Enzyme Science and Technology
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Module - III
Enzyme Production (Part 1: Cloning)
Lecture - 14
Polymerase Chain Reaction

Hello, everyone. This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And, what we were discussing? We were discussing about the different aspects of the Enzymes in the course Enzyme Science and Technology.

So, far what we have discussed? We have discussed about the History of the Development of Enzymology, then we in the previous module we have discussed about the Structure of the Enzymes and how we can be able to determine the primary structure, secondary structure, tertiary structure and quaternary structures.

And, if you recall in this particular module we are discussing about how you can be able to isolate the gene of your interest, so that you can be able to use that gene to clone it into a suitable vector and that can be used for the protein production or the enzyme production.

So, if you if you recall in this particular in the previous two lectures we have discussed about the two approaches. In approach 1, we have said that you are we are going to prepare a genomic library, so that the library is going to represent the every gene in that particular in the form of the clones and then you can use the different screening method to isolate the gene of your interest.

The alternate approach is that you can actually be able to prepare the CDNA library, and CDNA library is going to represent the expression status of that particular cell. So, and then you can be able to screen the CDNA library with the help of the antibodies or the enzymatic method. And, you can also use the DNA as a probe as well to screen the CDNA library and that is how ultimately you are going to get the gene of your interest.

And, then you can isolate the gene of interest from the library and then you can use that for cloning into the suitable vector, so that you can be able to use that for protein production or the enzyme production. These are the approaches when people is very popular when people were not having the information about the genes or the genomic sequences.

So, before the people pre genomic era when the people were not aware of the genomic sequences the utilization or the exploitation of the genomic library or the CDNA library was very very popular, but now since the we normally most of the time we normally use we know the genomic sequence of the organisms.

So, we can be able to use the straightforward approach and that is called as the polymerase chain reaction. So, we can actually be able to amplify the gene with the help of the polymerase chain reactions and that is how you can be able to use that amplified product and you can clone it into the vector and that is how you can be able to get the purified enzyme.

So, in today's lecture we are going to use and we are going to discuss about the polymerase chain reaction.

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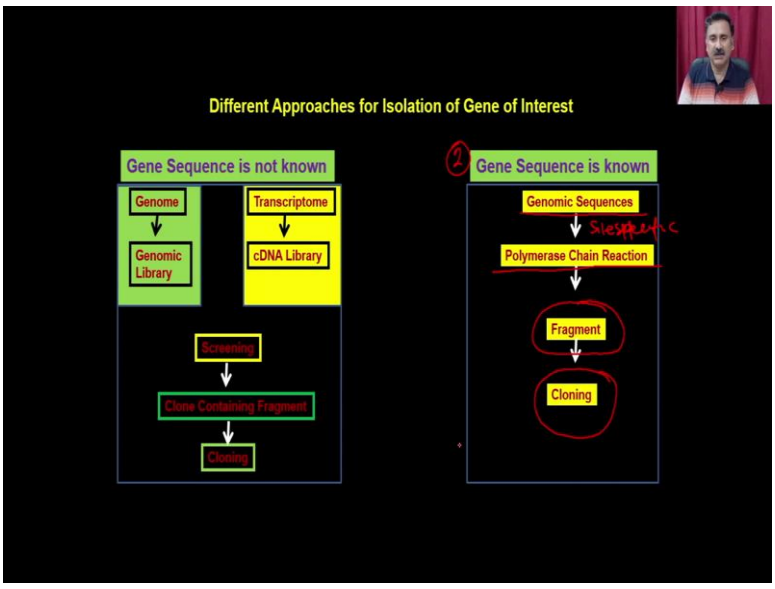

So, these are things we have already discussed. We have discussed about when the genomic sequence is not when the genomic sequence is known or the approaches when

you are going to use the gene sequence is known. So, in the when the gene sequence is known that is the approach number 2, you are going to take the genomic sequence, you are going to design the site specific primers and that is how you are actually going to get the you are going to perform the polymerase chain reaction and that is actually going to give you the amplified product.

And, that is how this amplified product can be cloned into a suitable vector and that you can be able to use for the you know for the enzyme production.

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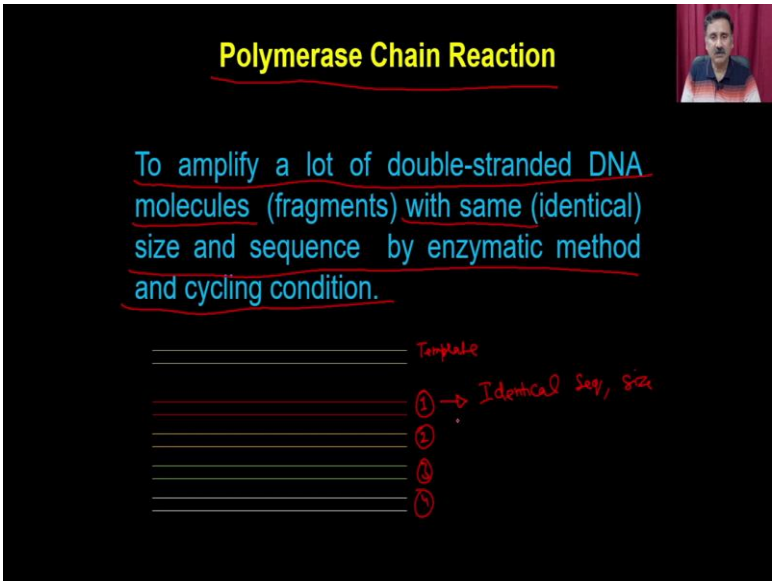

So, now, the first question comes what is the polymerase chain reactions? So, polymerase chain reaction is are the reactions which are actually going to help you to amplify a double stranded DNA with the same size and as the sequence by the enzymatic method and the cyclic conditions. So, what is polymerase chain reaction? Polymerase chain reaction is to amplify a lot of double-stranded DNA molecules with the same size and the sequence by the enzymatic method and the cycling.

So, for example, if this is the template DNA, then with the help of the polymerase chain reaction you can actually be able to make the multiple copies of the template DNA like this is the copy number 1 2 3 4 and all these four copies are actually going to be identical in terms of the sequence and as well as in terms of the size.

So, how you are going to achieve this? You are going to achieve this because the DNA is a very unique molecule and DNA itself provides you the information and as well as the tools to perform this particular task.

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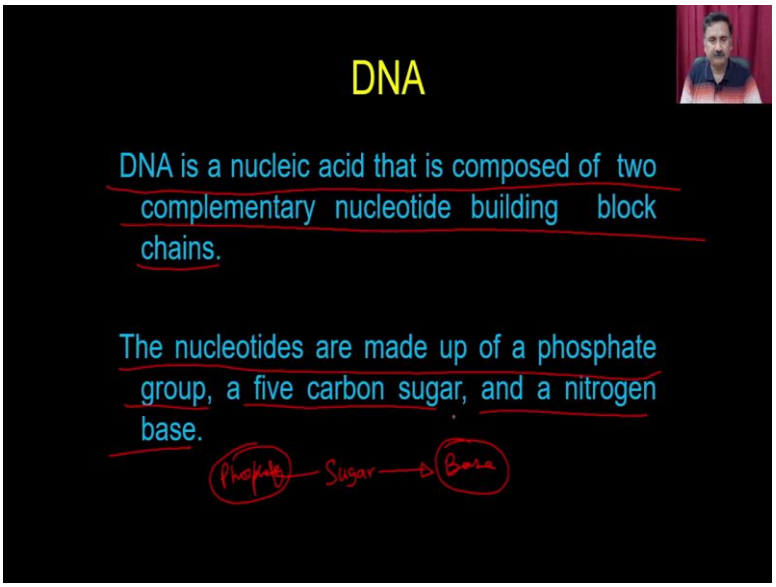

So, what is DNA? DNA is a nucleic acid that is composed of the two complementary nucleotide building blocks chains, right. You know that the DNA is double helix and it is made up of the nucleotides and the nucleotides that are made up of the phosphate group a five-membered carbon sugar and a nitrogen base which means a nucleotide is consist of the five member sugar, right.

And, the sugar is attached on one side it is attached to the base and on the other side it is actually attached to the phosphate group. And, this is actually going to help in formation of the DNA. So, you are actually going to have the different types of nucleotides which are actually going to be responsible for the DNA formation.

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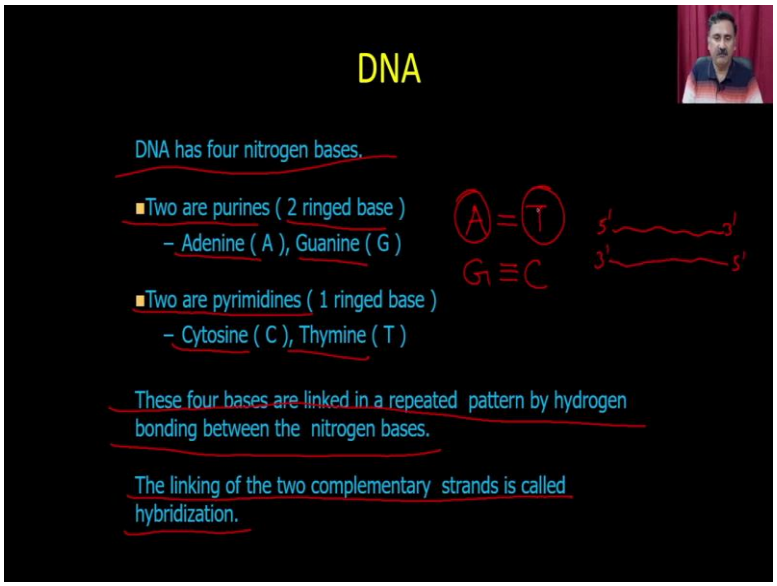

So, DNA has the four nitrogenous bases you have the two purine bases which are called 2 ringed bases. These are called adenine and guanine you have the two pyrimidines. So, these are called cytosine and thymine, and these four bases are linked in a repeated pattern by a hydrogen bonding between the nitrogen bases. The linking of the two complementary strand is called as the hybridization.

