Genetic Engineering- Theory and Applications
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Module 4
Basics of Cloning (Part 2)
Lecture 11- Molecular Tools for Cloning

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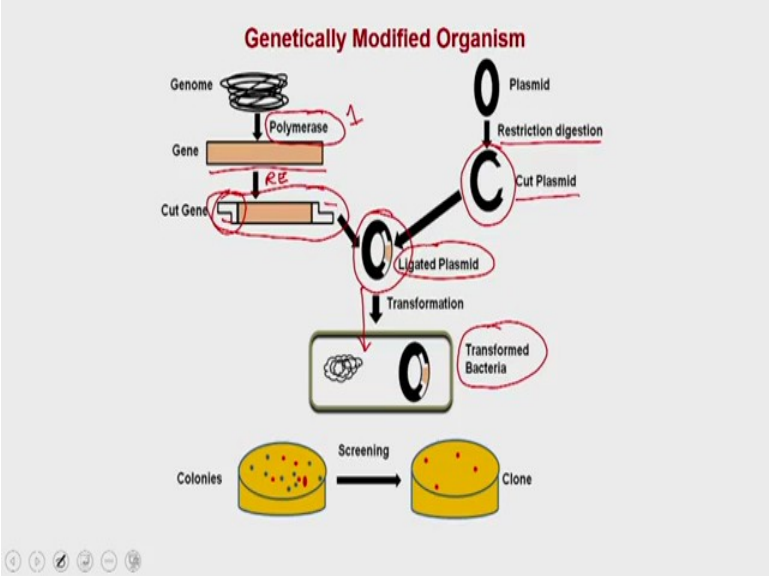

Hello everybody this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT Guwahati and what we were discussing, we were discussing about the generation of genetically modified organism and in the scheme of genetically modified organism, what we have discus so far we have discus about the different force strains they are physiology the biochemical reactions what they catalyzed and what is a relevance of this biochemical reactions for organism as well as for the industrial significance and in continuation to that discussion we have also discus about how to isolate gene of interest from the particular organism so that you can use the in change fragment into the downstream cloning reactions and to generate the genetically modified organism,

So what we have discuss so far is that if you have a genome and from which if you use the polymerase reaction to generate the gene, you can also get this fragment by the either by the genomic libraries or the (())(01:46) libraries, so whatever the sources after that you have to digest these gene with the restriction enzymes and what will happen if you do the restriction enzyme it you are going to generate the exquisite and both sides and the similar kind of procedure you have to do for the transforming genes such as the plasmids.

And then you have to put it for the ligation and then you will get they ligated plasmids this like it plasmids you have to put it into the host strain and that is are you are going to get the transform bacteria or the genetically modified organisms and so during this whole procedure of a generating a genetically modified organism you have the multiple enzymes which are being used in this complete scheme and in the todays lecture what we are going to do is we are going to discuss about the different enzymes what we used in the cloning reactions and their mechanism as well as how to perform the or how to use this enzymes in the lab as well,

So as you can see that we have the four major enzymes one is the polymerase this polymerase enzyme we have discus in our previous lecture the second enzyme is called as restriction enzymes. These restriction enzymes are also being required to generate the cohesive ends and third enzyme is the enzyme which is required it for legating the your insert as well as the plasmid DNA.

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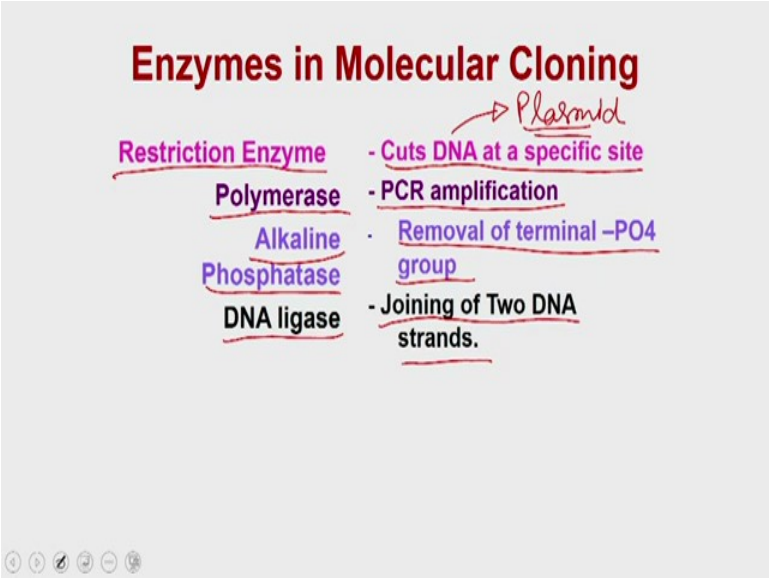

So in totality what you have is? you have the four major enzyme which are having the applications in the molecular cloning. The first class is called as the restriction enzyme the purpose of the restriction enzyme is to cut the DNA at specific side this DNA could be the plasmid or could be the you gene of interest what you have isolated after the PCR then you have the polymerases in the previous lecture we have discuss about the PCR amplification and utilizing the polymerases which are belonging to the tact DNA polymerases.

Then we have the alkaline phosphatase, the purpose of alkaline phosphatase is to remove the phosphate group and that also has the specific application in the molecular cloning and then the last enzyme is called as the DNA ligase, the purpose of DNA lag is to join the two DNA

strands in this case one could be the plasmid the other one could be your insert which you have digested with the restriction enzyme let us start with the restriction enzyme first.

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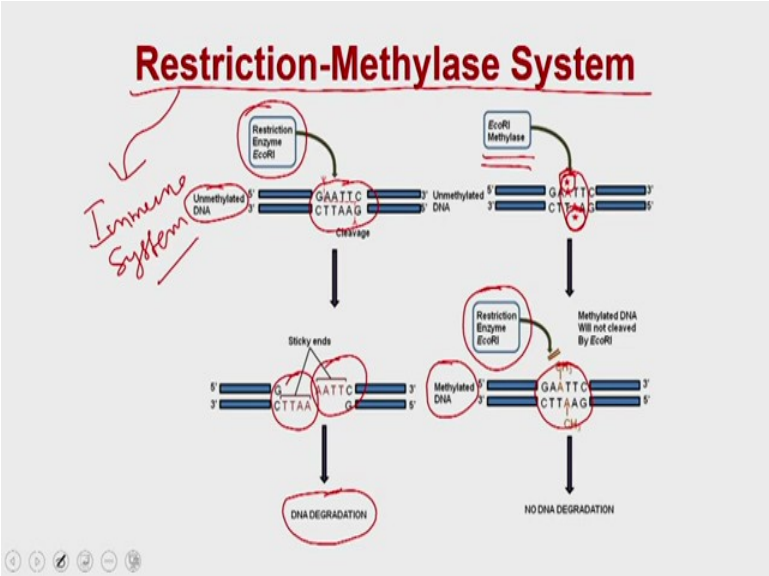

So the restriction enzyme is a part of big system which is called as the restriction methyl system and restriction methylase system is like a immune system of the bacteria or the lower organism, so as you know that what is the function of the immune system in the higher organisms, the purpose of the immune system of the higher organism is to not allow the external organism to grow or to proliferate that is being done by simply having the different types of barriers for example in a human being you have a skin which actually protects your body from getting the entry of bacteria fungus and all other kinds of infections organism.

