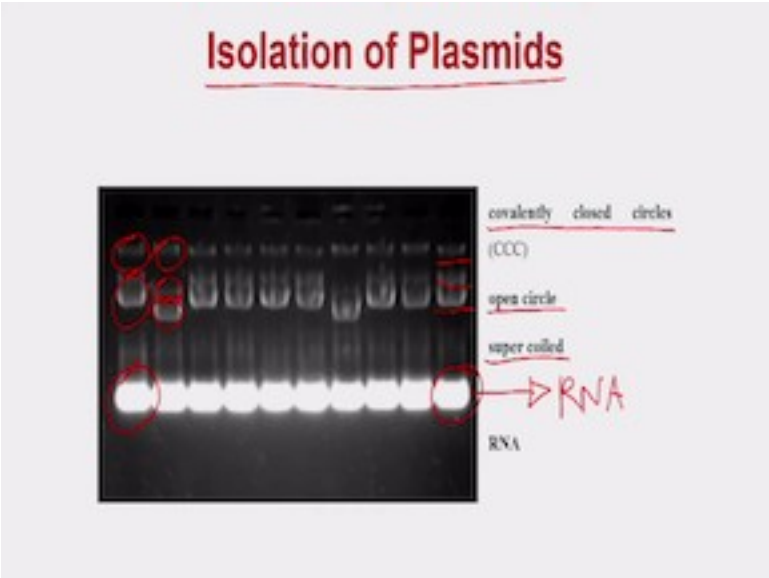

Now once you isolate plasmids what you will see is you will see a typical plasmid will give you the three different types of DNA and this is what is another one ok so what you have is one DNA, another DNA and the third DNA and if you do not do the resuspension or if you do not do the RNA treatment properly you will also going to see the RNA this is the RNA what you are going to see if the RNA is what you have added in the resuspension buffer is either not working or you have not perform that particular step properly.

Apart from that you are going to see the covalently closed circle model, covalently closed circle you are going to see open circle and as well as you are going to see the open super coiled DNA you are going to see all the three forms of plasmids into the

agarose gel when you analyze these plasmids from the bacterial cell and, so this is all about the bacterial plasmid which we have discussed so far.

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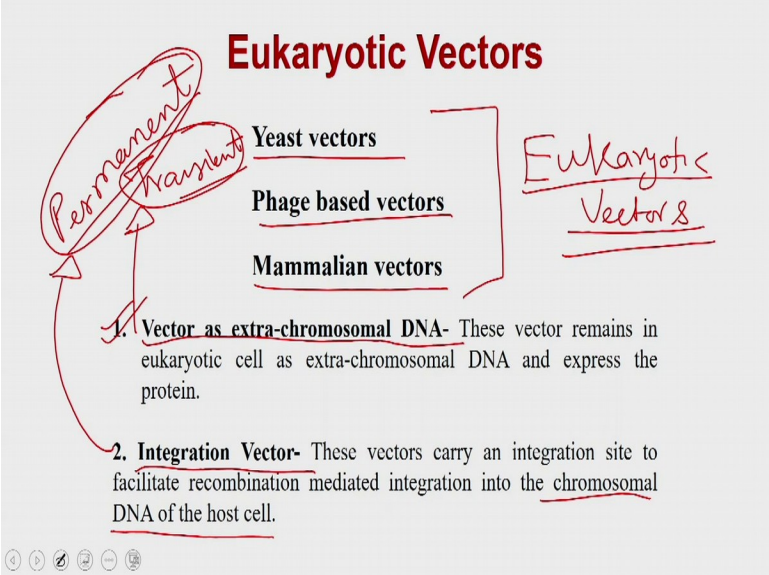

Now we will move on to the other types of vector, which we are, which we, which we are going to discuss. So the other vector we have to discuss is yeast vector, phage vector as well as the mammalian vector all these vector as we said in the beginning itself are the Eukaryotic vectors which means these vector are going to can be used for transfecting the Eukaryotic cell.