And, I am sure you all know that there is a strict base pairing between the purines and pyrimidines and that is why A is always making a pair with T with the help of the two hydrogen bonding whereas, G is making a pair with C with the three hydrogen bonding. So, every purine is making a bond with the pyrimidine.

And, so, because of this particular unique character of the DNA molecule, the molecules can be complementary to each other which means if you have a sequence on one strand for example, if you are having a strand from 5 prime to 3 prime, the second strand is actually going to be predicted based on this base pairing informations.

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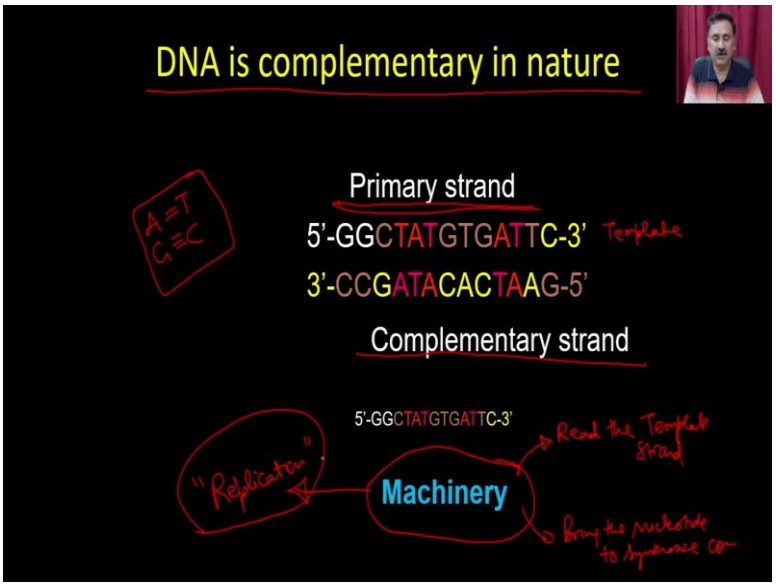

So, DNA is complementary in nature, right. So, if you can have the primary strand so, this is going to be running strand. Suppose, you can imagine that this is a template strand. So, this is the; this is the template strand, this is going to be the complementary strand.

So, wherever you have the G, it is going to have C and wherever you have the C, it is going to have the G; wherever you have T, it is going to have A and wherever you have A it is going to have the T which means wherever you have A it is actually going to have T and wherever you have, G it is actually going to have C because the A and T and G and C are having the strict relationship or strict base pairing informations.

So, what we are supposed to do is you are going to have the primary strands right available and then you are only you have to generate the complementary strand. This means you are looking for a machinery which actually be able to perform this particular task. So, what this machinery is going to do is, it is actually going to read the template strand and so, it is going to read the template strand right and then it is actually going to bring the nucleotides to synthesize complementary strand, ok.

So, this is what you are supposed to do, right and this machinery is readily available in the biological system. You remember that when we when the DNA is replicated, it is actually going to be performed you utilize this particular machinery for replications. So,

taking the inspiration from the biological system right people have also discovered the machinery which can be used under the in-vitro conditions.

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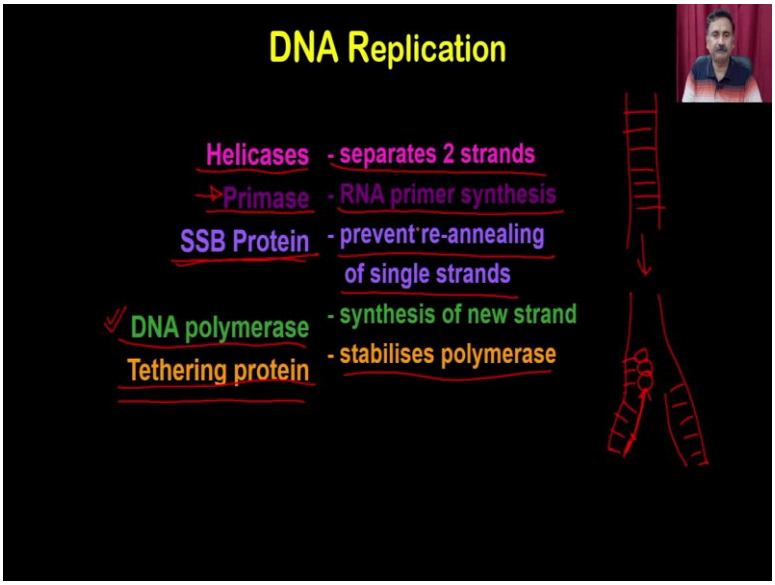

So, if you see the biological machinery, biological machinery had the different components: you have the helicases, you have the primase, you have the SSB proteins, you have a DNA polymerase and then you also have the tethering proteins. And, all of these machine proteins which are part of the machinery are having a designated specific role. For example, helicases. Helicases are going to separate the two strand because you know the DNA is double stranded and it is connected to each other.

So, in the step one you are actually going to make the two strands separate, so that the base pairs are ready and these base pair once these base pair exposed then only the you know DNA polymerase can be able to read this information and that is how it can be able to synthesize the complementary strand, right.

Then you have the primase. So, the purpose of the primase is to synthesize the RNA primer because the DNA polymerase cannot start from the nascent chain. So, it requires partially double bond structure, so that it can be able to use that information and then extend the DNA strands then you require the SSB proteins. So, SSB proteins are the protein which actually will prevent the reannealing of the single stranded DNA.

This means all these because once you once the helicase is going to generate the single stranded DNA the single stranded DNA will automatically will come and bind to each other. So, that is why the single stranded proteins are actually going to sit on top of this, so that it will not allow the nucleotides to interact with each other and that is how they are actually going to prevent the reannealing of the single strands.

Then you require the enzyme, the DNA polymerase which actually going to read these sequences and that is how it is actually going to start the synthesis of the new strands. And, then you also require a tethering protein which actually going to stabilize the polymerases. So, in taking the inspiration from the biological system, people have also started designing the in vitro reactions so that you can be able to perform the same task without utilizing the so many different types of enzyme.

Because coordinating these enzymes within the cell is a very very easy task because you have lot of you know regulatory proteins and all other things so that you can be able to regulate the activity of helicase, primase and SSBs and DNA polymerase as well. But, when you are trying to do this in the in vitro reactions it is very difficult to do that and that is why you have to you know bring the machinery in such a way that you can actually be able to replace some of these functions.

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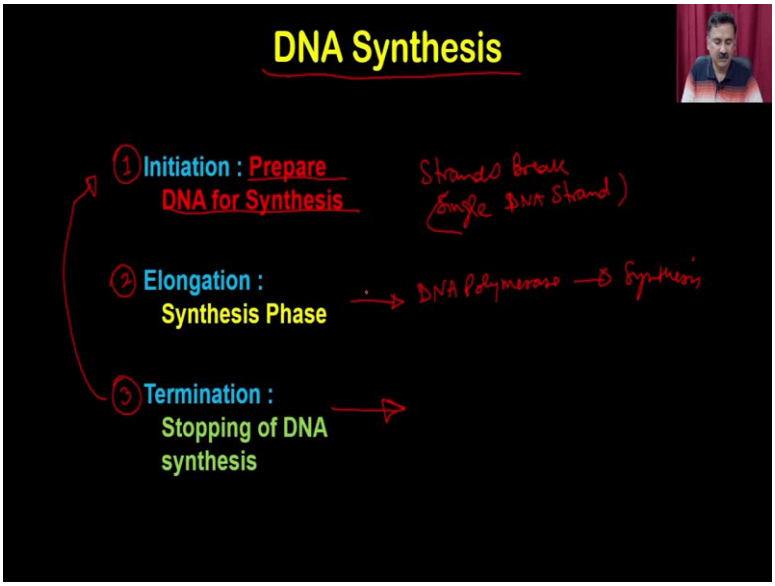

So, ideally if you want to do a DNA synthesis under the in vitro conditions what you are going to do is you are going to have to perform these three steps under the in a repeated

manner, right. After this termination you are again going to do this and then again doing like this, ok. So, this you have to do in a cyclic manner. So, initiation you are going to prepare the DNA for synthesis which means you are going to break the strands, right.

So, strands you have to break the strands, so that you are going to get the single DNA strand. So, in the step one you are going to bring the single DNA strands, right. Then, you are going to bring the DNA polymerase and it is actually going to give start the synthesis. So, that is going to start the synthesis and in the step 3 you are going to stop the DNA synthesis, ok.

So, these tasks were achieved not in a single day, but there was a concerted or there are lot of efforts people have made then only they could be able to get to the machinery which actually can be able to perform these task. So, these are the historical development in which the PCR is being developed.

