Experimental Biochemistry
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Lecture - 44
Basics of rDNA Technology Part – II

So, in today's lecture I am going to talk about the remaining part of rDNA Technology.

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So, today I will go through a one specific example and try to show you how to design a cloning experiment. What I am going to do is a very basic experiment, where we are going to clone a particular gene in to vector and then insert that into a bacterial system. So, I will talk about designing a PCR reaction. So, this is the crux of this experiment and then I will talk about how you do restriction digestion, ligation and finally transform this ligated product into a bacterial cell and as usual I will talk about the practical aspects of these experiments.

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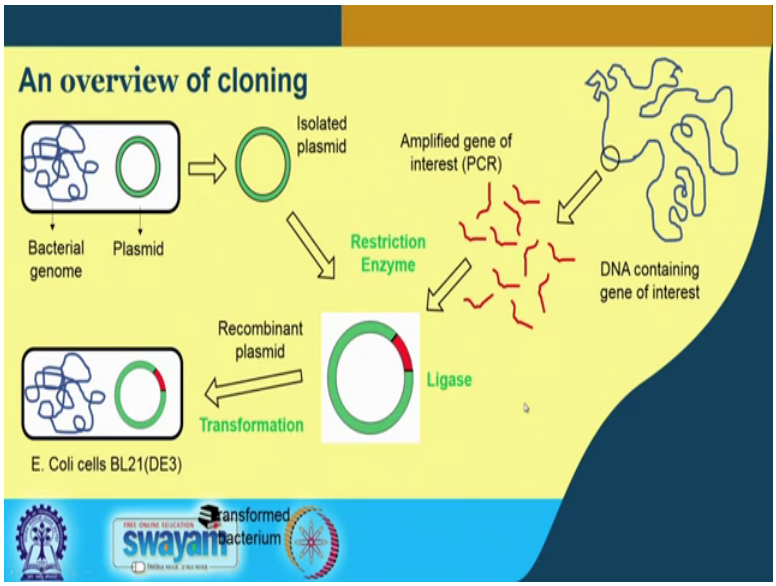

So, this is again the overview of this cloning process. This is the source of the DNA. So, it can be a human cDNA from which we want to extract out a particular gene sequence and that we will do by PCR. So, today we will see how you design primers; so, that you can specifically extract out this particular gene from this cDNA.

We will also get our plasmid or vector from a bacteria and both will be digested using restriction enzyme and then finally, joined together using our glue; enzymatic glue which is ligase to get this vector that will be inserted into the bacteria. So, we will use a particular string BL21 DE3 which we will express the protein and if you want to purify that protein you can do that from here. So, this is our complete cloning experiment.

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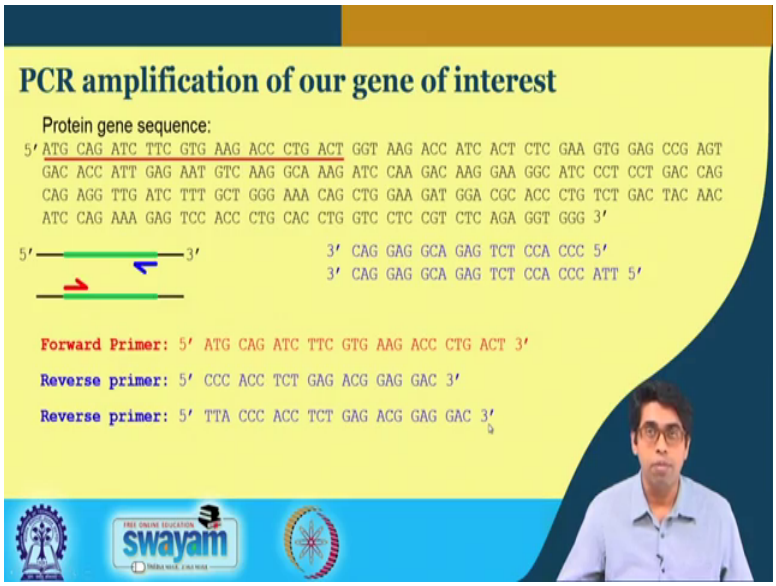

So, here we start with the sequence of our protein. So, the sequence of the protein is shown here. Again this is the 5; so, this is the gene sequence and this is the 5 prime end of the gene and this is the 3 prime end of the gene. And the nucleotide sequence is divided into 3 blocks; so, each of these is a codon with codes for a particular amino acid. So, if you count there are 77 amino acids in this gene sequence. What will and remember that this is part of a much bigger DNA; so, it does not end here in these two regions.