In the case of Eukaryotic vector you could have the vector of two different type the vector which are as a extra chromosomal DNA which means the vector which could work as a plasmids which will replicate along with the host strain and as a extra chromosomal DNA or you could have the integrating vector where the when the when you put the vector into the host cell it will integrate within the genome of that particular host cell and that is how when the host cell is going to produce or will going to make the copy of its own host its make going to make the copy of its genome then it is also going to carry forward.

Which means either it is going to be as a, as a plasmids which is means it will be you are going to have a vector which is extra chromosomal DNA so that actually is going to be the vector which you use always for the transient expression where as the integrating vectors which you are going to use for the permanent equation which because integrating vectors are going to integrate your foreign DNA into the

chromosomal DNA of the host cell and because of that it actually going to completely changed the host cell and host cell will replicate along with that the foreign DNA is also going to the replicate.

This means with the integrative plasmids you are going to generate the permanent transient organisms where as if the vector is present at the extra chromosomal DNA it will let you to produce the modified organism but that will be the transiently expressing your gene.

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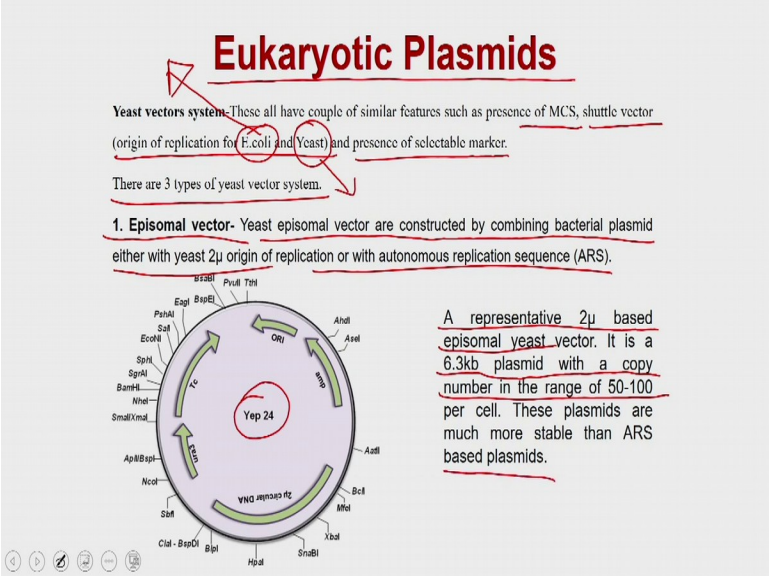

So lets start with the yeast, eukaryotic plasmids from the yeast. In the eukaryotic system from the yeast has the similar kind of features such as the it contains the multiple cloning sites, it could be shuttle vectors which means it is going to have the origin of replication for E coli and the yeast and it also going to have the selection marker.

So most of the Eukaryotic vectors are actually containing the origin of replication for the bacterial system which means E coli as well as for their own host strain at that actually important because most of the molecular biology manipulation before you test these vectors into their original host strain you actually try to do the molecular biology manipulation into the bacteria because the bacterial manipulation or the transformation into the bacterial cell as well as the molecular cloning is related steps are much easier in performing into the E coli compared to the original host strains.

That is one advantage the other one is that E coli life cycle or the E coli doubling time is less so whatever the procedure you will do in E coli is going to be faster compared to the yeast or the other hosts strains to you have three different types of the yeast vectors.

Number one is called as the episomal vectors, episomal vector the yeast episomal vectors are constructed by combining the bacterial plasmids either with the yeast to micron origin of replication or with autonomous replicating sequences. So either of these will be combined with the bacterial plasmid and that actually give the yeast episomal vector.

One of the classical example of the yeast episomal vector is the Yep 24 and the Yep 24 is a 2 micron yeast based episomal vector. It is actually a 6.3 kilo bar plasmid with a copy number in the range of 5200 and these plasmids are since these are the 2 micron origin of replications these plasmids are much more stable compared to the plasmid which are been derived from the autonomous replicating sequences.