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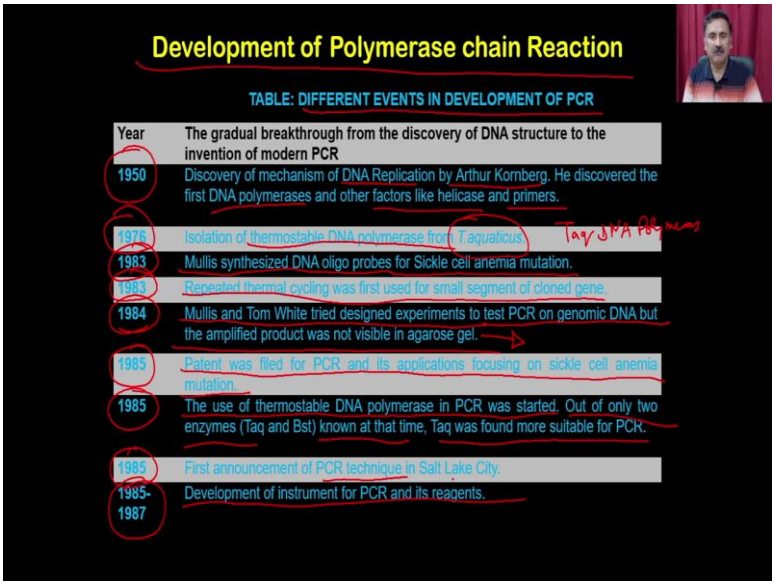

So, there are different events in the development of PCR. For example, in the year of 1950 the people the Arthur Kornberg actually discovered the DNA replication and that is how we could be able to know that what are the different proteins are involved and how the DNA polymerase is synthesizing the DNA. So, he actually discovered the DNA polymerase and helicases and primers and that is how we know that there is a you know all these enzymes are involved.

Then in the year of 1976 the thermostable DNA polymerase from the thermus aquaticus was discovered which is called as tag DNA polymerase. And, and then in the year of 1983, the Kary Mullis synthesize the DNA oligo probes for the sickle cell anemia mutations. And, in the same year repeated thermal cycling was first used for small segment of the cloned gene and that is how the people have this thought that maybe we can do the repeated cycling and that is how we can be able to amplify the small stretches of DNA.

And, in the year of 1984 the Kary Mullis and Tom White tried the different designed experiment to test the PCR on the genomic DNA, but the amplified product was not visible in the agarose gel because of the simple reason that the efficiency was not very high.

Then in the year of 1985, the patent was filed to the PCR and its applications focusing on the sickle cell anaemia mutations. And, in the year of 1985 the use of thermostable DNA polymerase in PCR was started out of only two enzymes Taq DNA polymerase and Bst known at that time.

So, Taq was found more suitable for the PCR because what will happen is when you go for this timer cycling, it actually denatures the enzyme. So, that is how you have to keep adding the enzyme every reaction every cycle. So, when we people have discovered the Taq DNA polymerase the that problem was being overcome. Then in the year of 1985 the PCR techniques was discovered, and then in the year of 1985 to 87 people have also discovered the thermal cyclers.

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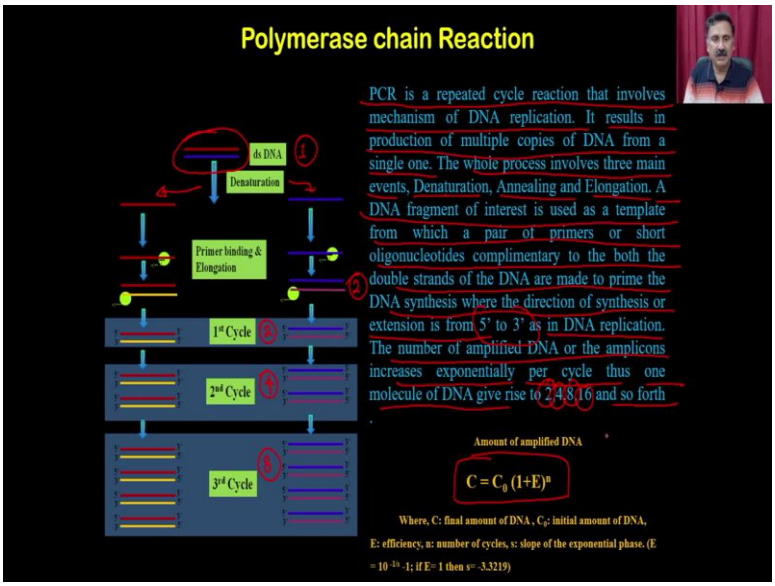

So, in a typical PCR what happened is that PCR is a repeated cycling reaction that involves a mechanism of DNA replication. It results in the production of multiple copies of DNA from a single one. The whole process involves three main events – denaturation, annealing and elongation. So, denaturation is the first step which is actually very close to as initiation step; then annealing where the primers are going to anneal and then the DNA synthesis will start so, that will be and then we are also going to have elongation.

DNA fragment of interest is used as a template from which a pair of primers or a short oligonucleotide complementary to both the DNA strand are made to prime the DNA synthesis where the direction of the synthesis or the extension is from 5 prime to 3 prime as in a DNA replication. The number of amplified DNA or the amplicons increases exponentially per cycle. Thus, one molecule of DNA will give rise to 2, 4, 6, 15 and so forth.

And, if you want to calculate the amount of amplified DNA, you can actually be able to use this formula. So, what happened is that it started with a single template. So, you have a single template in the step 1, there will be a denaturation. So, denaturation is actually going to separate out the template and you are going to get the two in single strands, right.

And, once the single strands is there then you are going to allow the you know you lower down the temperature. So, that is why you are going to allow the binding of the primers

and once the primer is bound the DNA polymerase will sit and it is actually going to synthesized the second strands. So, for one template you are going to get two fragments and from the two on the two fragments you are going to have the synthesis of the strands. So, this means you started with the one fragment and now you are going to have the two fragments.

So, at the end of the two cycle you are going to have the two fragments one of this and one of this. Now, in the second cycle this one is also going to serve as a template, this one is also going to serve as a template and that is how you are going to have the four molecules which are going to be synthesized. And, that is how in the third cycle you are going to have the 8 what is going to be synthesized and so on.

So, this is actually going to give you the exponentially increasing number and that is how the amplification is going to be very high in few reactions.

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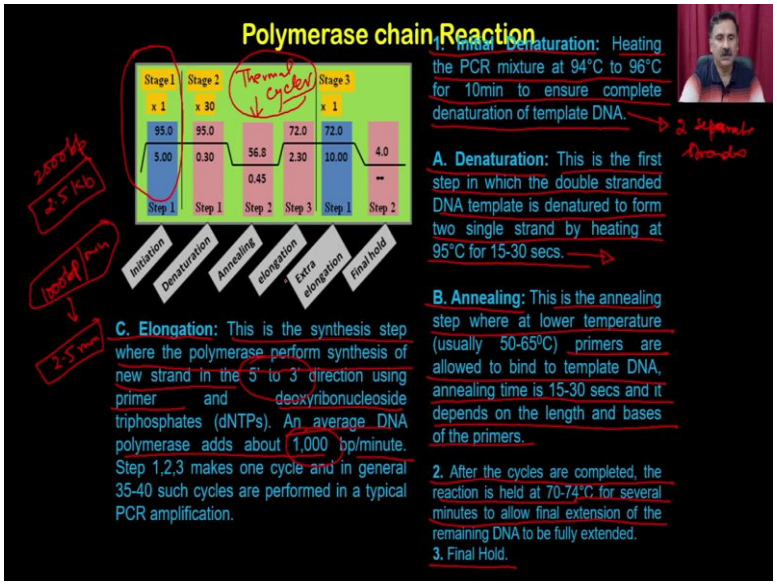

How you are going to perform the PCR reactions? So, you are going to set up the PCR in the following steps, right. First is you are going to have the initial denaturation. So, this is the initial denaturation in the stage 1, right. And, initial denaturation you are going to heat the PCR mixture at 94 to 96 degree Celsius for 10 minutes to ensure the complete denaturation of the template DNA which means you are going to have the two separate strands.

Then you are going to have the initial. So, first step is the initial denaturation in the stage 2 you are going to have three events in denaturation, annealing and elongation. So, in the denaturation this is the first step in which the double stranded DNA template is denatured to form the two-single stranded DNA by heating at 95 to 50 to 30 seconds.

And, then you are going to have the annealing step. So, at this stage you are going to have the temperature at 95 degree Celsius and then you will going to lower down the temperature at 90 56 degree Celsius. So, this is the annealing temperature where the lower temperature will usually allow the primers are allowed to bind to template DNA and annealing temperature is 15 to 30 seconds. And, it depends on the length and the bases of the primers.

So, and then after this you are again going to increase the temperature. So, you are going to enter the into the elongation phase. So, this is the synthesis step where the polymerase is going to perform the synthesis of new strand in the direction of 5 prime to 3 prime using the primers and the dNTPs what you are going to add into the reactions. An average DNA polymerase adds about 1000 bases per minute. So, you can actually be able to calculate.

So, taking this into account like when you are adding the 100 base pairs per minute you can be able to calculate what is the elongation time. So, suppose you are working with a with a gene which is 2.5 kb; that means, 2500 base pair. So, you can actually be able to keep the annealing temperature as 2.5 minutes or 2 minutes 30 seconds.

So, after this you are going to have the final elongation. So, in the stage 3 you are going to have a final elongation. So, after the cycles are complete the reaction is held at 70 to 74 degree Celsius for several minute to allow the final extension and then you are going to have the final holds which means the final hold at 4 degree.

Now, these are the these are the things what you have to set up on the PCR machines, so that you can be able to perform the reaction. So, these machines are called as the thermal cycler right and in a typical thermal cycler you are going to have the you know you can have the flexibility of varying the temperature.