So, this extends in this direction and this also extends in this direction. So, we have to design a PCR reaction so, that we can extract out exactly this part from this very big chromosomal DNA. And we will do that using these two primers. So, again this black lines represent the two strands of this very long DNA and this two green lines represent the gene of our interest. So, the first one the red one is the forward primer which is complementary to the so, this top strand is the coding strand which is this sequence ok. So, the bottom strand is complementary to the top strand or this actual gene sequence.

Now, this primer is complementary to the bottom strand which means that this primer sequence will be exactly the same as this top strand. So, designing this forward primer is very easy, all you have to do is take a sequence from this 5 prime end of your gene and the length of the primer will depend on the melting temperature of that particular sequence. So, we want the melting temperature to be somewhere close to 60 65 degree centigrade between 60 to 65 degree centigrade.

So, in this case what I have done is I have chosen a primer up to this point. So, this is 1 2 3 4 5 6 7 8 9 so, that is 27 nucleotide long primer. For the reverse primer it will be complimentary to the coding strand. So, this is 5 prime, this is 3 prime it means this will be 5 prime and this will be 3 prime and that is exactly what I have written here. So, it will be towards the 3 prime end of your gene and this is the 5 prime end; so, all you have to do is simply do a compliment ok. So, again I have taken a primer which is this long and then if you want to order this primers normally we send them as a 5 prime to 3 prime end sequences.

So, the forward primer sequence is very easy, it is exactly the same as the beginning of your gene sequence. While the reverse primer is the complement and then you have to reverse it because we want to write the 5 prime towards left end and 3 prime towards the right end. So, that is why it is called the reverse complement. So, this is the reverse primer and now you have these two primers for your PCR reaction, but this is not all because you will notice that there is no stop codon at the end of this gene.

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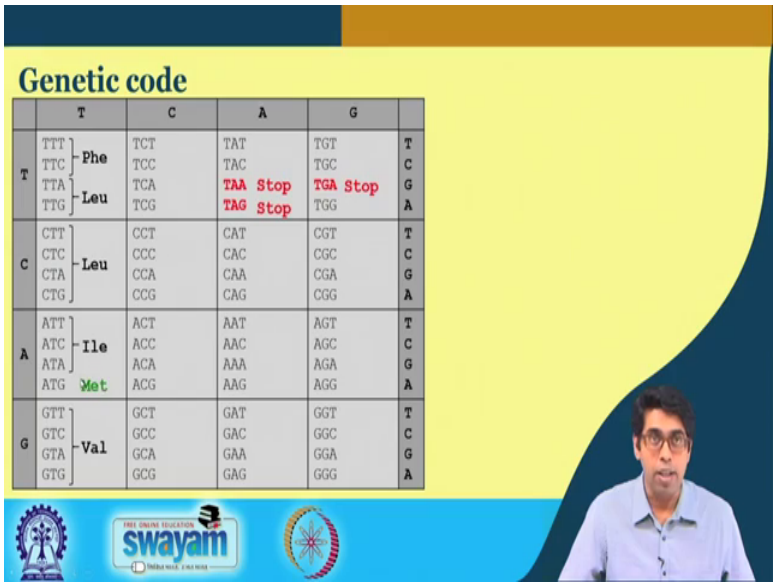

So, where is the genetic code and these are all the codons that are listed remember that the first one is methynin so, if I go back you can see that the first one ATG codes for methynin so that is the start codon. And then there are this three stop codons, but all this three stop codons none of these are present at the end of our gene of interests. So, we

will have to introduce this stop codon and we can use our reverse primer to introduce this stop codon.

So, what I will do is I will use this TAA as our stop codon. So, again that stop codon will come after this last amino acid. So, it should be TAA since I am doing it as a compliment this will become ATT because here it is TAA. So, the compliment is ATT. So now, my primer will have these extra sequences from the 5 prime end which is TTA.