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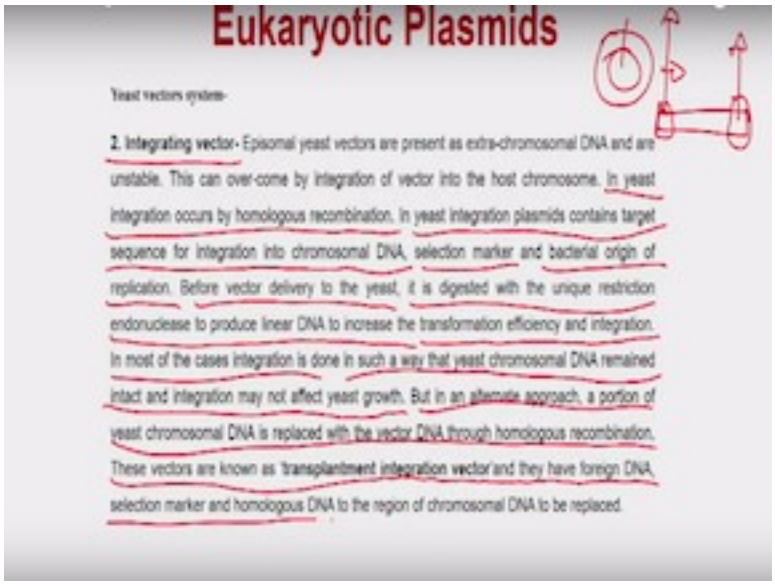

Now the other one is the integrating vector, these integrating vector as I said integrating vector will let you to integrate that particular foreign DNA into host genome. So in yeast the integration occurs by homologous recombination and in yeast integrating vector contains the target sequences for integration into the chromosomal DNA, selection markers and the bacterial origin of replications as well as it will contains the yeast replication activator before, so these are the circular plasmids, Ok.

But before you put them into the host or for the delivery of this particular integrating vector into the yeast it is digested with the unique restriction endonuclease to produce the linear DNA to increase the transformation and efficiency as well as the integration.

So what will happen if you cut with restriction enzyme that is how you are going to create the linear DNA and that linear DNA you will transform into the yeast because the linear DNA will have the exposed homologous sequences and that actually will allow its efficiency into the integrating into the genome.

In most the cases the integration is done in such a way that the yeast chromosome DNA remain intact and integration may not affect the yeast growth. So this is very important that when you do the integration of any foreign gene into the host chromosome you should ensure that it should integrate into the region which should not affect the growth or the viability of the host because if that happens then the corresponding host strain may not survive and it will be going to be a ledal integration or the homologous recombination.

In an alternate approach a portion of the yeast chromosomal DNA is replaced with the vector DNA through homologous recombination. These vectors are known as the transplant integration vector and they have the foreign DNA selection marker and so these are the different category of the integration vector where what you will do if you will also going to have the cassettes for the host as well and the host will it so in that case what will happen if when the integration vector is going to insert the DNA along with its cassette you are also going to provide the essential genes, so that the yeast is also not going to be affected by doing this particular type of homologous combination.

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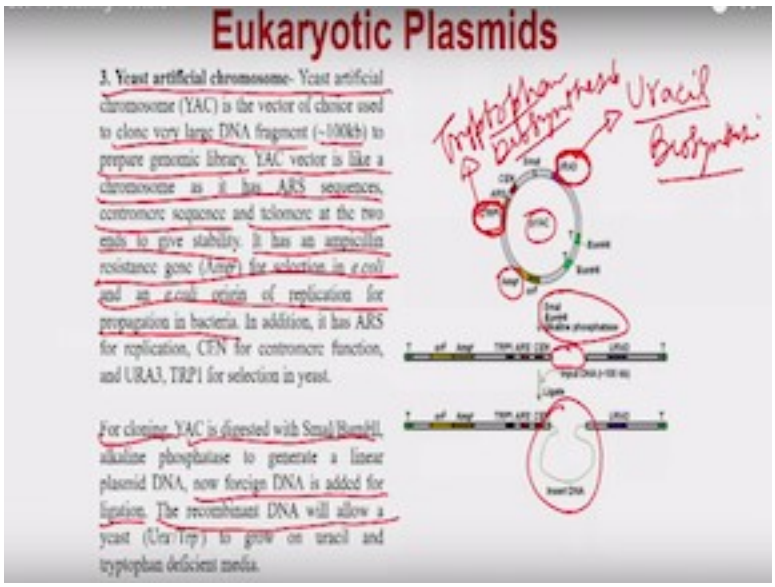