Below to this skin you have the first layer of defense which is mediated by the macrophages or other kind of immune cells and after that you have the real immune cells which are like b sell d sell and all those kind of t impolite and b impolite and all these impolite are having similar functions that they are not going to allow this particular infections organism to grow into your back cell and what is the advantage of having this system, because it will not allow the opportunistic infection or the other kinds of infections organism to draw the nutrition from the host.

Similarly the Bactria as well as a smaller organism have the restriction methylase system to not allow the propagation of the particular DNA of the external sources, so how this restriction methylase system works is that every restriction enzyme for example, in this case we have taken an example of eco oven has specific site which is called as g g a a t t c so it is

having a specific site means specific sequence of nucleotide and that would be cleaved by the eco r one but under the two conditions.

One either the DNA will be un methylated or the DNA would be the methylated if the DNA is un methylated which mean that this particular DNA is not going to belong to that particular organism and if the system will see un methylated DNA the echo r one will going to cleave this DNA and will generate the sticky ends. So by doing those it is actually going to generate the external DNA into the DNA fragments but suppose this particular DNA if belonging to the economic r one or the eco la itself in those cases what will happen is, that the eco r one methyl is enzyme which is actually also this part of the restriction methylate system is going to methylate the specific site on to the restriction site so in that case what will happen is that is this DNA belongs to the eco lite cells then what in the eco la itself then what in the eco la what will happen is that the within this recognition sequence the crucial (())(8:23) going to be get methylate by the enzyme and because of this methylation group what will happen is, that the restriction enzyme eco r one will not be able to bind the DNA.

And we will not able cleave and once it is not done, then it is not going to degrade the DNA which means that restriction methylase system is also has spasticity it also has ability to recognize what is cells and what is non cells. For example, in this case you might has seen that this particular DNA is considered as the non sells, simply because it does not have the methylation on the admin groups, whereas the DNA containing the same restriction side but methylated on the add in group is a consider to with a cell DNA and that is actually a unique mechanism to distinguish between the self DNA verses the non self DNA.

Now how the enzyme will be going to know which one is self which is non self is because the restriction methylase system has the three different activity associated within the single enzyme one is called s activity the other one is called as the methylase activity and the third one is called as a restriction methylate activity.

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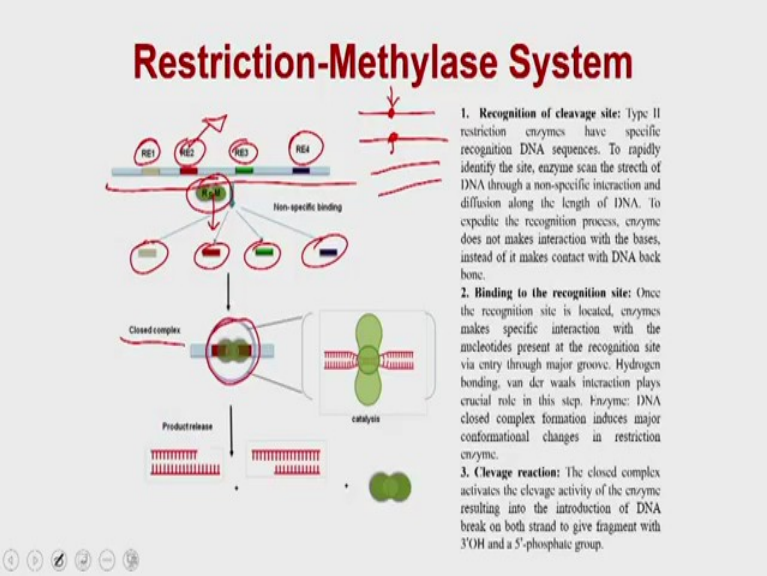

How that happens that, suppose imagine that you have a peace of DNA with four restriction sides RE1, RE2, RE3 and RE4. Now you have the four sides and you have added the restriction enzyme or restriction methylase system and which actually contains the three activities the s activity r activity and m activity. The s activity which actually going to recognize the specific sequence on to the DNA and it will, then launch the restriction enzyme on to the DNA of your interest.

So what will happen is this enzyme is going to interact with the RE1, RE2, RE3 and RE4 and all this four sequences are different so that is why the s activity will going to intact and while it is interacting it either will form the open complex or the close complex, once it is binding it may have the very high affinity for the RE2 because this is a side which is actually being recognized by s activity within the restriction enzyme.

So once it is going to recognize it will go and bind to the RE1, now you can imagine that the enzyme has been launched on to the DNA with the help of s activity, once the s activity will in launched then it will insert its m activity into the enzyme to detect or to check whether the DNA is methylated or hemi methylated or the un methylated.

If the enzyme if the DNA is un methylated, if DNA is methylated the m activity will find these methyl group and then it will not be going to give the access to the restriction activity to come into the picture but, if the DNA is hemi methylated which means that the if you have the side and one of the side it is methylated the other side is non-methylated in that case what will happen?

That the m activity on to this strand is going to insert whereas on this side m activity is going to generate the methylation, so if the DNA is hemi methylated the restriction enzyme system, restriction methylene system will say that okay this is the DNA which is being generated from the parent DNA and actually being our self DNA and because of that what will happen is that restriction methylase system is going to add the methyl group on to the next strand.

Just so that it will be get immune from the getting the cutting of DNA but you can imagine that if you have the restriction inside and it is non-methylated so s activity is being used to provide the side where the DNA is going to bind by the enzyme. The m activity will detect whether the methylation is there or not, if there is no methylation then m is also get inserted into the DNA and then the r activity or the restriction activity will come and bind form the crossed complex and once it is from the closed complex which means the r activity can actually be able to access the DNA then it is actually going into cut the DNA into the two parts and these two parts are going to be released.

Once these two DNA fragments are going to be released the enzyme is also going to be released into the solution and it will again be ready for the next cycle of catalysis. So restriction methylase system is very very preside system adopted from the microorganism or the smaller organisms to protect them self from the propagation of bacteria or viral DNA or other kind of DNA from the other organisms.

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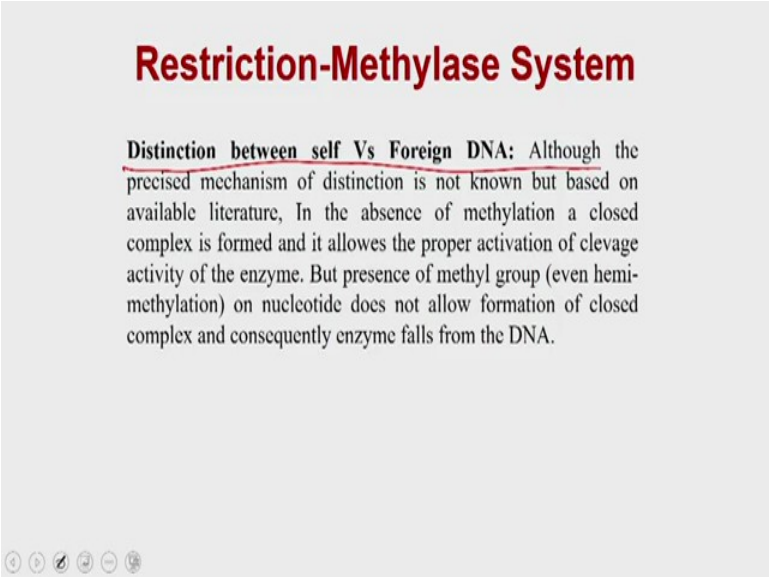

Restriction methylase system is also going to distinguish this is what we have already discus between self and the foreign DNA simply because the foreign DNA is going to be un methylated compared to the self DNA which is either going to be methylated or the hemi

methylated so if the same side is present into the hemi methylated form or the methylated form it is considered to be self DNA. If it is the same side which is present as the un methylated form, then it is considered to be the foreign DNA and that is what the way restriction methylase system is distinguish between the self or the non self DNA.