So, now I write my reverse primer as TTA and then this part remains exactly the same. So, now I have using my reverse primer I have added this stop codon which was missing in the original gene sequence.

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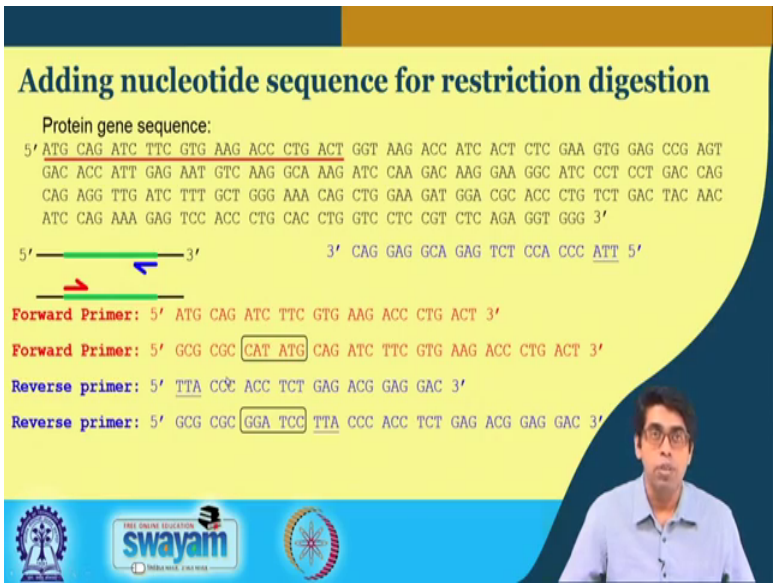

Now, the next so these are the two primers that I have.

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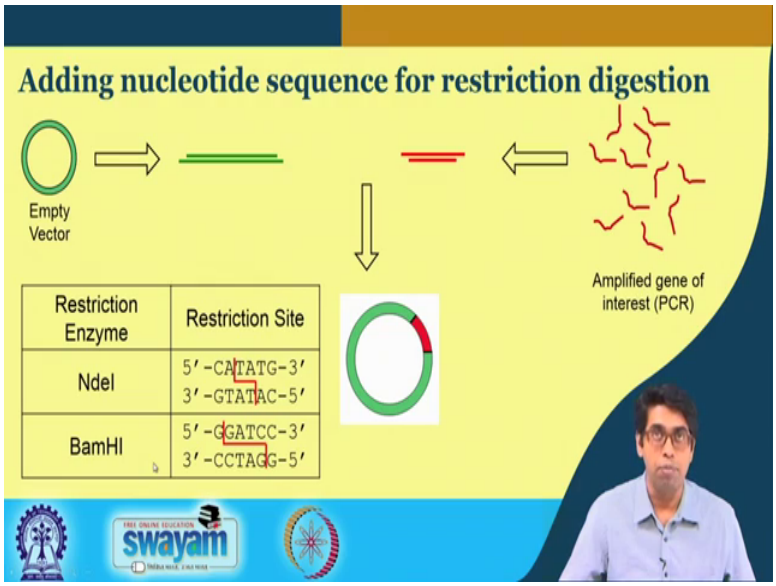

So, again going back to our cloning strategy, once you have the PCR product and once you have the empty vector so, this is we call them; call it as a empty vector because it does not have our gene of interest encoded in it. We will have to ligate this into this, and to do that we have to first digest both the empty vector and the PCR product by a set of two restriction enzymes. The reason we used two restriction enzymes is that then, these restriction enzymes will cut somewhere here which is the multiple cloning site as I discussed in the last lecture. So, the multiple cloning site encodes for different restriction enzymes.

So, if we use two restriction enzymes then what you get is two different overhangs in this end. So, you can see clearly that this overhang is different from this overhang so, that it will not be able to go and complete this plasmid. So, it will not again go back to a circular plasmid. By using the same set of restriction enzyme on our PCR product, we will generate a restriction digested PCR product.

Now, you can see that this compliments very well with this and this compliments very well with this so, that when you put them together, they can form the correct hydrogen bonds and you add ligase to form this circular vector with your gene of interest.