Now the third degree is called as the yeast artificial chromosome. So yeast artificial chromosome if you remember the yeast artificial chromosome is the choice of the vector for producing the genomic library. So human genomic library is been produced within the yeast artificial chromosomal system because they can actually take up very large DNA approximately up to 100 kilo base DNA and that is why they are the most preferred vector for preparing the genomic library.

YAC vectors are just like a chromosomal, chromosomes because it contains the ARS sequences, centromere sequences and the telomere at the two ends to give the stability. It has an ampicillin in this gene for you can see that has the ampicillin resistance gene and it also for selection in E coli where as it has the E coli original replication for propagation in bacteria. So these are also called as the shuttle, shuttle plasmids because they also going to have the original replication for the yeast as well.

For cloning the YAC is digested with a particular combination of Sma1 and BamH1 so for once you want to use them for the cloning purposes what you have to do is digest this particular plasmid with Sma1 and BamH1 and alkaline phosphatase.

Once you do this type of treatment what will happen if the plasmid it is going to be linearized and it also going to create a space which is actually going to have the space where you can actually put the foreign DNA and the foreign DNA you will put along with the legislation reactions, so the foreign DNA is going to get ligated.

Now what you do is you put this particular recombinant DNA into the selection marker. So in the case of the YAC plasmid what you have is you have the two genes, which you can use for selection, one is called URA3 the other one is called as the TRP1. So URA3 is for the will allow you to for the uracil biosynthesis pathway and where as the TRP1 tryptophan for the tryptophan biosynthesis.

So you can use either of these depending on the place where you want to use. So what you have to do is because once you disturbed these genes the resulting recombinant DNA will not be able to, will not going to provide these enzymes, which are required either for the tryptophan biosynthesis or for the uracil biosynthesis, so you have to grow these yeast on the uracil or the tryptophan deficient media and because the plasmid contains tryptophan or the uracil gene.

So they will be able to grow if they will get transformed where as the non transformed E coli will not be able to grow in the uracil or in the absence of all uracil or the tryptophan. So these are the two genes, which are present on to the particular YAC plasmids and they will be used as a selection marker.

So these are the two genes, which are going to be synthesized for tryptophan biosynthesis as well as for the uracil biosynthesis and you have the ability or you have the choices to use tryptophan as well as the uracil deficient media to select the transformed cells.

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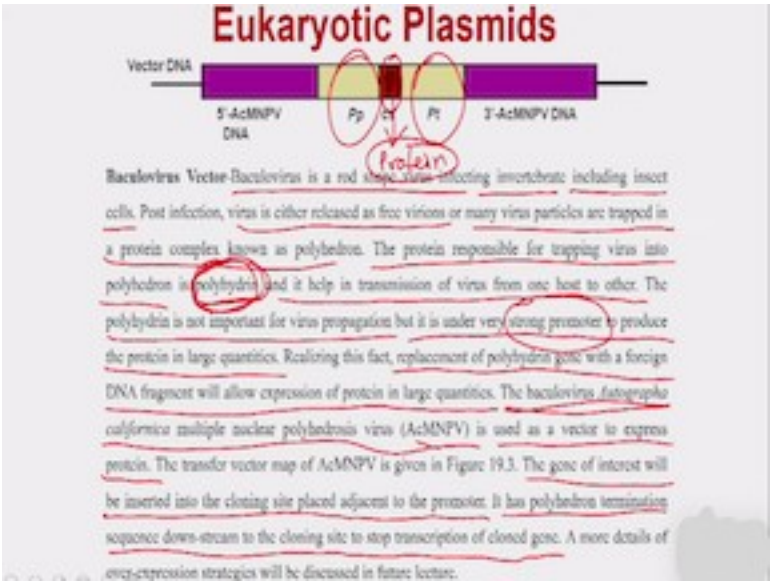

Now will move on to the another plasmid that is called as the baculovirus plasmids. So baculovirus is a virus, which actually infects the invertebrates including the yeast. So baculovirus is a rod shaped virus, which actually interfect the invertebrate including the yeast cells post infection the virus is either release as a free virions or many virus particles are trapped in a protein Complex known as the polyhydrin or polyhedron.