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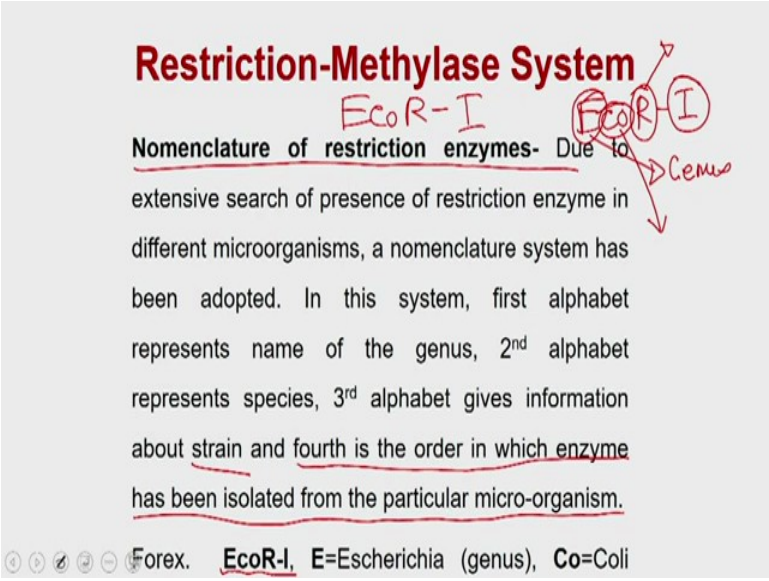

Now, since restriction enzymes are being discovered there is classical or there is a particular rule which people are following write the name of the restriction enzyme so if you can they see an example what you can see in example you see the example of the eco r one is one of the enzyme okay, so the nomenclature is that the first letter you will take as the genes of that particular organism.

The second is the spacious whereas so in this case eco r one e is ecola or Escherichia cola so e is Escherichia the c o is coming from the coli and then r is actually the information about the strain okay. So for example in this case you have the r strain and the fourth letter is the order in which the enzyme has been isolated from the particular organism which means that you if you have isolated the eco r one, the first time this is going to be the first enzyme from the e coli strain r.

And so that all the people have started naming the different different restriction enzyme for example, you have the bamech one you have hin three, you have h e three. You have xho one and so on. So these all these restriction enzymes have a critical pattern in which they are name is being put and so that actually helps in getting the information about the enzyme it also getting the information about the source from which you have isolated the enzyme.

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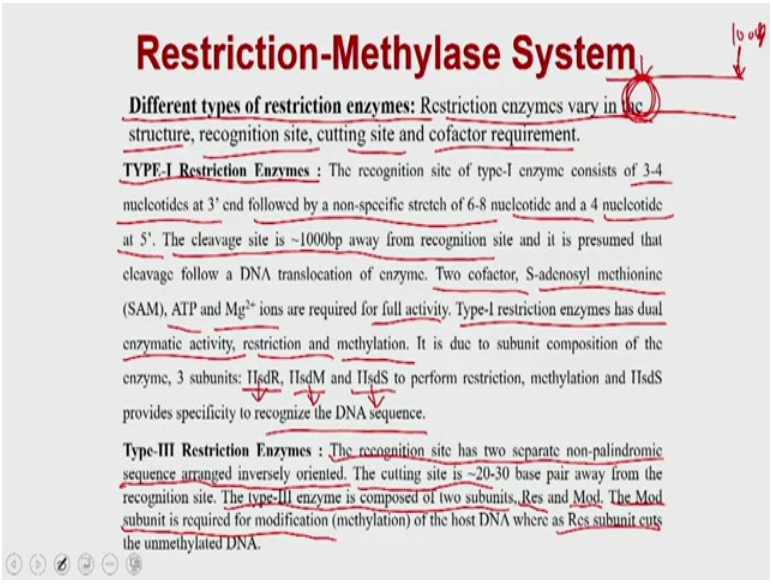

There are different types of enzymes, different types of restriction enzymes. So restriction enzymes are varying in they are structures recognition site and cutting site, cofactor requirement. Based on these parameters the restriction enzymes are of three different types, one is called as the type one restriction enzyme,

The type one restriction enzyme are the recognition site is consist of three to four nucleotide at the three prime and followed by a nonspecific stretch of six to eight nucleotide and the four nucleotide at the five prime, which means the type one restriction enzyme recognition side is going to be a very very long and nonspecific and then this is the recognition site. Once it is the recognizing the cleavage site is approximately hundred base pair away from the recognition site.

Which means that the enzyme is actually recognizing here and binding here and whereas it is cutting the DNA away. Which means the type one restriction enzymes are first of all have a very very un-recognized and loose restriction sites and then where they will cut is actually the one thousand base pair away from the actual recognition sites.

They also require the co factors one is called as the s- adcnosyl methionine atp and magnesium for their activity and the type one restriction enzymes has the dual enzymatic activity which means they can be able to do restriction as well as the methylation and the enzyme which that is because the enzyme has the three sub unit one is called as hsdr hsdm and hsds whereas hsdr is for the restriction m is for the methylation and s is for the detecting the recognizing the particular DNA sequence.

Apart from that you have the type three restriction enzyme that type three restriction enzymes are having the restriction side, which has the two separate non palindromic sequences arranged inversely oriented the cutting site, so cutting site in this case is twenty to thirty base pair away from the main recognition site and the type third restriction site also composed of two sub units, it is restrictions site as well as the modification site.

Mode activity or the mode sub unit is required for the modification which means the methylation of the DNA where as res subunit is required to cut the un methylated DNA so whether it is the type one site or the type three site. Both are this restriction enzyme are recognizing at particular sequence but they are cutting at a different site. Or the cutting at a very distance site.

In the case of type one enzyme it is actually cutting at the thousand base pair where as in the case of type three it is cutting approximately twenty to thirty base pair away from the their recognition site.

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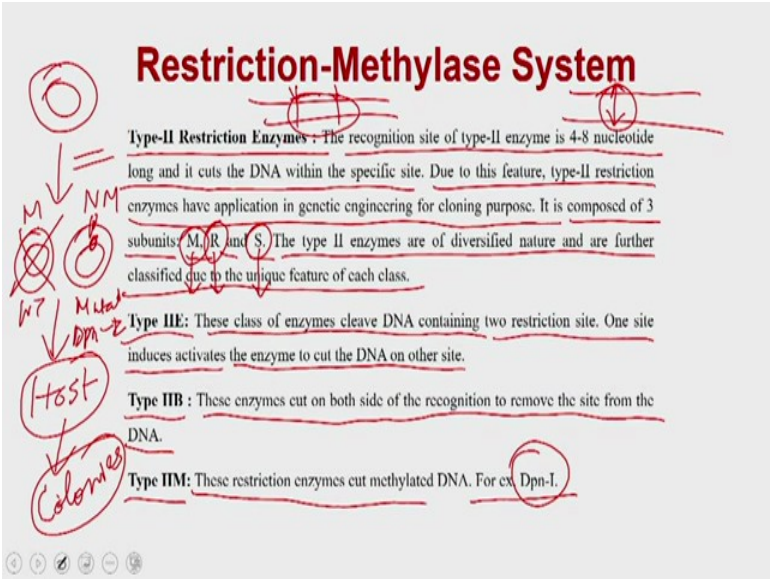

Now the type two restriction enzyme is the recognition site of type two enzyme is four to eight nucleotide long and it cuts the DNA within the restriction site. Which means that if you have this DNA and enzyme is recognizing this particulars starch of DNA it cuts the restriction site within that particular site, due to this particular unique feature the type two restriction site have an application in the molecular cloning because you will need the un enzyme which actually precisely cuts where it actually goes and bind and you should know that what sequence is cuts and what sequence is binds.