So, in this example if you go through any vector you will see there are so many different restriction enzymes. So, you will have to choose restriction enzymes whose restriction sites do not appear in your gene of interest, otherwise these restriction enzymes will cut

in the middle of your PCR product; so, you do not want that. So, you have to be very careful in choosing restriction enzymes which will not cut anywhere between your PCR product or the gene of interest. So, these sequences should not be present in your gene of interest. So, we have done that and we have chosen these two particular enzymes. So, Nde1 cuts like this; so, there will be a small overhang of two bases and BamH1 cuts this so, that there will be an overhang of four bases.

So, you can see that one side will have two over base over hang and the other side will have four base over hang and they will not be able to recombine. So, that will prevent the spontaneous cyclization of our empty vector once they are digested by this two restriction enzymes. We are going to use exactly the same two restriction enzymes to digest our PCR product. So now, how do we insert these restriction sites in our PCR product? So, again we are going to do that by our designed primers. So, one of the restriction in sites will be encoded before the start of the gene sequence so, towards this 5 prime end and the other one will be encoded after the stop codon so towards this.

So, in the gene it will be towards the 3 prime end but even in the 5 prime end of our reverse primer; so, it will be towards the 5 prime end of both primers. So, here is the now, redesigned forward primer so you will see that, starting from this ATG to this ACT is here, ATG to this ACT. What we have done is we have added these extra nucleotides towards the 5 prime end so, this CAT and these two. So, this CAT and ATG is it codes for Nde1. So, we have the Nde1 restriction site in our forward primer ok.

Similarly, for the reverse primer we are using the other restriction site. So, again from TTA to GAC is TTA to GAC and we have added these two sequences which codes for the other restriction enzyme which is BamH1 GGATCC GGA TCC and then again we have this extra sequences. So, you will see that this is GCG CGC. So, GC's form three hydrogen bonds and that stabilize the annealing of the primer with your DNA sequence. So, these so we put this GC clams towards the end of our primer. So, that the annealing is much better.

So now we have our forward primer with the design restriction site and the reverse primer with a stop codon and another restriction site. So, now, you are ready to do the PCR experiment.

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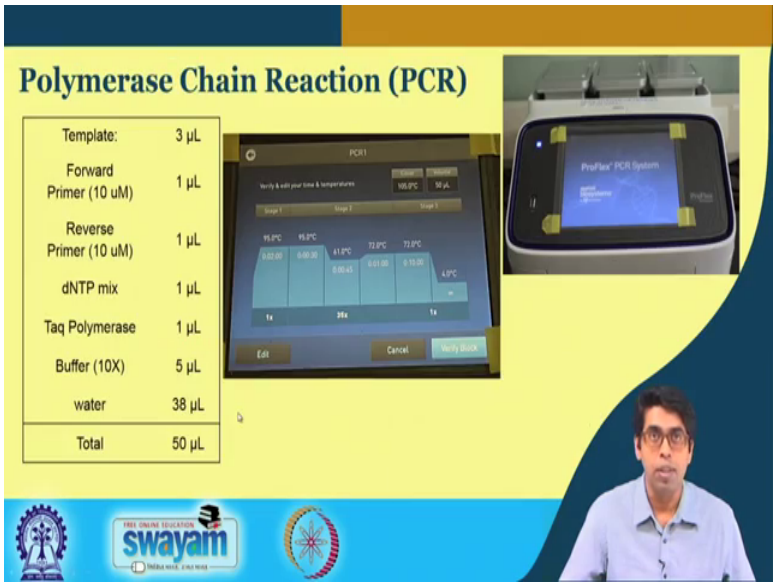

So, here we have a typical PCR setup so, this is a PCR thermocycler. So, all this instrument does is basically it will go through different temperatures in a cyclic fashion. The way you will setup your experiment is you mix all this different components in a small append of tube or any tube any PCR tube these are the standard size is 200 micro litre tube and then you make up a total volume of 50 micro litres.

So, out of that roughly thirty eight micro litre is water. So, remember you start with this and then you add the 10X buffer, then you add your template the two primers, dNTP mix. So, dNTP mix is the mixture of ATP, GTP, CTP and DTP these are the monomers that make the DNA molecule. And in the last step when you have added everything you add the polymerase which is enzyme; so, that polymer is the one which is thermostable, you add it at the end and then you put it in your PCR instrument and start the reaction. So, this is again one of our a typical PCR cycle that we use. So, it goes in 3 steps.