The protein responsible for trapping virus into the polyhedron is called as the polyhydrin and it helps in transmission of virus from one host to another. So this means the virus is actually synthesizing a protein which is called as the polyhydrin and this polyhydrin job is to accumulate or to protect the viruses or trap the viruses so that they will be get transmitted to another host and the polyhydrin is not important for virus propagation but it is under very strong promoter to produce the protein in large quantities.

So this particular protein is only required for the transmission of this particular viruses from one host to another but this particular protein is not essential for the virus propagation and but this protein is been produced from the viruses in a very large quantity because it is under strong promoter

Releasing this what people have done is there taken this particular protein and what they have done is they have replace a large chunk of this protein with the cloning cassettes so what they have done is polyhedron gene is replaced with a foreign DNA which allowed expression of protein in a large quantities even imagine that if this is the cassette of the polyhedron gene, what do you if you remove this particular cassette along with the foreign DNA virus is going to produce this protein in a large number large quantities.

And because of that the people have developed the plasmids with all the vector which is called as the so they have taken out this particular gene from the Autographa Californica multiple, AcMNPV and it is been used as a vector to express the proteins.

Gene of interest will be inserted into the cloning site adjacent to the promoter site so this is the cloning site where you can put your foreign DNA this is the promoter site this is the termination site. So once you do that it also has the polyhydrin termination sequences downstream the cloning to stop the transcription and once you put that it is

actually going to produce the foreign DNA and it will going to produce that particular protein instead of the polyhydrin.

So that how you can use this particular plasmid or this particular type of vector to infect the insect cells and the insect cells will going to produce the protein in large quantity.

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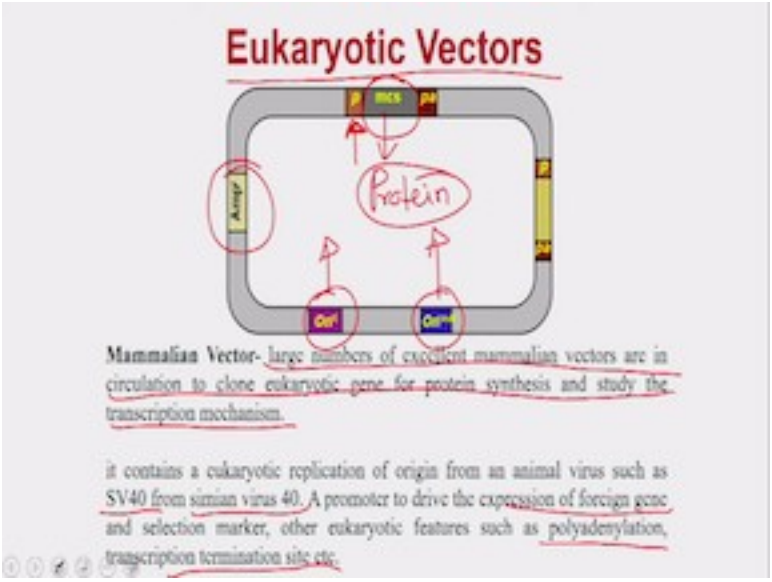

Now I will come back to the mammalian vector. The mammalian vector there are large number of excellent million vectors in circulation for protein synthesis as well as to study the transcription mechanisms mostly the mammalian vector are all going to contain the origin of replication from the from the animal viruses such as the SV40 or the simian virus 40, then it also contains the promoter to derive the derive the expression of a foreign gene which is this and it also contains the origin of replication for the E coli.

It also contains what origin of replication from SV40 and it also contains all other features such as polyadenylation site, transcription termination site and all that. And this is the multiple cloning site so you can actually express or replace your gene into this multiple cloning site the promoter is present next to that and that is how you are going to you can be able to use this for reducing the protein. You have the ampicillin resistance as well as the, so that you can use the mammalian expression vector for cloning purposes.