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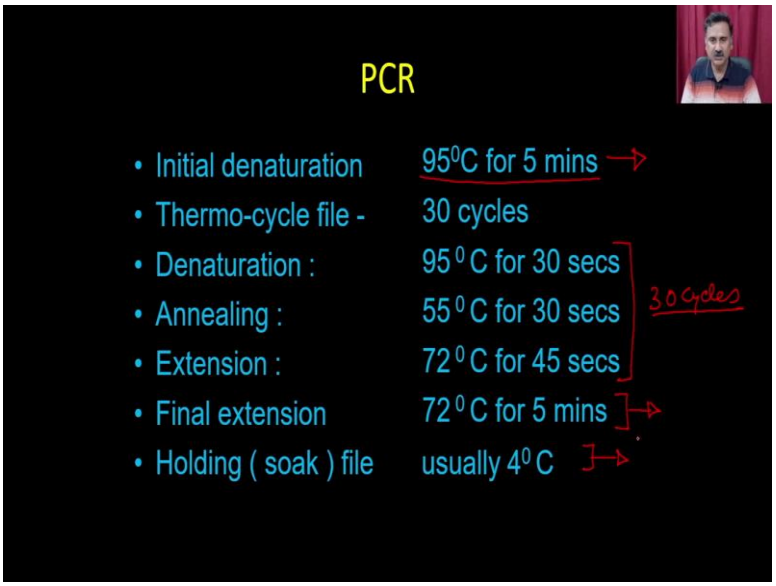

Then we are going. So, these are the cycles what you are going to do first is in the stage 1 you are going to have the 95 degree Celsius for 5 minutes and then this these are the things what are going to repeat it for 30 cycles. So, in each cycle the molecules are going to be amplified exponentially and that is how it is actually going to give you the amplified product.

And, then this is going to be the final elongations and then once the final elongation is over then you can read keep the reaction at 4 degree, so that it should not have the any kind of degradations.

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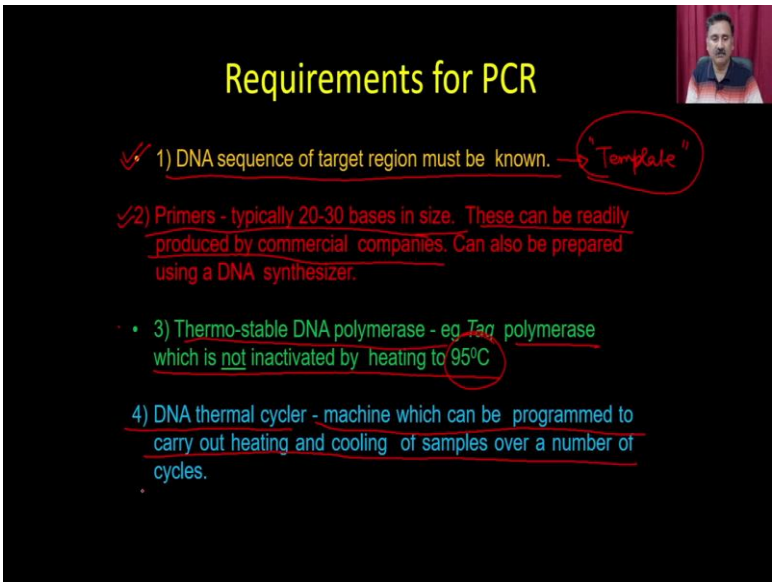

Now, if you want to perform the PCR what are the different products what required? So, what are the requirements? You require a sequence DNA sequence of the target region is known. So, that is going to give you the template. So, you should know the information about the template from where you are going to do the amplifications.

Then you require to have the primer. So, taking the sequence from the template you can be able to design the primers and these can be readily produced by the commercial companies. So, once you design the primers so, primers are nothing but the oligonucleotide sequences. That you can actually be able to use for you know designing the primers and then you can give those sequences to the companies and they will give you the you know synthesized oligos.

Then the third is thermal stable DNA polymerase. For example, Taq DNA polymerase so, which is not inactivated by heating even at 95 degree Celsius. So, that is what you require. And, then you also require a machine which is called as DNA thermal cycler. So, machine which can be programmed to carry out the heating and cooling of a sample over a number of cycles.

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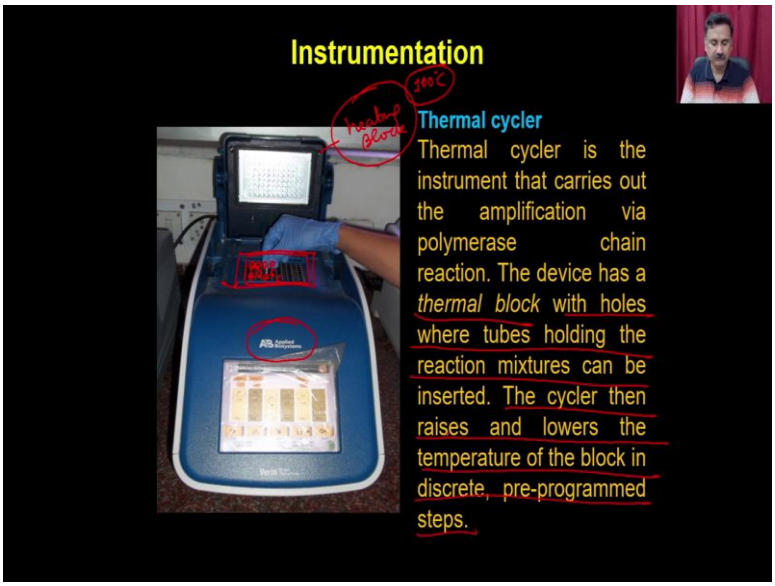

So, this is what you are going to see this is the thermal cycler from the applied bias system and what you are going to do is you see these are the; these are the blocks where you can actually have the holes. So, in these holes you can be able to keep your append off or reactions and then this is called as heating block. So, this heating blocks you are going to use to close the lids so that and this heating block is going to be at 100 degree Celsius.

So, this actually ensure that there should be no evaporation of the material from the reaction vials. So, thermal cycler is an instrument that carry out the amplification by polymerase chain reaction. The device has a thermal block. So, this is the thermal block right what you see with the holes where tubes holding reaction can be done and then the cyclers, then raises the and lower the temperature of a block in a discrete pre-programmed steps.

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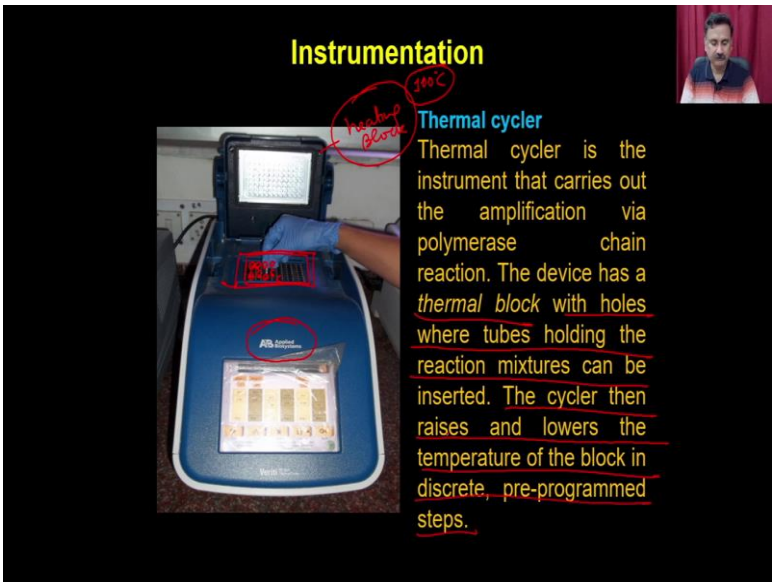

And then you have to have to set up the reactions. So, as I said you know you are going to have the multiple components like the template DNA you are going to have the primers, you are going to have the dNTPs, going to have the Taq DNA polymerase and you are also going to have the water. So, you are also going to have the autoclave water which you are going to use, right.

So, template DNA if template DNA you have to you have to use as per the source of the template DNA so, for example, if it is a viral or the short templates you can use the template DNA in the range of picogram to nanogram, but if it is a genomic DNA then you might have to use into the micro gram range.

Primers you are going to have two primers the forward primer and the reverse primer and that you have to used in the range of 0.1 to 0.5 micro moles. Then you also require the magnesium chloride. So, magnesium chloride is in the range of 1.5 to 2 millimolar and then you also require dNTPs; dNTPs means the all the nucleotides. So, you acquire a dATP, dGTP, dTTP and dCTP ok. So, all these are going to be present into this dNTPs mixture so that you can use as 200 micro molar.

And, then you also require the Taq DNA polymerase. So, Taq DNA polymerase you can use at the 0.5 to 2 units for the 50 micro liter reactions. And, then ultimately you are going to add the water so that you can be able to make the reaction as 50 micro liter. So, as far as the sequence in which you are going to add these so, you are going to first add

the water, then you are going to add the buffer, then you are going to add the dNTPs you are going to add the template DNA at the end.

And, once you have all these reactions you are going to set up you are going to put that into a thermal cycler and that is how it is actually going to give you the amplified product. So, we have prepared a small demo actually to demonstrate you these events and this demo is being prepared for by in my laboratory by some of the students and what they are going to do is, they are going to show you how to set up the PCR reactions and how you can be able to perform the PCR.

Hello, everyone my name is Sooram Banesh, a research scholar at Department of Biosciences, Bioengineering at IIT, Guwahati. In this video, we will show you how to set up a PCR reaction and what are the precautions we have to take while setting up the reaction and how to analyze the PCR design using agarose gel electrophoresis. So, let us start it.

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Hello, everyone. In this video will be demonstrating how to set up a PCR reaction and analyze the results using agarose gel electrophoresis. PCR are polymerase chain reaction is a widely used molecular biology technique to amplify a particular segment of DNA. It is also employed in biomedical research and forensic medicine. The main application of this polymerase chain reaction is chlorine [FL].

To set up a PCR reaction we need template DNA, site specific primers, dNTP mix, nucleus free water and Taq polymerase. For a 50 micro liter reaction in a typical concentrations of 10 to 100 nanograms of template DNA used and 5 picomoles of each primer will be used.

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This is a earlier version of thermal cycler which contains display unit where we can observe the parameters and change the parameters. This is a hard shield, this is sample holder and inside there is a peltier system which can maintain the temperature fluctuations.