Whereas if you see that in the type one and type three they were binding to somewhere and cutting to somewhere so that is why you cannot predict or you can not generate thus you cannot use those enzymes for the molecular cloning.

It is composed of three subunits which is called m r n s so, similar to the type one enzyme t m r and s have the similar kind of functions m is going to do the methylation or the modifications are if for the restriction n s is for the recognition of the particular DNA sequence.

The type two enzymes are also of diversified functions so they are also being classified further due to unique feature of each class so in that type two e this class of enzyme contain the DNA which actually contains the two cleave at site. Which means that if you have the one cleave at side this type two every enzyme will not be going to cut the DNA. Whereas if you have the two site, where first site is actually being required to induced the activation on the and then the enzyme actually cuts on to the DNA on the other site.

Similarly, you have the type two b and these enzymes actually are cutting the both sides of the recognition side to remove the recognition sides from the DNA which means that if you have the restriction side like this it actually cuts on both the sides and that is how it actually removes that fragment of that particular DNA.

Third is the type two m the type m is the restriction sides which actually cuts the methylated DNA. So the coeternity to other restriction methylase system these enzymes are actually cutting the DNA when it is methylated, the one of the classical example is dpn one and the dpn one has the very very classical usage because of this unique property that it actually cuts the methylated DNA.

You might as we discus like methylated DNA is immune from getting the cut from the restriction methylase system but in this particular class of enzyme the enzyme cuts the DNA because it is methylated and what is the application of this particular enzyme this particular enzyme is always been used to detect or destroy the host gene when you are actually generating metalations.

How we use this enzyme is that suppose you are generating a side directed mutant into a particular type of while type gene. In those case should what will happen is that you use the primers to muted the side okay? so in that case you what will happen is that you have taken a plasmid and you did the pcr with the muted primers in those cases what will happen is that

you are going to get the two plasmids one were which is going to be the while type the other one is going to be the while type and the other one is going to be the muted.

Once you got these two kind of species you will transform this particular reaction into the host and both are these species are capable enough to grow into the host and will give you the colonies. So the stud n what is the difference between the while type and muted is that the muted is non methylated whereas the while type is methylated so ion these kind of situation what the people do is the treat this reaction after the pcr you got the this two plasmids and what they do is the just treat this reaction with the dpn one and what will happen is the dpn one is going to cleaved or will going to destroy the while type enzyme whereas keeping your mutated plasmid intact.

And then if you transform that into the host you are going to get the colonies that colony is going to contain only the plasmid for the mutant then you can take out the colonies and do the sequencing to conform that the mutation is been done. So that is what is the application of this restriction enzymes which are belonging to the type to m.

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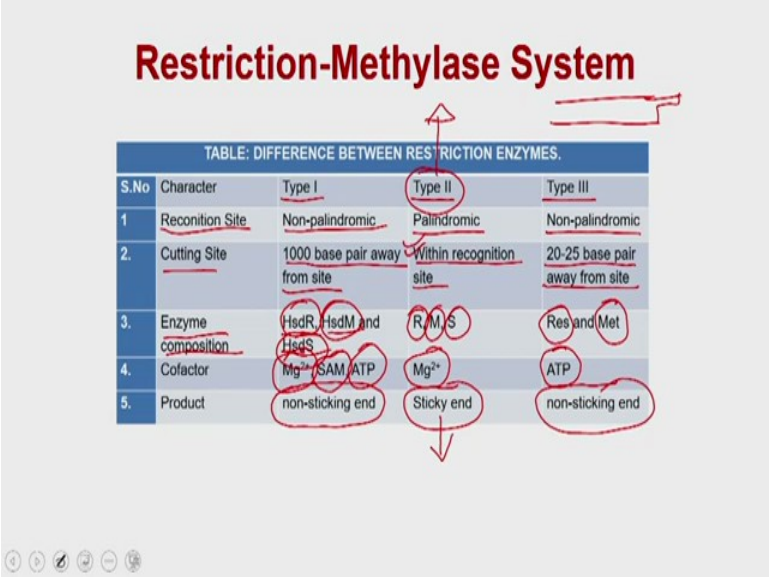

Now let us see what is the differences between the different restriction enzymes so you have the type one type two and type three as well as the recognition side is concerned the type one is non palindromic okay? We will discuss about what is palindromic and what is non palindromic type two is palindromic and type three is also the non-palindromic. The cutting side as we discuss is one thousand base pair from away from the recognition sides where as in the case of type two is within the recognition sides and in the type three it is twenty two twenty five base pair away from the recognition side.

The enzyme composition in the case of type one you have the three sub units one is called hsdr hsdm and hsds and all these individual sub units have their exclusive functions where as in the case of type two you have the rmns subunits where as in the type three you have the two subunits one is called res and the other one is called the met co-factor in the case of type on you have the requirement of 3 co-factors.

One is called magnesium other one is called as sa methionine and other one is called atp where as in the case of type two you required the magnesium and in the case of type you required the atp. The product what is being generated in the case of type one is non-sticky end what is mean by the non-sticky end is that you are not going to generate the sticky fragments where as in the case of type two you are going to generate the sticky ends and in the case of type three also you are going to use the non-sticky ends.

Which means the type three or type two restriction enzyme are actually been having the relevance applications in the molecular cloning because of the only reason that it actually going to recognize within the restriction sides and actually cuts with the recognition site and it generates the sticky ends.

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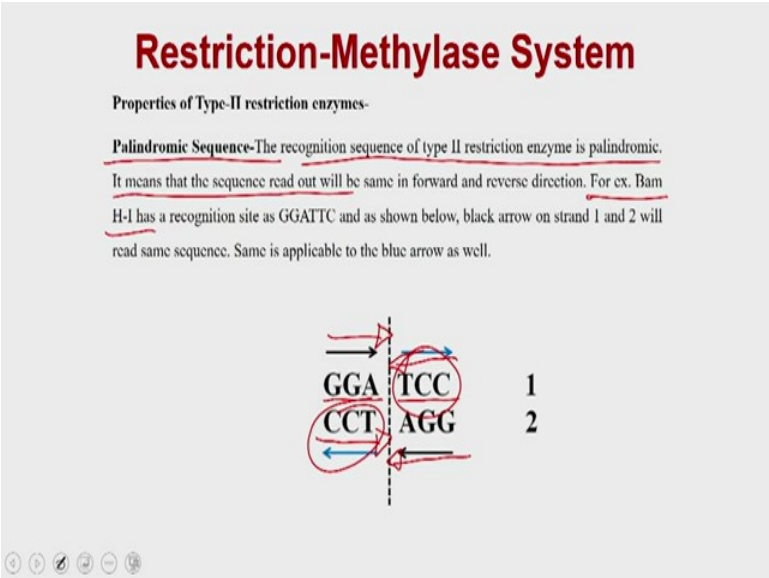

So in the case of type two restriction sides you have the two properties one is that is recognises the palindromic sequences.