The first one is where the temperature is increased to 95 degree centigrade. So, at this high temperature all the DNA molecules are denatured meaning the double strands come apart. So now they become single stranded. So, this is just done once and then these 3 steps are repeated 35 times. So, in these 3 steps the first one is again denaturation; so, we heat our reaction mixture for 30 seconds. So, that all the DNA molecules come apart there is no hydrogen bond between them.

Now, you cool it down to 61 degree centigrade, this is the annealing temperature and you have to be careful about this annealing temperature. So, this is something that you set based on your primer sequence. So, there are many websites where you can put your primer sequence and it will tell you the melting point of your primer. So, based on the melting point of the forward and the reverse primer, you will set this annealing temperature and that is set for 45 seconds.

And then the temperature is raised to 72 degree centigrade which is optimal for this polymerase enzyme. So, the temperature is raised to 72 degree centigrade and it is run for 1 minute. Now, again this timing is set based on the length of your gene of interest. So, every for every enzyme it will tell you how long it takes to synthesise a particular length of a DNA.

So, based on that information you will set this elongation time and then this cycle is, these 3 steps are repeated 35 times and finally, we have a longer elongation time. So, that all the products which were half done will get completed and then you bring the whole system down to 4 degree centigrade. So, a typical reaction for this type of cloning will take somewhere between 1 and half hours to 2 hours. So, you can set up this experiment, wait for 2 hours come back and then you have to check whether your PCR experiment worked or not. To do that, we have to go back to gel electrophoresis.

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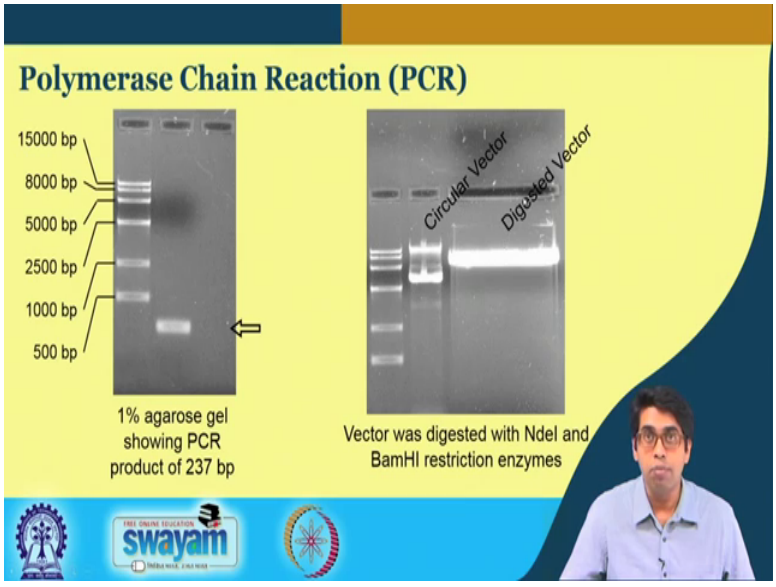

So, once we have done PCR using these two primers, we have again a colourless liquid and to check whether our PCR reaction worked, we have run this agarose gel. Remember this is DNA; so, for DNA we use we have to use agarose gel and these are much bigger DNA molecules.

And since, we want to look at a PCR product which is very small, the expected length of our PCR product is 237 base pair. So, that is why we have used a dense agarose gel so, it is a 1 percent agarose gel. This first length is the molecular standard, molecular weight marker and you can see all the molecular wrights are listed here and this, in this length we have run our PCR reaction; a small part of our PCR reaction. And you can see that there is nothing else and there is a very clean band which means that the reaction has worked really well and it is less than 500 base pair. So, it is 237 base pair that is expected.

So, most likely this is the correct product. So now, we can go ahead with this and do the restriction digestion. So, you have to take this PCR product add the two restriction enzymes incubate it for some time so, these are all prescribed by the enzymes where that you buy it is there it will be in the enzyme catalogue and then you will have your restriction enzyme digested PCR product. The other part that you need is the circular vector which should be also digested by your restriction enzyme. So, you have to set up two reactions of restriction digestion one is for this PCR product and then the other is for your circular vector and whether that reaction worked or not is again tested by another agarose gel electrophoresis.