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Now we will talk about the bacteriophage lambda based vectors. So bacteriophage are the virus using bacteria as their host for replication. Bacteriophage lambda is the virus of E coli and has been in used for developing the vector for genetic engineering before I get into the detail of how it has been used for molecular cloning or the developed for expression of the protein into the host strain lets go with the biology of bacteriophage plasmids.

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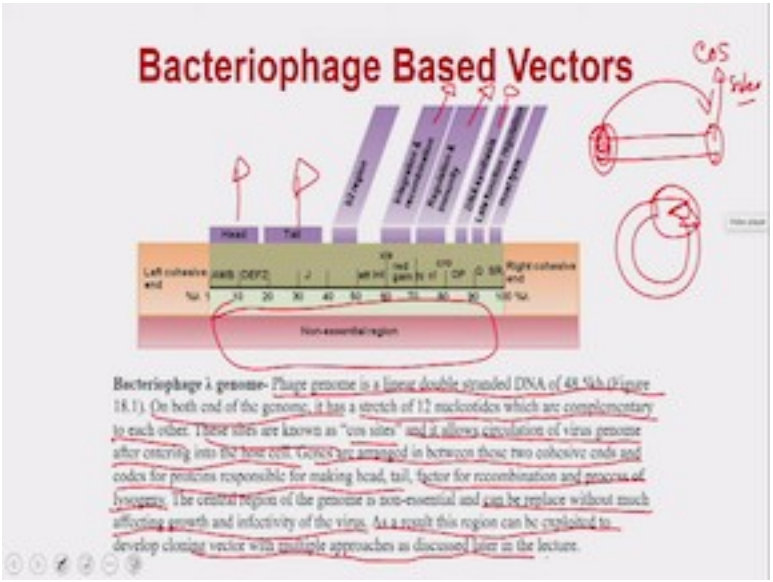

So phage genome is a linear double standard DNA of 48.5 kilo bar DNA in both the ends of genome it has a stretch of 12 nucleotides, 12 nucleotides which are called as

the complementary to each other. Each site are known as the cost site, so what you have is genome and on the corner of the genome you have the tall nucleotide long chain which is actually complementary to each other so because of that what will happen is if you put them together these two sites will come and will stick to each other because of that these things are called as the cost sites.

And it allows as I said it allows the circulation of virus genome after entering into the host cell so what will happen if the virus will enter into the host as a linear DNA and then once it enter into the host the course site are been exposed and these cost sites resupplies the genome and gene are been arranged between these two cost sites or the cohesive site and it codes for the protein which are responsible for making the head, making the tail.

You have the gene which are required for integration as well as recombination, the gene which are regulating the immune system of the host then you have the gene which are required for DNA synthesis and so on and vector for recombination and the process of lysogeny, ok.

The central region of the genome which is this portion is non essential and can be replaced without much affecting the growth and the infectivity of the virus as a result this particular region can be exploited to develop a cloning vector.

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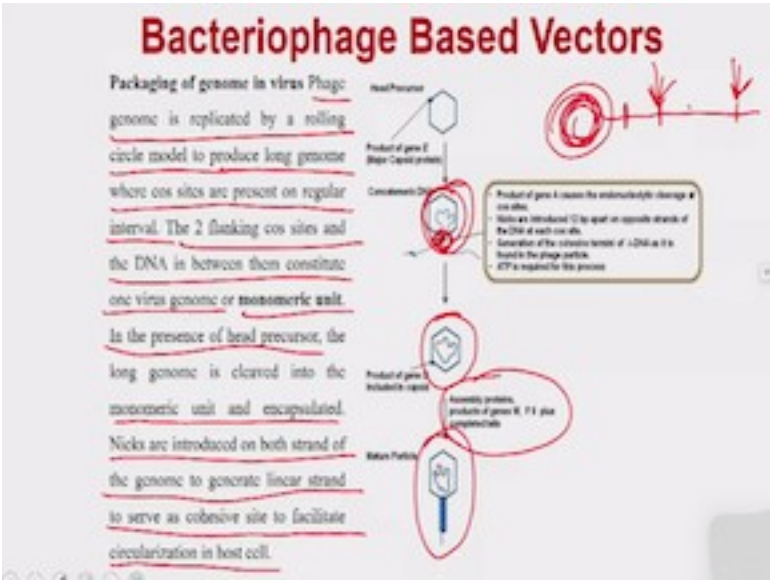

Now let us see how the genome is packed into the virus. So phage genome is replicated by a rolling circle model to produce the long genome whereas cost sites are present on the regular intervals.