The palindromic sequences are the recognition sides for the type two restriction enzyme and what is mean by the palindromic sequence is that the palindromic sequences are you read from the forward or the backward direction they remain as such because as you can see here

this is one of the restriction side which is called gga tcc okay? so in this case what you see is if I reading in this direction or if I am reading in this direction it is actually the same which means that if you cut the recognition sequence from the middle it actually is going to be the mirror image which of each other.

You can see again if it is cct from this side it is actually the cct from this side which means this particular thing is the mirror image of this and that is what is called as the palindromic sequence. Non palindromic sequence means that this is not going to follow. In this case we have taken an example of bam h one which is another restriction enzyme.

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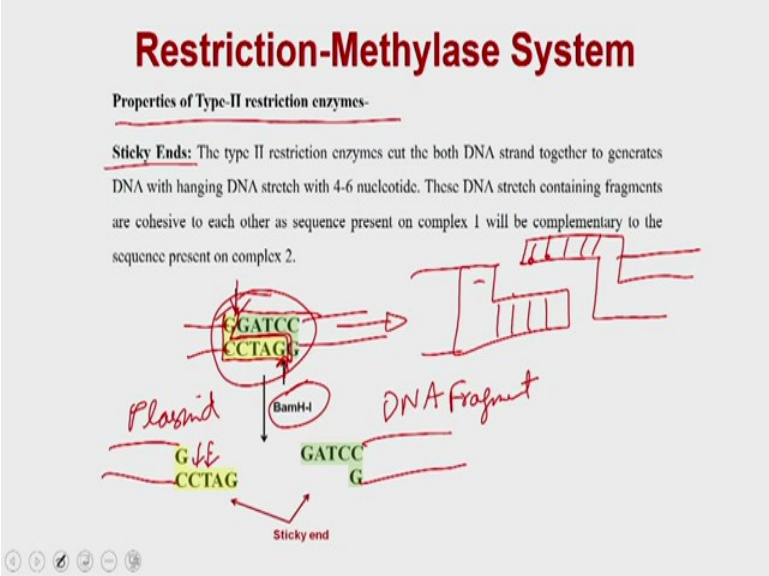

Second thing is that the type two restriction enzyme are also generating the sticky ends what is mean by the sticky end is that if you cut a fragment for example in this case again we have taken a bam h one as an example so you can imagine that you have this DNA which contains this particular restriction side and the bam h one cuts just after the g.

So if you cut just after the g in both the direction what will happen is that this particular fragment will go with this DNA and this particular fragment will go with the other DNA so that all you will see this is the DNA what you are going to have which means that you are going to have a DNA like this in one side the other one you are going to have a DNA like this.

Which means you are actually generating the cohesive ends which means you are actually generating the sticky ends and these sticky ends are going to be complementary to this one. Which means if you have these two fragments and you put them back together they will

spontaneously come together and will bind to each other because the nucleotide what you have here is going to have the complementary to this one.

This one is going to have this one. So they will actually is spontaneously will find and bind the will form the hydrogen bonding and that has a very very big advantage because if you cut any fragment so you can imagine that this actually belongs to the plasmid and this actually belongs to the DNA fragment or the gene and suppose you cut the fragment with bam h one, you cut the plasmid with bam h one you are going to generate this kind of franking sequence or the sticky end on to the plasmid side this is the sticky end you are going to generate on the fragment side and once you put them together.

This particular g is going to have the affinity for c and a is going to have the finite for t and so on. So instant they will come together and they will form this and that is how you are going to insert this particular fragment into this particular plasmid which actually the transforming agents and that is how you are going to generate a chimeric DNA and once you put this chimeric DNA in to the host you will be able to generate the genetically modified organism.

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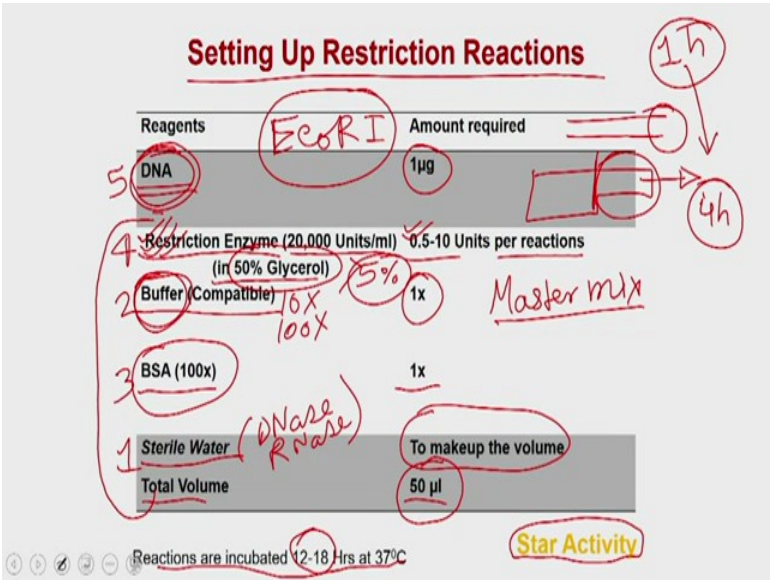

Now let me tell you how to setup the restrictions reactions so in the case of restriction reactions what you are required to have the DNA which you want to cut with the restriction enzymes you can get the one micro gram or you can take more depending upon the amount of DNA what you want as the cutting as for as the final product so you can take the 100 nano grams 200 Nano grams you can take one microgram you can take two micrograms and so on.

Then you have to take the restrictions enzyme most of the companies what they supply is they supply restriction enzymes at a concentration of twenty thousand units per ml and that restriction enzyme is always being supplied in fifty percent this role so that these restriction enzyme should not be freeze when you store them in minus twenty so can add a restriction enzyme which is actually from point five to ten units per reactions then they also going to provide a buffer which is actually where the particular restriction enzyme is going to give the best activity.

That you have to add as one x so the buffer what you going to get is either ten x or the 100 x so depending on what is composition of your buffer you can add the buffer accordingly to make it one x, then they will provide you the bsa which is 100 x and that also you have to add in one x in some of the restriction enzymes not in all the restriction enzyme you have add the bsa but in some of the restriction enzymes you have to add the bsa and then you have to add the sterile water which is actually going to the free off DNA ses and rna ses .

Dna's and rna's are the enzymes which actually degrades the DNA or the rna and you know that DNA is actually cuts the DNA from the xo nucleus. So it actually cuts the DNA from one end of the DNA. So if you have any kind of DNA's or the rna's contamination the DNA is going to chew up the DNA and you are not going to get the final product.

Because once you generate this it actually going to eat it up your sticky end and as a result of DNA contamination you are not going to have this sticky ends once you do not have a sticky ends this particular fragment is not going to be useful for downstream cloning reactions.

Then the total volume so your total volume is the fifty micro liter normally we do the restriction enzymes reactions in the fifty microliter. You will add the sterile water to make of the volume so the sequence in which mostly people add these reactions components are that start with the water then buffer then bsa then restriction enzyme and the last they will add the DNA of interest.