So, here another agarose gel is run is shown. So, you can see that the same molecular weight marker is run here this length is the intact circular vector and this length is the digested vector. So, this lower band originates from this circular vector because since it is circular is actually runs faster and when this vector is cut open it becomes slower. So, it is migration becomes slower and you get a band which is slightly shifted upward. You will notice that these lengths are much smaller and this length is much bigger. So, what has been done here is 2 or may be 3 lengths like this have been joined together to create a much bigger length.

So, that all of the reaction product can be run at the same time, the reason we do that is because when you set up a digestion reaction you will have it will not be 100 percent. So,

in this gel we actually see a single band we do not see a band down here. So, it is almost very close to the completion, but in many cases you will see two bands which means that the digestion reaction is not complete.

So, in that case you will have to separate out these digestive vector from the undigested vector because if the undigested vector is there then it will also get transformed into your bacterial cells and there will be no way you can tell which cells has which cell has your vector with your gene of interest and which cell has the empty vector. So, in that case if you see two bands then you will have to cut out this band and purify the digested vector from this band. So, that is why we run a gel where we fuse several lengths together and run all the digested product. So, this band was cut out and purified using standard kits where you can extract the DNA from this agarose gel.

So, now, you have your digested vector, you have your digested PCR and you can put them together.

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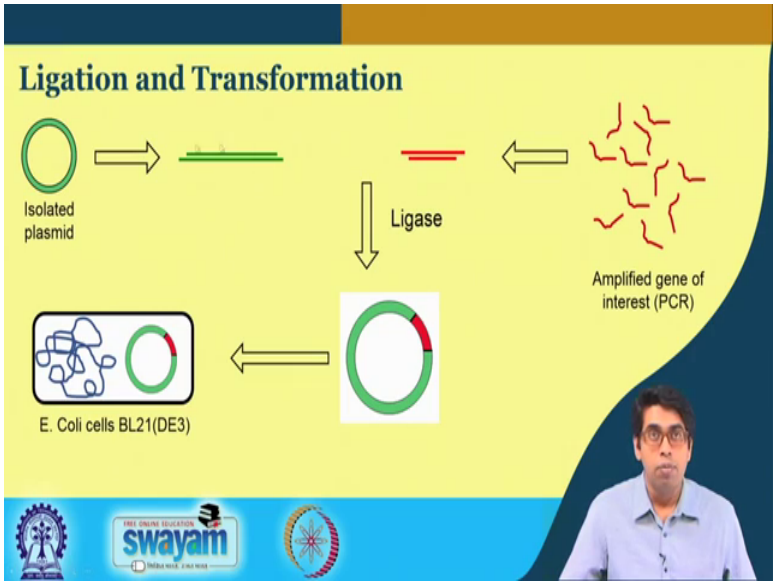

So, we have our digested vector, we have our digested PCR product, we can add them together and add our ligase and again this reaction takes time some time. So, depending on the enzyme that you use you will have to set up a reaction. And it is always advisable to use different ratios of the vector and the PCR product and set up at least two and if possible 3 or 4 reactions with different ratios, because you do not know which one is going to work. And then, you take your ligation product and transform it into bacterial

cells. And you plate those bacterial cells so, the way this is done if you remember from the last lecture, these vectors have a selectable marker.

So, in this case let us say the selectable marker is kanamycin. So, only those bacterial cells which take up this vector will have that selectable marker. So, in our agar plate we have we add kanamycin. So, the equalise cells which have this vector will survive and all other cells which do not have this vector will die off. So, you transform your bacteria with this vector, you plate it on another plate put it in an incubator at 37 degree centigrade overnight and come back next morning to see whether your whether you get colonies.

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So, there are two types of bacterial cells that we use, one is DH5 alpha and the other one is BL21 lambda DE3. So, the DH5 alpha bacterial cells are the ones which are used when you do this cloning reaction. So, you will add your ligation product to these bacterial cells plate them and next morning when you come back, you see this white spot these are the colonies that have grown overnight.