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For setting up a PCR reaction initial denaturation at 95 degree Celsius 3 minutes and these steps we will use 30 repeats where initial denaturation will be 30 seconds and annealing it. Extension time should be given 1 minute per kb.

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And, here final extension should be given 10 minutes and hold it 4 degree Celsius 10 minutes.

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Once the PCR reaction is completed, we have to analyze the results for amplification. Further we need agarose and DAE buffer. First, we have to weigh agarose and mix with the DAE buffer. It will not dissolve easily. So, we have to heat it in microwave oven until it get dissolved. Now, agarose got dissolved in DAE buffer. We have to let it cool down up to 50 degree Celsius.

Now, before pouring we have to add ethidium bromide for detection purpose. Now, the gel got solid bed we have to take out the gel and keep it in the electrophoretic apparatus. We have to gently remove the con, loose the nuts and keep the gel in the apparatus. Make sure that the buffer is submerged the gel. We have to fill the remaining part with 1X TAE buffer.

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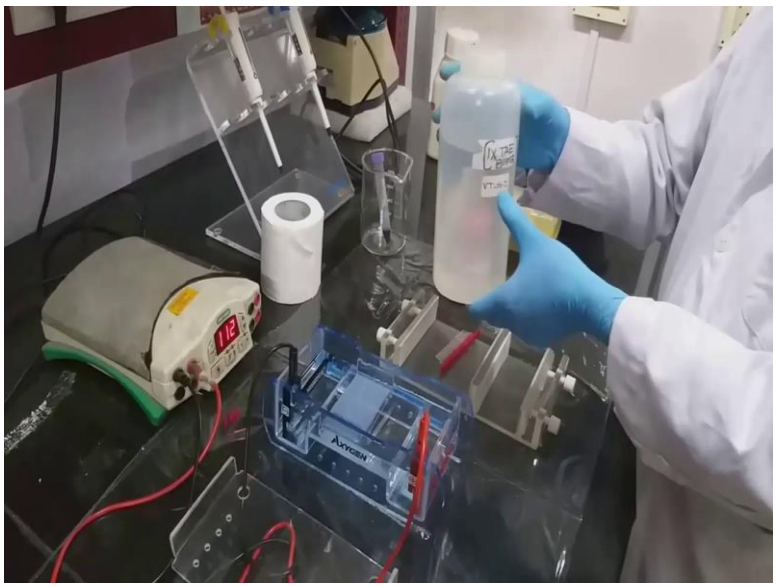

Generally, for analyzing the DNA samples we will use agarose gel electrophoresis. This is the power pack and this is the electrophoretic apparatus. This is a negative electrode and this is a positive electrode. We can change the voltage from here for loading gap sampling. We have to mix PCR reaction mixture with phi x loading time.

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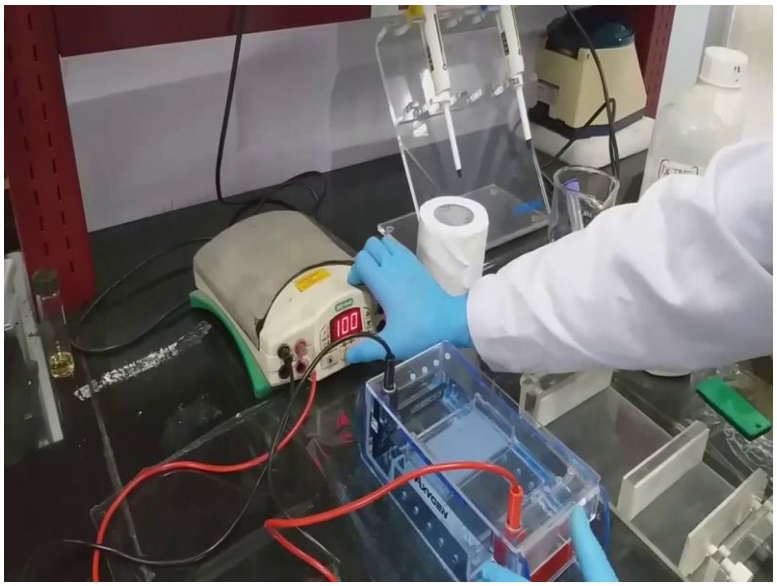

The loading is over. We have to cover the electrophoretic apparatus with the lid and we have to adjust the voltage, then start Run.

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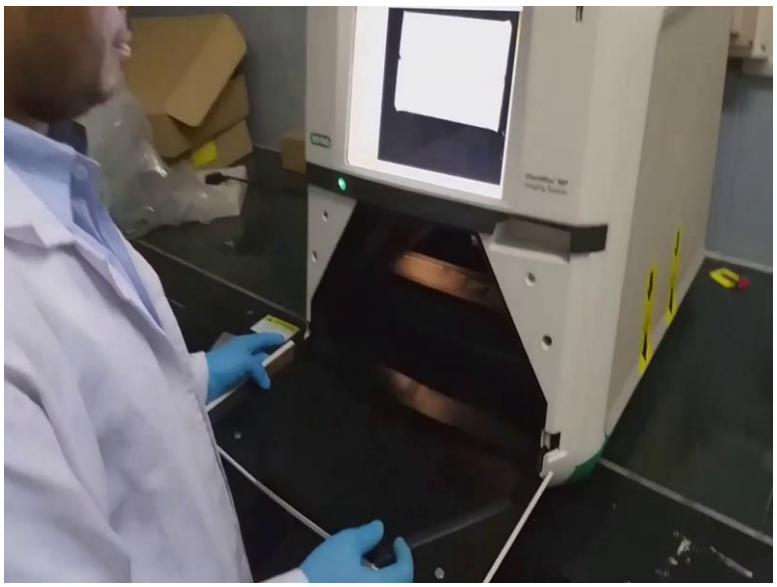

After the agrose gel electrophoresis, we have to visualize the amplified product. This is the ChemiDoc MP, where we are going to visualize the amplified product.

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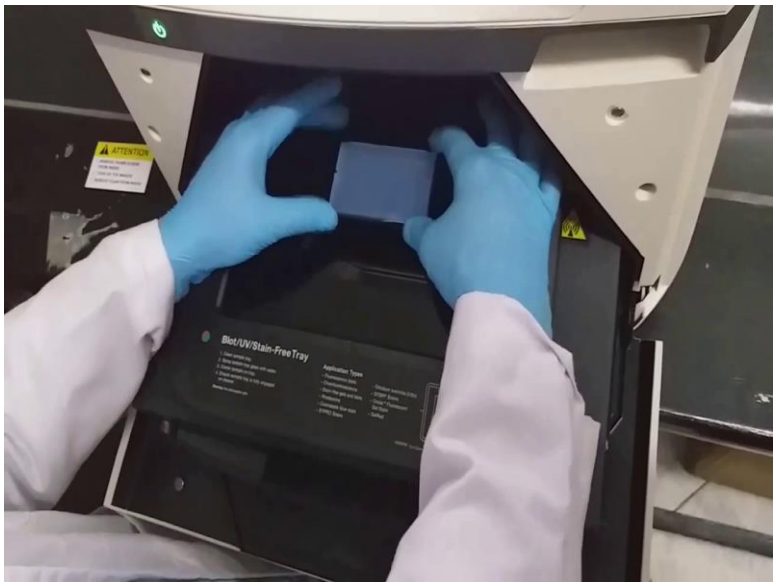

Now, we have to keep the gel, then close the tin.

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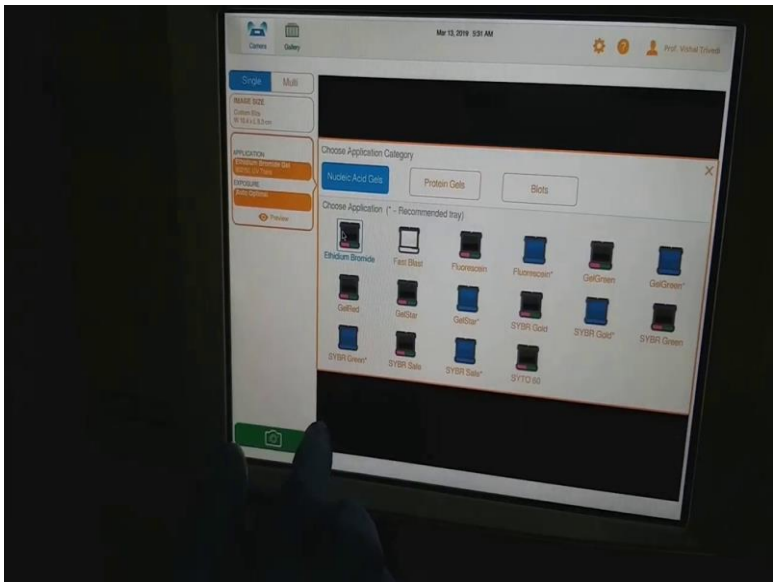

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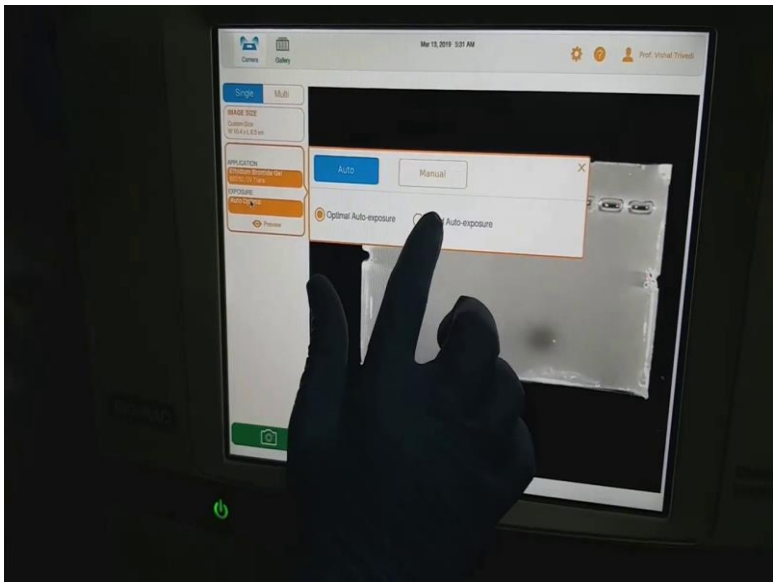