In some of the cases what people do is they also make the master mix which means they actually add all these component from and make up us a cocktail kind of thing and then they dispense that and then add the DNA in to different reactions because many times what happen is that adding the ten unit or 0.5 unit of this restriction enzyme is very very difficult because you cannot dispense 0.2 or 0.5 microliter and in those cases people try to make the master mix of ten reactions 20 reactions and then they will going to dispense eventually and then add the different DNA to set up the restriction side or restriction reactions.

Once you set up the restriction reactions then you increment this restriction reaction at thirty-seven degrees Celsius in the water bath for twelve to eighteen hours what people do normally is that when they suppose they are working with the new enzyme or working with the new particular type of DNA fragment and they do not know how much time it will take what they do is to start with one hour and they go up to the four hours.

To see what is the restriction or what is the cutting they are getting and based on this only they actually optimized the time of cutting. The other thing also important is that whether you are using the single restriction enzyme or the double restriction enzyme, because sometime it happens that you may be looking for cutting a particular DNA with the two restriction enzyme in those cases the buffer what you are going to use.

So most of the companies provide a table in which it is saying that if you are using the combination number one and two you have to use the buffer number one. Or if you use the combination number one and four you have to use the buffer number two. Like something like that. So they also provide the company also provides a table which buffer you have to use for a certain combination of the restriction enzymes.

In some cases, they also say that, for example in the case of eco r one, so most of the enzymes if you are doing a double digestion and you are using the eco r one as an enzyme. What companies recommend that you use the buffer for what is mean recommended for eco r one. Simply because of one point that most of this enzymes like eco r one hin there or bam h one they normally also show the star activity. What is mean by the star activity? Star activity is the activity in which the enzyme actually misbehaves or he does not cut as per its recognition sequence and sometime the other enzymes also show the star activity.

Because of the two reason one that if you have the glycerol concertation the final glycerol concentration more than 5 percent into the reaction. So as I said the enzyme what you are going to get it will be stored in fifty percent glycerol so this fifty percent glycerol reaction you have to use the enzyme in such way that the concertation of glycerol should be less than five percent.

But if it is more than five percent then the enzymes actually start showing the star activity which means they do not recognize the sequence of their own but they recognize some random sequences and then they cut at the random sides. So because of that they are not going to give you the desirable products.

So to avoid the star activity some time the company also ask you to add the bsa. The other parameter which also be in contributing to the star activity is the addition of too much enzyme compare to the amount of DNA what you are adding in this reaction. So if there is a lot of enzyme and very little DNA then also the enzyme is actually also showing the star activity and once you set up the restriction enzyme, the restriction reactions you should avoid the conditions where the enzyme should show you the star activity.

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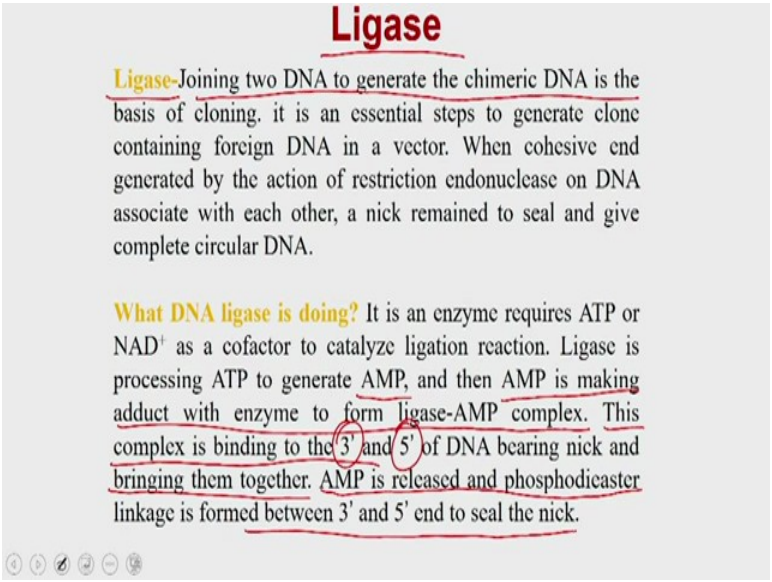

The other enzyme which we also having a role in the molecular cloning is the ligase , the purpose of the ligase is to join the two DNA to generate the chimeric DNA and it is actually being is become one of the essential enzyme for making the cloning possible, so what happen is that the enzyme is having the either using they atp or the ned plus as a co-factor so in both the cases what happen is that enzyme is generating the amp and that amp is binding to the enzyme and that is all the it is making the adept with the enzyme.

And once the it is making an adapt with the enzyme the amp is being transferred on to the nucleotide and then the amp is getting in corporate and it is actually sealing the links or it is joining the DNA fragments. So this is what is written here, that amp is making and adapt with the enzyme to form the ligase amp complex this complex is binding to the three prime and the five primer of the DNA, which actually has the nick and then bringing them together once you bring them the three prime and the five prime end of the DNA they will get form a phosphodiester linkage.

Once the phosphodiester linkage the amp is going to be released and it is going to seal the nick between the three prime and five prime end.

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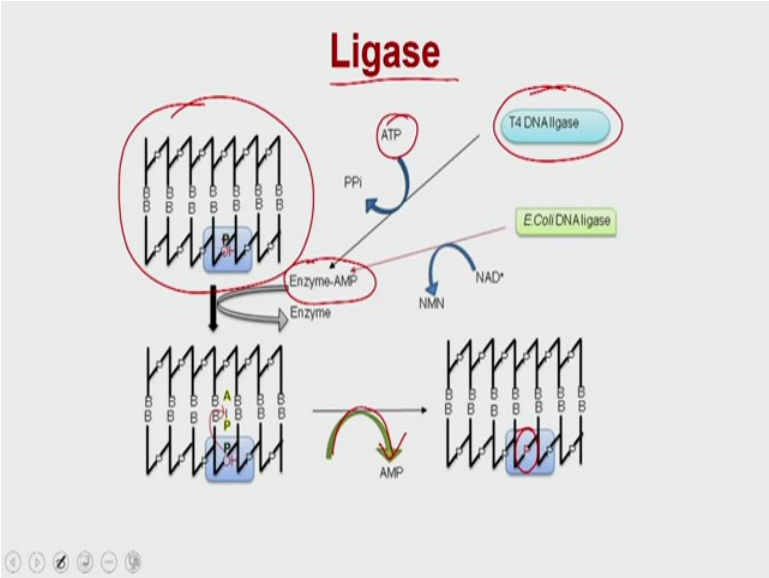

Let us see how it is done suppose this is the DNA what you are putting for the ligation reaction and what you are going to do is add the atp and add the enzyme which the t four DNA ligase and as well as you add that the enzyme amp complex is going to be form and that actually what we will do is the amp will go and bring the phosphate as well as the three prime and five prime phosphate and the oh group together and once they come together the amp will be removed and you will going to have the linkage between this nick. And that is how the ligase is actually jointly the two DNA.