So, these are the cells which took the plasmid in and have survived in this agar plate which also has kanamycin because the plasmid has the selectable marker. So, then you pick one of this colonies, you can run a PCR again with the primers and if that PCR works you know that the plasmid is inserted in this and then you can keep that as a stock in the lab.

This type of cells are typically used to maintain a plasmid library because they; so, these we do not use these DH5 alpha cells to make our protein, to make proteins to over express the protein, we use a different cell which is BL21 lambda DE3. So, you have to extract out the vector from these bacterial cells and transform them into this type of cells and again, the process is same you wait incubate it at 37 degree centigrade overnight; next day, you come back and you will see colonies like this and you can pick one of these colonies and then you have your clone, that you can use to express your protein of interest. So, all the other experiments that you want to do will be done using this clones that is picked from a plate like this. So, this is the overview of our cloning experiment.

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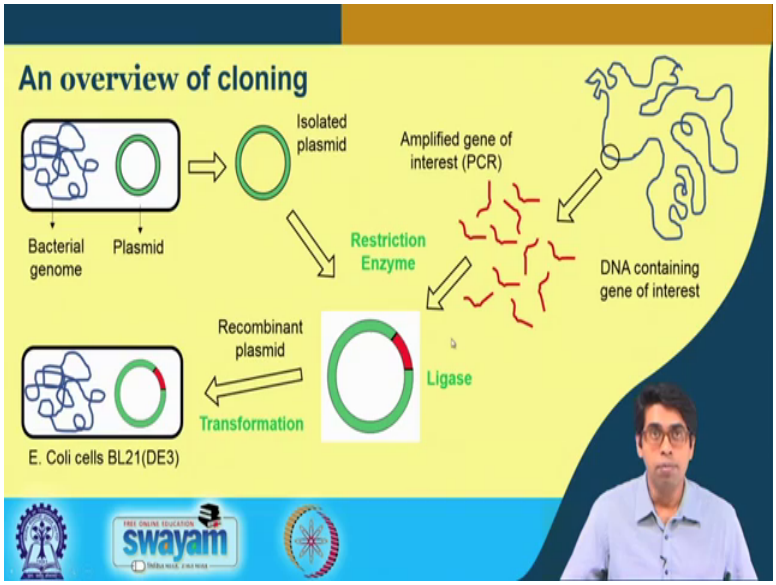

So, today what we have done is we have designed the PCR experiment; we have designed primers to do the PCR experiment. So, that we can extract out our gene of interest and the way we design the primer is such that we extract out the gene of interest, we amplify it, we also encoded a stop codon and we also designed sites for restriction digestion, And then, we used that two restriction enzymes, to digest our PCR product, digest our vector then, we used ligase to ligate this to digested products and then transform them into our bacteria of interest for production of protein.

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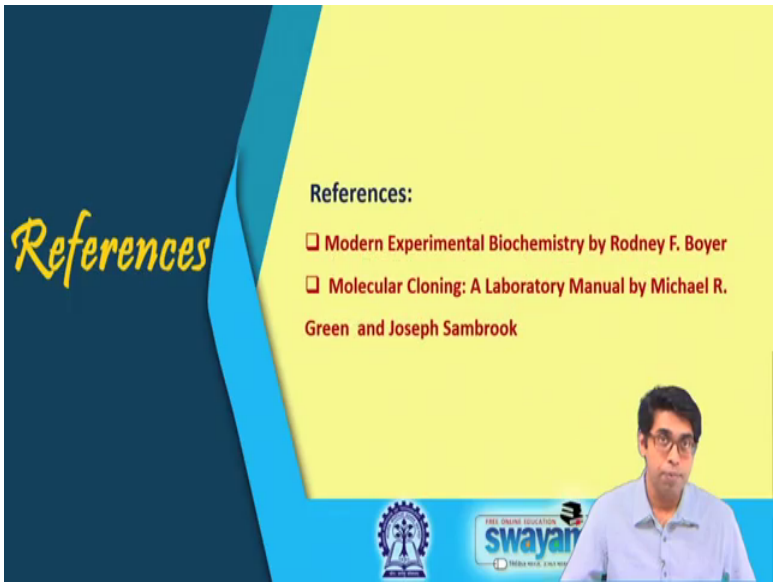

So, again these are the books that we have followed and.

Thank you very much.