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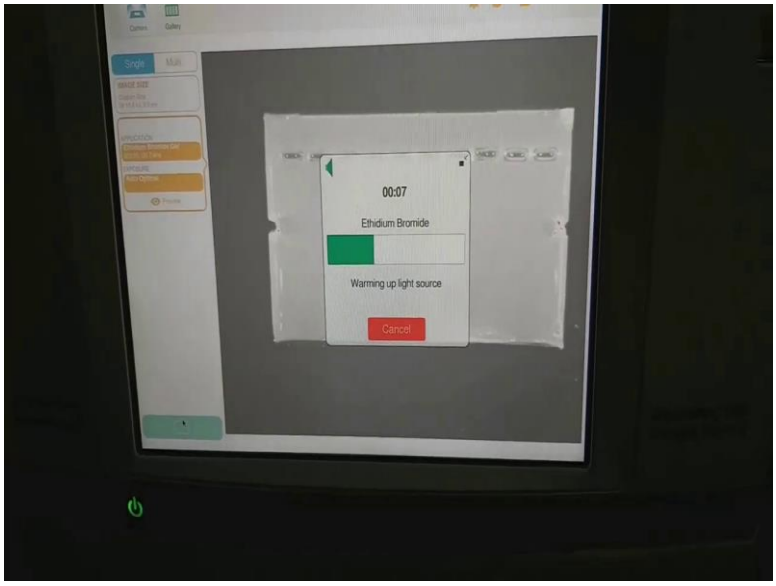

We have to select here application, Nucleic Acids, Ethidium Bromide. EXPOSURE, Optimal Exposure or we can select Manual, also then we will acquire the images.

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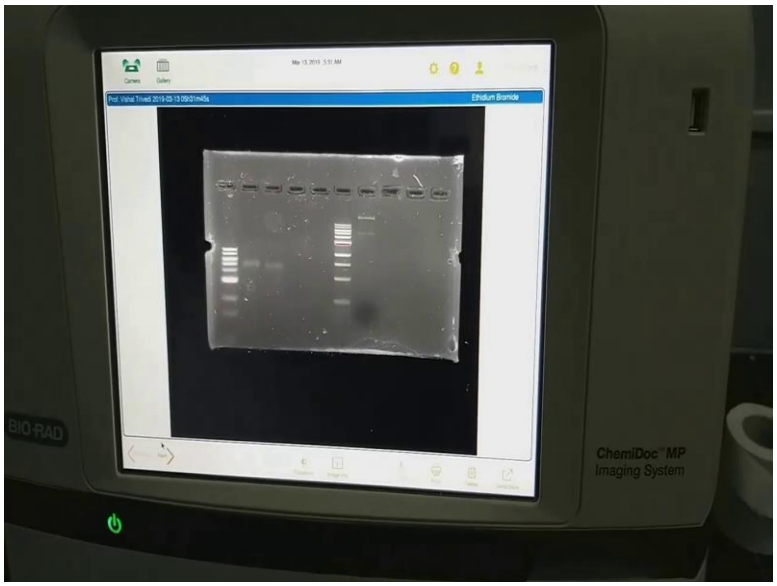

Now, we can find here. This is the DNA ladder; this is the PCR amplified product. We can transform it into transform or save this image into jpg at. In this video, we have discussed how to set up a PCR reaction and how to analyze the PCR result. We have also shown how to use a thermal cycler and what are the components of thermal cycler. So, during all this process we have to take some precautions to get better results like all the time you have to keep polymerase enzymes and your primers at on ice.

And, other thing we have to remember is while running the agarose gel electrophoresis always wear the gloves to prevent any contamination with the ethidium bromide. So, ethidium bromide is a carcinogen. So, it is not very likely to cause any cancer, but we have to make sure that we are avoiding this kind of touching with the ethidium bromide. So, in this demo we have discussed about the different events and what are the precautions you should take and all that.

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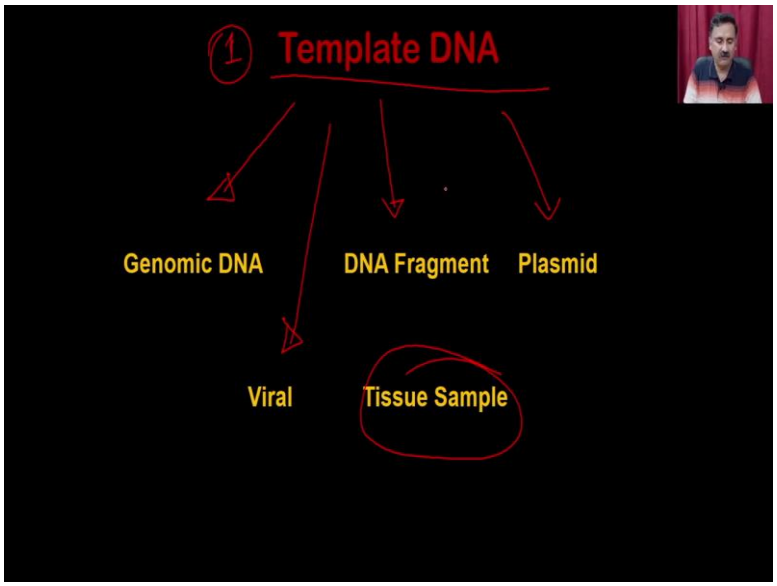

Now, as far as the template DNA is concerned the template DNA could be of any type. It could be genomic DNA, it could be DNA fragments, it could be plasmid, it could be viral sequences or it could be the tissue samples, ok. So, that is the template DNA. So, depending on the source you can actually be able to choose the amount of template DNA what you require for the reactions.

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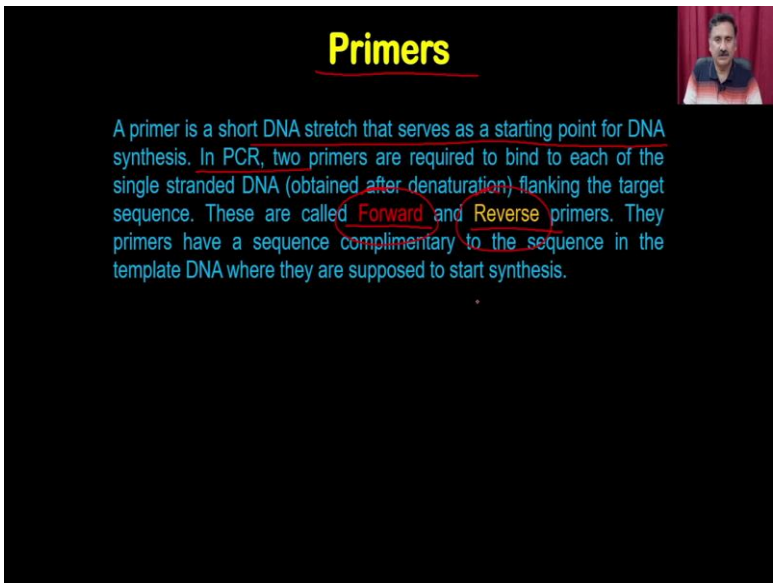

Then you also require the primers. So, primers are the short DNA stretch that serve as a starting point for the DNA synthesis. In the PCR you require the two primers you require

the forward primer and the reverse primers and primer designing is very very crucial and important for achieving the best results during the PCR. So, you have to design the forward primer and as well as the reverse primers in such a way that they should actually going to give you the best amplified product.

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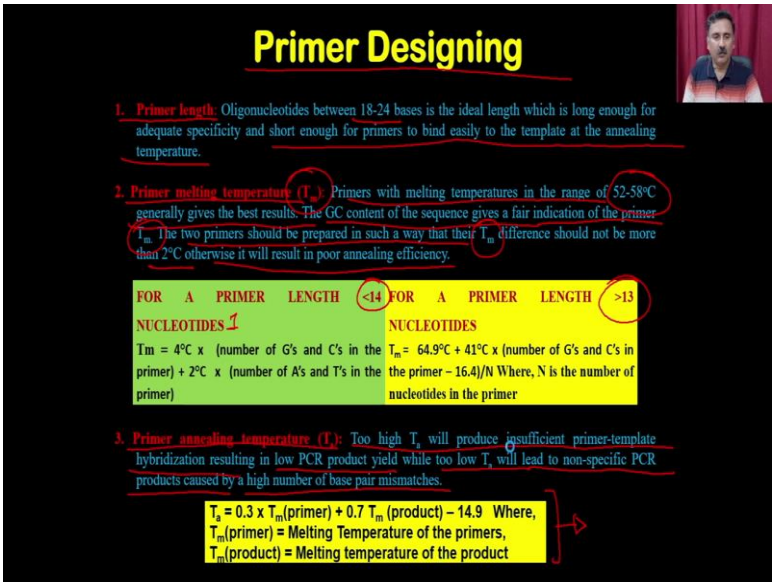

So, what are the criteria you are going to adopt when you are going to design the primer. So, first is the number is primer length. So, primer length can be 18 to 20 bases and it is the ideal length which is long enough for adequate specificity and short enough for primer to bind easily to the template at the annealing temperature.