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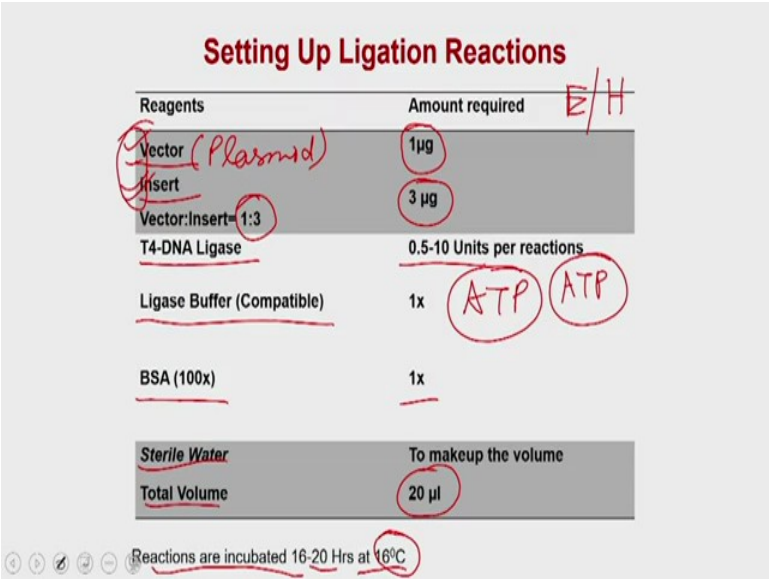

How to setup the ligation reactions for the ligation reaction you are going to have the two different types of DNA, one is called the vector DNA which is actually could be the plasmid

DNA which you have already digested so both are these vector as well as the insert you have the digested with a certain set of restriction enzyme for example you might have the just digested the vector and the insert with the eco r one and henry and the basic understanding about mixing these 2 is that they should be mix in a ratio of one is two three which means if we taking one microgram of vector you will take the three microgram of the insert.

Then you add the t4 DNA ligase, the ligase you will put one and two ten reactions ten units per reactions you will put the ligase buffer, this ligase buffer will contain the atp so you have to store the ligase buffer on to the minus twenty so that they will be enough amount of atp present sometime this atp you have to add the atp externally as well as if the ligation reaction are not working then you also have to add the bsa and sterile water and the total volume.

The total volume in the case of ligation reaction is always been very low and it is twenty microliter compared to the restriction reactions which is actually the fifty microliter. Why it is so, because you want these molecules to interact very strongly with each other that is why you want as lower volume as possible and that is why you are putting the ligation reaction in a smaller volume because, you want this oh and po phosphate group should come as closer as possible and if you put it a lot of water the water will also come and play, will contribute into the reaction. So you want less water you want less volume so that the amount of vector and insert what you are going to add you will have a highest chances of interacting with each other.

Then reactions are also been set up at for sixteen to twenty hour at sixteen degree Celsius so you can see that even the molecular Dynamics also been slow down simply by reducing the temperature if you remember in the restriction reactions we have done the increment as 37 degree Celsius where as in the case of ligation reaction you have to do the increment as 16 degree Celsius, simply because you do not want the lot of moment within the reactions so that these enzymes or the components will get enough time to seal the nick.

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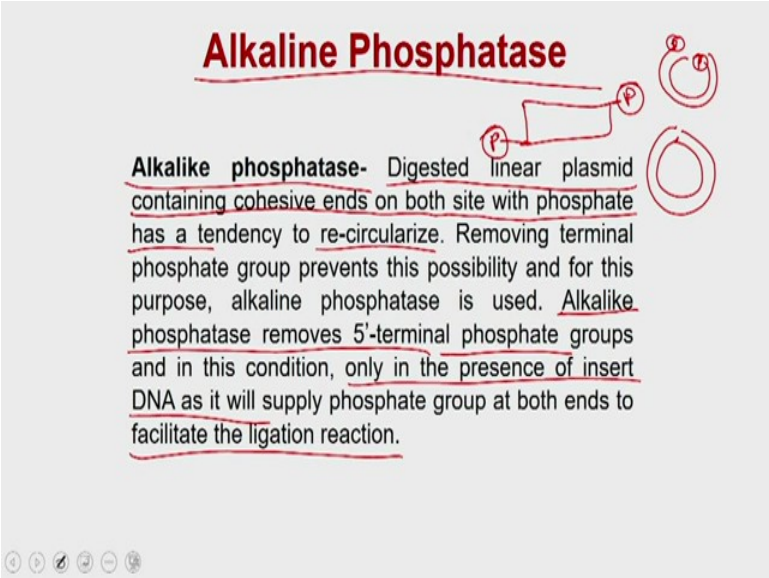

And then the third enzyme is alkaline phosphate, so alkaline phosphate has a very specific sequence or a specific purpose, so what happen is that when you are actually digesting a linear plasmid containing the cohesive ends on both the site and what will happen is that you have the phosphate on both the sites. If you have the phosphate on both the sides what will happen is this phosphate and this phosphate will come together and will re circularize and will not insert to come into this, so you can imagine that if you have a plasmid like this and if you have phosphate on both the side.

Then this plasmid itself will re circularize and it will not going to let the addition of the insert. So what you can do is, you can use the alkaline phosphate is which actually going to remove the five-point terminal phosphate group and in that condition only in the presence of insert DNA which actually going to provide the phosphate and oh both it is going to facilitate the ligation reaction and that is how you are going to get the clone.

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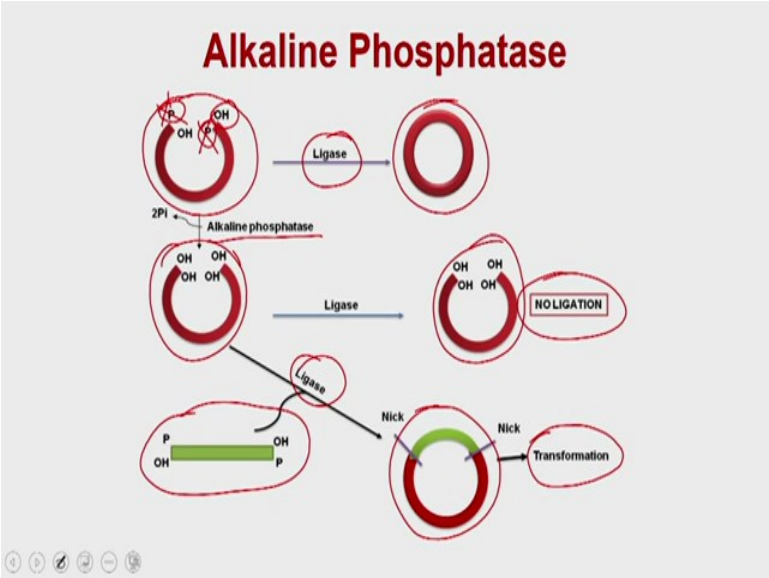

This is what we are trying to say is that if you have a plasmid which actually contains the phosphate and oh both and suppose you put the ligation reactions, this oh and phosphate will come together and will give you the ligation product or the recircularization of the plasmid, where as you want the insert to be incorporated but without having the insert this particular thing is going to re circularize and will give you the false colonies.

Whereas what you can do is, if you take this particular product along with the alkaline phosphate is it actually going to remove the phosphate from the plasmid and it will generate the oh, free oh group on both the things if you put the ligation reaction for this it is not going to re circularize because, you need the phosphate to form phosphate as the linkage.

So there will be ligation reaction but if you insert which will go and will be compatible with this particular type of sticky ends and if you put the ligation reactions this phosphate and this oh will come and will be form a nick and this nicks will be sealed by the ligase and it will give you the cymeric DNA.