So what happen is the virus is using the rolling circle model and that so it is producing the large linear DNA or linear DNA which contains the cost sites on regular intervals and these cost sites just next to the cost sites are, so you have two flanking cost sites and the DNA between them are constituting the two virus genome.

So this is the virus genome or one monomeric unit. In the presence of head precursors, so once the head proteins are been synthesized the long genome is being cleaved at the cost sites and encapsulated.

Nicks are been introduce and at the both strand of the genome to generate the linear trend to work as a cohesive site to facilitate the circularization. Once this genome is inserted and the head is been synthesize the Nick is been generated on to the virus cohesive ends and the cohesive ends come together and the genome is been circularized. Then you, then the assembly protein as well all other protein come and then the mature virus particle is been produced.

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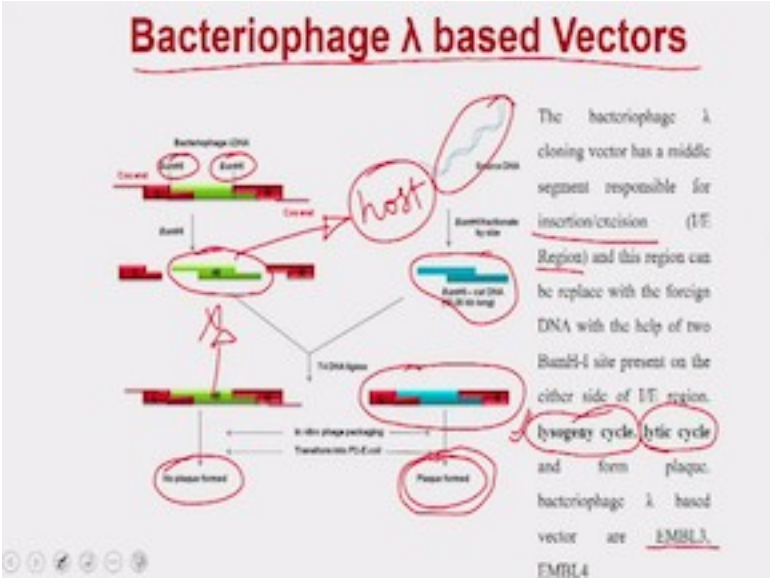

Now taking this into the account the people have developed the bacteriophage lambda based vector, so how the vector works is that it has the two BamH1 site which can be used to insert the foreign DNA. So you can imagine that you have the two scenarios,

in one scenario what you have done is, so under the normal circumstances what you have is you have the insertion or excision region which is been inserted between the these two cost site, ok, in the vector.

So as long as these sites are present it will integrate along with the host and it will not going to create any platformation instead it is going to go through with the lysogenic cycle but once you insert the foreign DNA which you have taken out from the host ok and once you produce this foreign DNA and inserted that into the bacteriophage lambda based vector what will happen is you are going to lose the I by E region which means this particular type of virus is not going to integrate or will not going to perform the lysogenic cycle in stead it will go to the lytic cycle.

And once it go through with the lytic cycle it is going to generate the plaques and these plaques formation can be identified simply by looking at the cell under the microscope and that is how this actually plaque formation is this selection pressure or selection criteria when you use the bacteriophage lambda based vector. Two classical examples are EMBL3 and EMBL4.