Then you require the primer melting temperature so, T m, right. So, primer with the melting temperature in the range of 52 to 58 degree Celsius generally gives the best results. The GC content of the sequence gives the fairy indication of the primer T m melting temperature the two primer should be prepared in such a way that the melting temperature differences between them should not be more than 2 degree Celsius otherwise it will results in the poor annealing efficiency.

And, you can be able to calculate the primer melting temperature by using this particular formula. So, if the primer is having the length which is less than 14 you can use this formula and if it primer is having the more than 13, then you can actually be able to use this formula.

And, the third is the primer annealing temperature. So, too high annealing temperature will provide insufficient primer template hybridizations results in the low PCR yield while the too low annealing temperature is actually going to give you the nonspecific PCR product caused by the high number of base pair matches. And you can be able to use this formula to calculate the primer annealing temperature.

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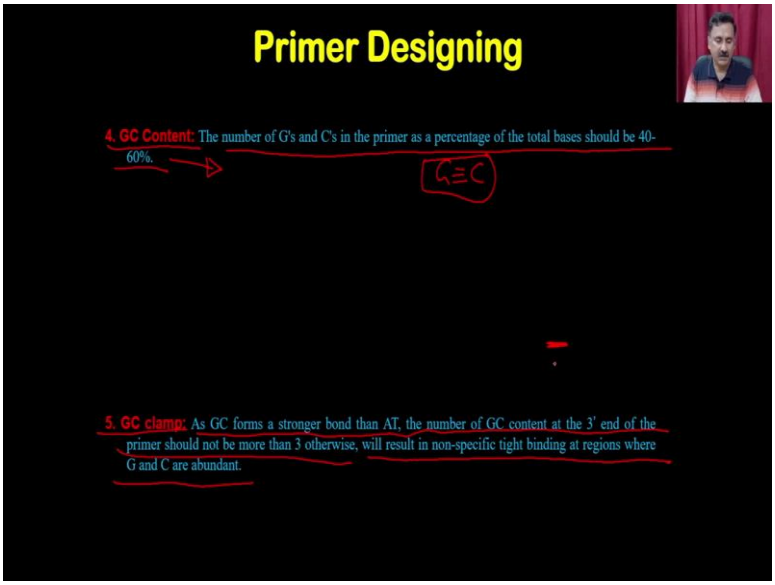

The fourth is you are also have to take care of the GC content. The number of GCs in the primer as a percentage of total base should not be should be between 40 to 60 base pair because the GC actually is a having the 3 base pair, right. So, every G is having the three base pair, 3 hydrogen bonding with the C and, that is why it provides better specificity and better strength for the primer to anneal to the template.

Then you also require to see the GC clamps. GC clamp is formed a stronger bond than AT then the number of GC present at the 3 prime end of the primer should not be more than 3 otherwise will result in a non specific tight binding in the region where the G and C are abundant.

So, GC clamp is going to be formed at the end of the template and we should avoid the lot of GC at the end because otherwise it is actually going to bind at the end ok and it is going to form the GC clamp.

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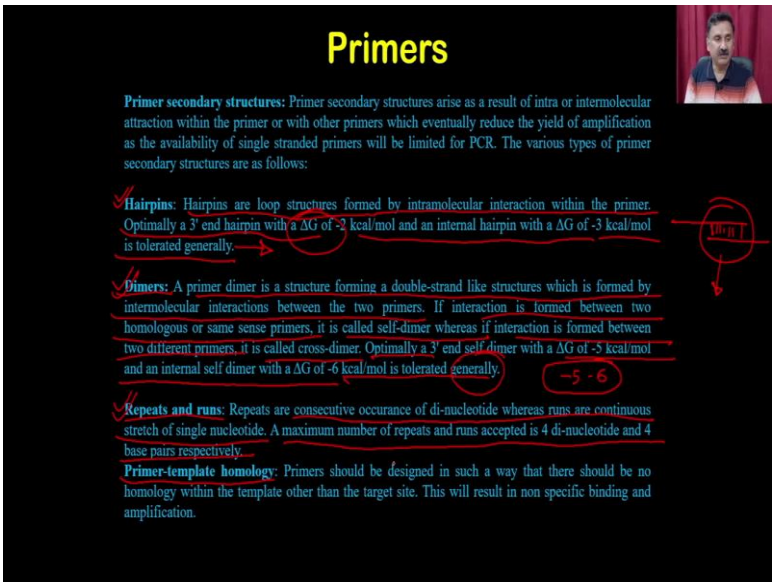

Then we also have to take care of the hair pin loops, dimers and the repeats, ok. So, hair pins are the loop structure formed by the intermolecular interaction with the primers optimally a 3 prime end hairpin with a delta G of minus 2 kilo calorie and an internal hair pin with a delta G of minus 3 kilo calorie is tolerated generally, ok.

So, if it is a having a hair pin with it is having a you know free energy in the range of minus 2 kilo calorie to minus 3 kilo calorie, it can be easily be managed because when you increase the temperature all these hairpins are going to be broken down.

Similarly, you have the dimers. A primer dimer is a structure form a double stranded structure which is formed by the intermolecular interaction between the two primers. So, you can have the two primers and they may have the overlapping region and that is how it is actually going to form the dimer.

This means this region is not going to be available for hybridizations with the template, right and that could be crucial sometime for providing the specificity and as well as for amplifications. So, if the interaction is formed between the two homologous or same sense primer it is called self dimer.

Whereas, interaction is formed between the two primer it is called cross-dimer or hetero-dimer optimally the delta G of 5 kilo calorie or internal self dimer with a minus 6 kilo

calorie is tolerated. So, if it having a minus 5 to minus 6 free energy kilo calorie per mole, then it is can be broken down when you increase the temperature.

Then you also have the repeats and runs and repeats are consecutive occurrence of di-nucleotide whereas, runs are continuously stretch of single nucleotide. A maximum number of repeats and run accepted is di nucleotide and 4 base pairs respectively and then you can also have the primer template homology. So, that is very very important and it will actually lead to the non specific binding and amplifications.

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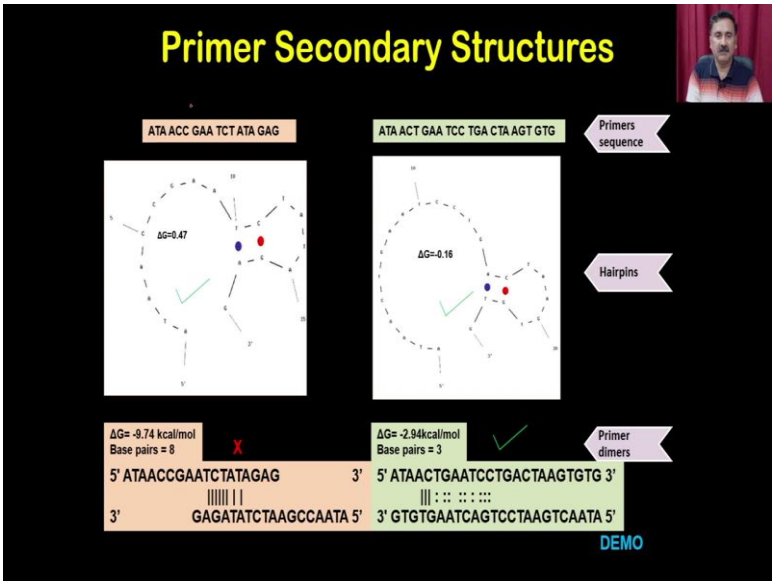

So, what you see is the primer secondary structures. So, these are the primary structure. So, for example, if this is the primer I have designed, it is actually going to form the you know so many secondary structure which are going to be stabilized by the these structures. So, this is not correct, ok.

So, what I what we have done is we have just changed little bit sequences and that is how you are going to broken down the hair pins and you are going to broken down the loops and that is how it is actually delta energy is very low and that is how it is actually going to be good.

So, we have prepared a small demo clip where I have we have explained how you can be able to design the primers and student have taken full care that they are actually going to first show you the manual method how you can be able to design the primers and then

subsequent to that they have also shown you the automated method, where you can actually be able to use the different softwares and they will actually give you the design primers.

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Primer Designing

Hello, everyone. In this video, I will show you how to design the primers and analyze them.

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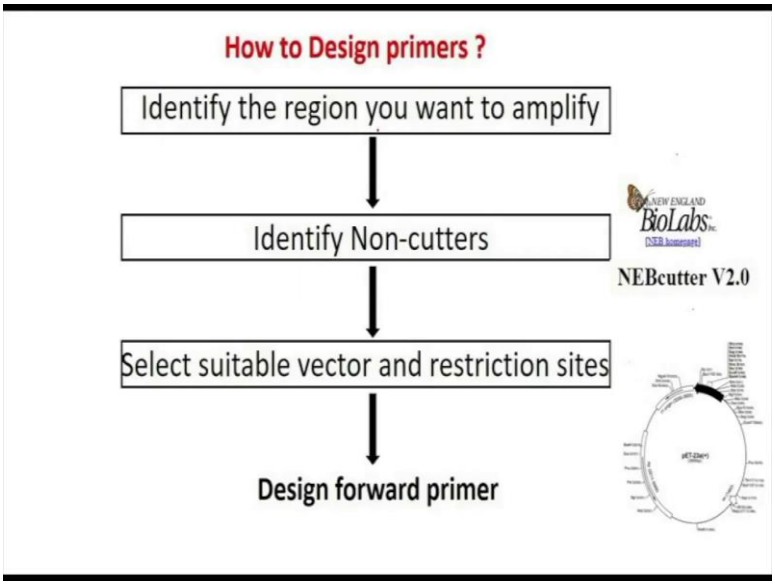

So, for designing primers first you have to identify the region of interest, your region of interest which you want to amplify from any vector or any sequence. So, in second step

you have to identify non-cutters. There are various softwares available, but we can use New England BioLabs NEBcutter version 2.0.