And which you transform into the bacteria and it will give you the colonies, so apart from these enzymes which are having the significant role in the molecular cloning, the people have also discovered many types different molecules which are also having the role in the molecular cloning. That also facilitate the insertion of the inserts in to the plasmids or the transforming a genes,

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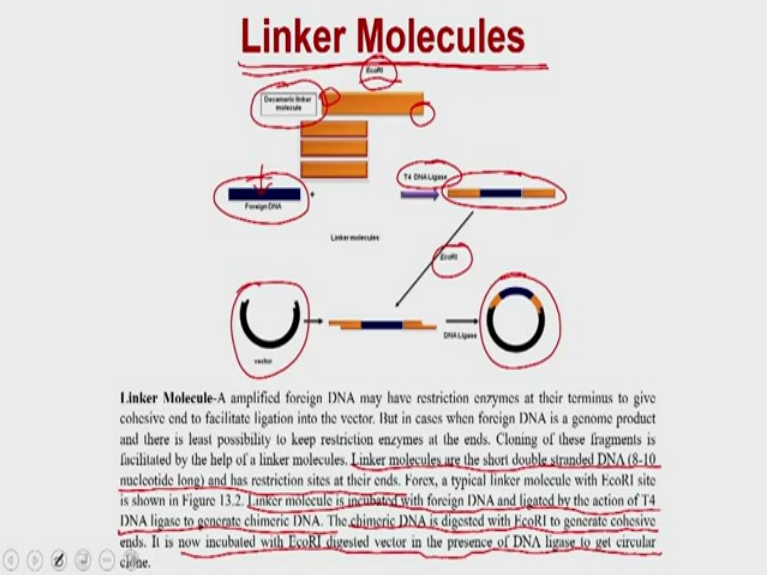

So let us discuss about these additional tools which people have developed to facilitate the cloning reactions, one of such molecule is called as the linker molecules, so linker molecule is being used in those conditions when you are generating a foreign DNA but that foreign DNA does not generate or does not have any kind of restrictions sites so that you could be able to generate the sticky ends or the cohesive ends.

So in those cases what you do is you take the linker molecules and you add those linker molecules to this foreign DNA for example in this case we have taken an example of the chimeric linker molecule which contains the restriction sites for the eco r one. So what we have done is we added this chimeric linker molecule to the foreign DNA which does not contain any restriction sites to generate the cohesive ends on to the corner, then you put a ligation reaction along in the presence of t four or DNA ligase and that will give you a linker molecule attach on both the sides of your foreign DNA.

Now once you treat this foreign DNA with eco r one, the eco r one will cut the sides on to linker DNA and that is how the linker DNA will generate the cohesive end similarly you can cut the vector with the eco r one to generate the linker to cohesive ends and then you take this vector and this modified foreign DNA and put it into the ligation reaction and it will give you the chimeric DNA.

So the linker molecules are short double standard DNA of 8 to 10 nucleotides and has the restriction side at their end, which means you have an eco r one sites on this side you have an eco r one on this side okay? Lnker molecules is incubated this foreign DNA and ligated by the action of t four DNA ligase to generate a chimeric DNA. The chimeric DNA is digested with the eco r one to generate the cohesive ends and it is now incubated with eco r one digested vector in the presence of t four DNA ligase to give you the circular clone.

Apart from the linker molecule in some cases what happen is that the foreign DNA does not have the ability even to take up the linker molecules and the restrictions sides what you are willing to choose are also being present inside the foreign DNA which means if you are trying to use a linker molecules or eco r one, that eco r one is also present within the foreign DNA in those kind of extreme cases what are supposed to do is you will use the another set of molecules which are called as the adapter molecule.

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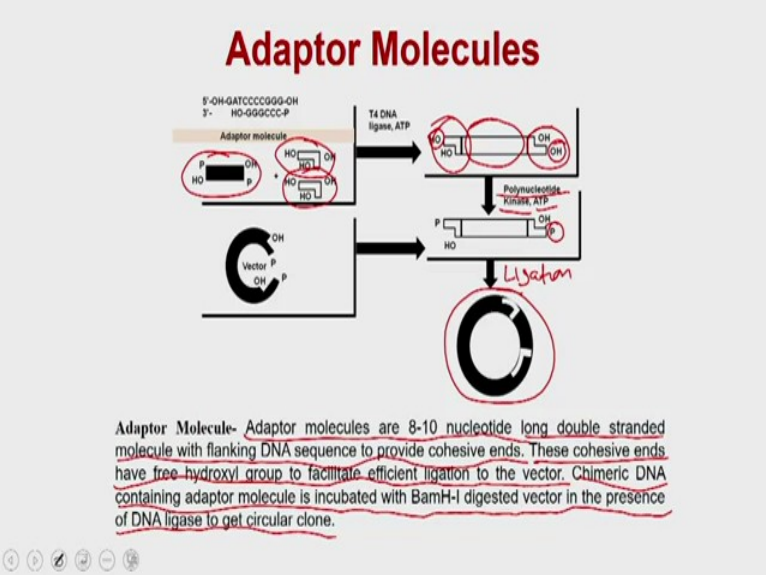

So what are these adapter molecules, the adapter molecules are the 8 to 10 nucleotide long double stranded DNA or double stranded molecule with the flanking DNA sequence to provide the cohesive ends and these cohesive ends have the free hydroxyl group to facilitated efficient ligation to the vector the, chimeric DNA contain the adapter molecule is incubated with the bam h one because in this case the cohesive end what you are going to generate in the bam h one and then if you generate and in cubed along with the t four DNA ligase , it will give you the required DNA.

So what is adapter molecule is that it is actually the molecule of eight to ten base pair long, eight to ten nucleotide long double standard DNA with the cohesive ends or with the sticky ends already being generated. What you do is, take this adapter molecule and put it for ligation reactions these molecules will go and bind on to your foreign DNA. And that is what is given here, this is your foreign DNA on one side it is adapter molecule is being formed. Other site it is another adapter molecule is formed an that is actually the bam h one adapter site and then what you do is you add this along with the…and since, these are containing the oh group you have to generate first rate group.

So that is why they have done, they have use the polynucleotide chines reaction in the presence of atp and what it will do is it will convert the terminal oh group in to the phosphate group because, this phosphate group on to the terminal side in required for participating into the phosphor dieted linkage formation and then you will put the ligation reaction and once you use the ligation reaction it will give you the circular plasmids.

So in today's lecture what we have discussed? We have discuss about the different types of enzymes which are having the role in the molecular cloning and then we have also discuss about the 2 different types of adapter molecules as well as the linker molecule which are also having the role in facilitating the cloning of foreign DNA into your vector of your choice and what we have also discuss about the different types of restriction enzymes their relevance in protecting the bacteria or the macro organism from the foreign DNA and what is the utility of these restriction enzymes in the molecular cloning.

Apart from restriction sides its restriction enzymes, we have also discuss about the ligase as well as the alkaline phosphate is. So with this we would like to conclude our lecture here and in the subsequent lecture we are going to discuss about the different types of vector which you can use for molecular cloning purpose, either it is for the prokaryotic system or the eukaryotic system and then subsequently we will discuss about the how to generate the chimeric DNA and how to insert this chimeric DNA into the host of your choice.

So with this I would like to conclude our lecture here, and in the subsequent lecture we will discuss about the different types of vectors which are available for cloning purposes. Thank You.