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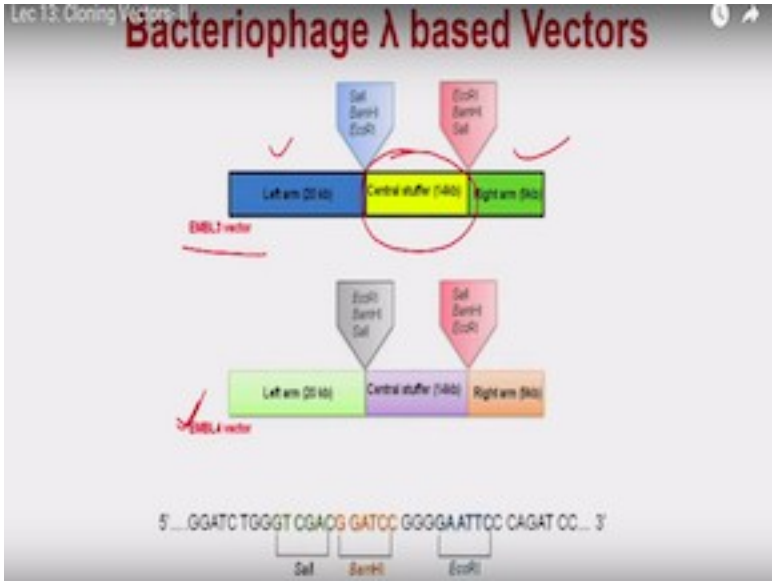

So these are the EMBL3 vector where you have this is the left cohesive site or this is the right cohesive site and within this you have this central shutter portion which you can use for replace this along with the foreign DNA and can be used for cloning purposes. Similarly you have the MBL4 which can be used under the similar conditions and.

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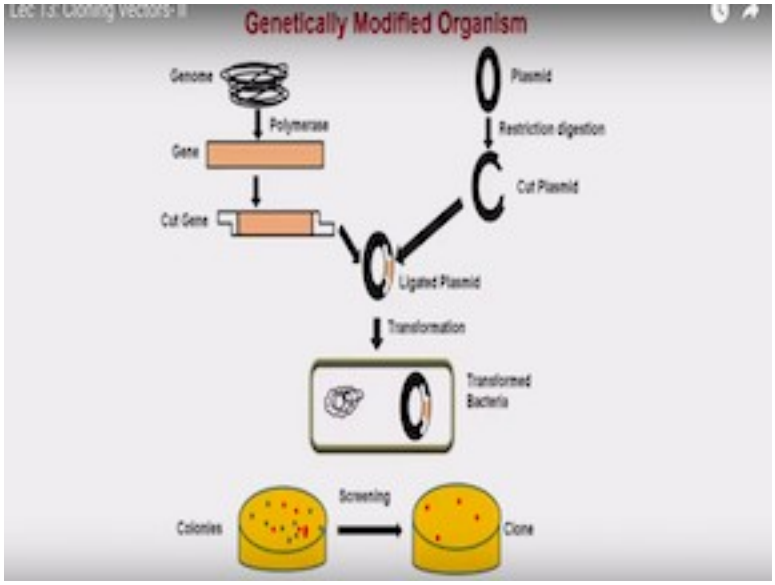

So with this we have completed our discussion about the different types of vector which are been found and which are available for cloning purposes and what we have discussed so far we have discussed about the plasmids which are been used in the bacterial host and the plasmids could be exist in multiple forms, either it would be close circular form or open circular forms or the super coil DNA.

We have also discussed about the how to generator how to design a new plasmids and use that for the cloning purposes and apart from the plasmids we have also discussed about the yeast, yeast vectors, we have also discuss about the bacteriophage lambda vectors, we have also discuss about the vectors which you can use to overexpress the protein in the insect cells and then at the end we have also discuss about the bacteriophage lambda based vector which you can use for the producing the protein or the transforming the cells in the host for the for the mammalian systems.

And with this we would like to conclude our lecture here and in the subsequent lecture we are going to discuss about the entry of the recombinant DNA into the host. What we are going to discuss in the next lecture is that how to generate the recombinant DNA and then how to transform or how to insert this recombinant DNA into the host cells. We will discuss about the prokaryotic, the methods which are popular into the prokaryotic system as well as a Eukaryotic system. So with this we would like to conclude our lecture here. Thank you.