After identifying non-cutters, you have to select a suitable vector in which you want to integrate this amplified region and suitable restriction sites. You will get suitable restriction sites from non-cutters, after that you can go for designing forward primer.

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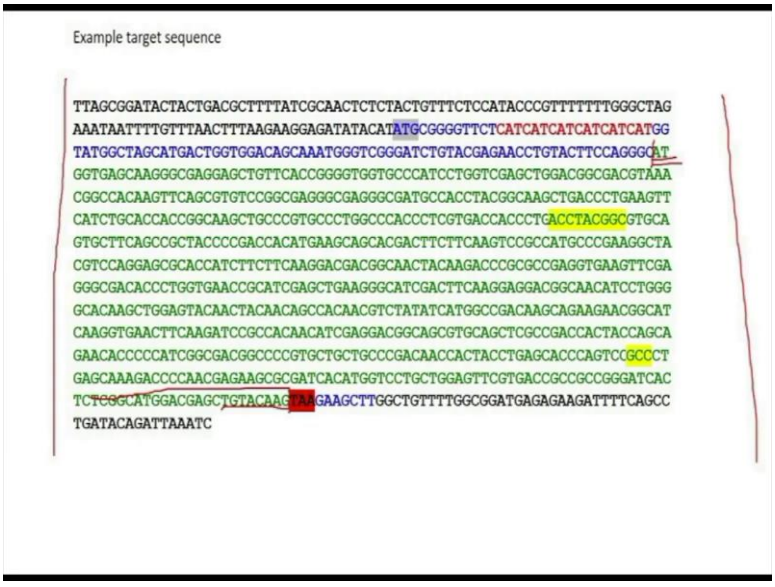

So, for understanding purpose I gave this sequence. So, I am using this sequence. I will use this sequence to design the primers and analyze the primers. So, this is the whole sequence, but I do not want to amplify whole region. I want to amplify the letters, the sequence which is highlighted in green. So, I want to amplify starting from here to here.

So, now, the question arises what are the non-cutters? So, you want to amplify this region and integrate into another vector for that you have to identify which are non-cutting restriction enzymes. So, what I will do? I will copy this sequence into NABcutter and identify what are the non-cutters.

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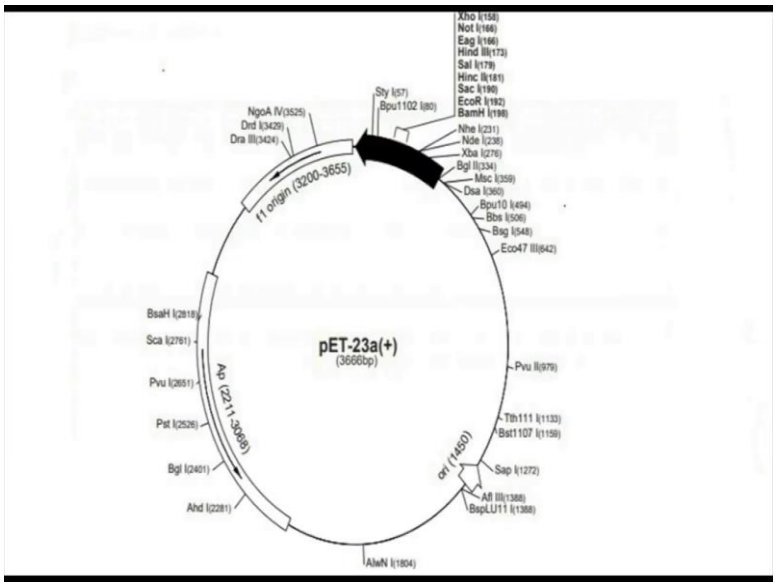

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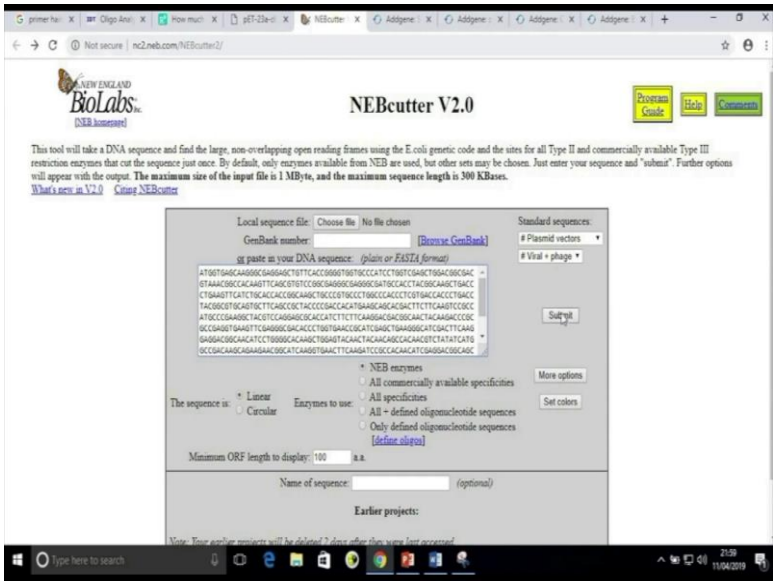

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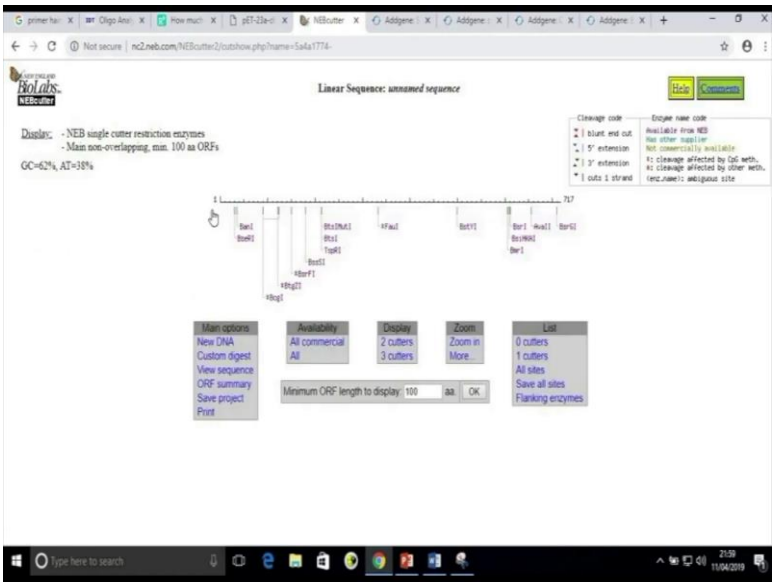

So, I just copy the sequence paste here and I will ask submit. So, it will analyze the sequence and give non-cutter. These are the enzymes cutting inside the sequence, but we are interested in which are non-cutters.

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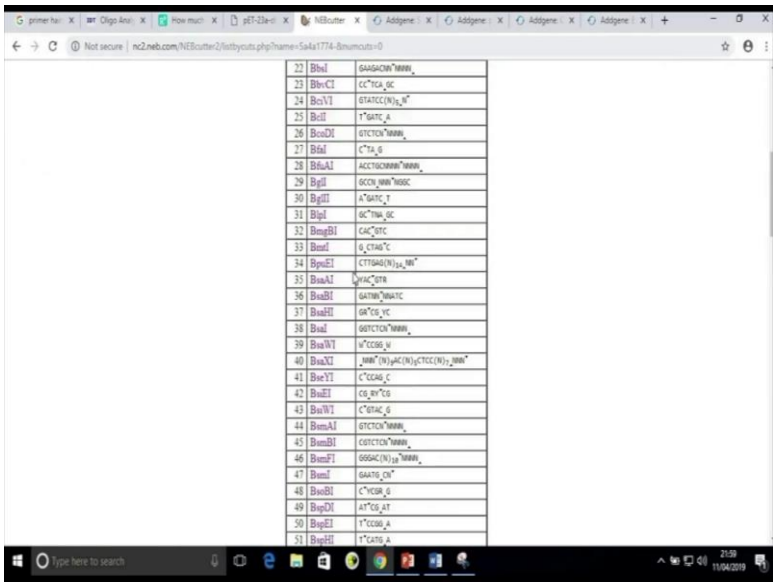

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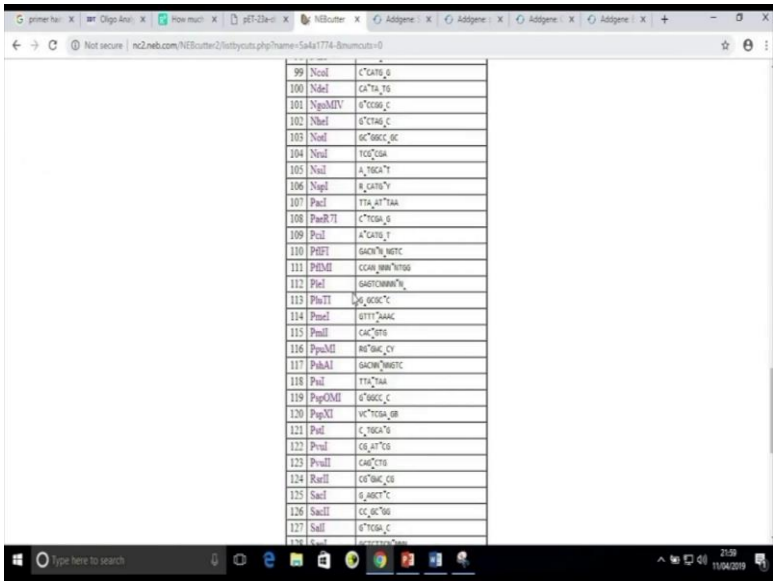

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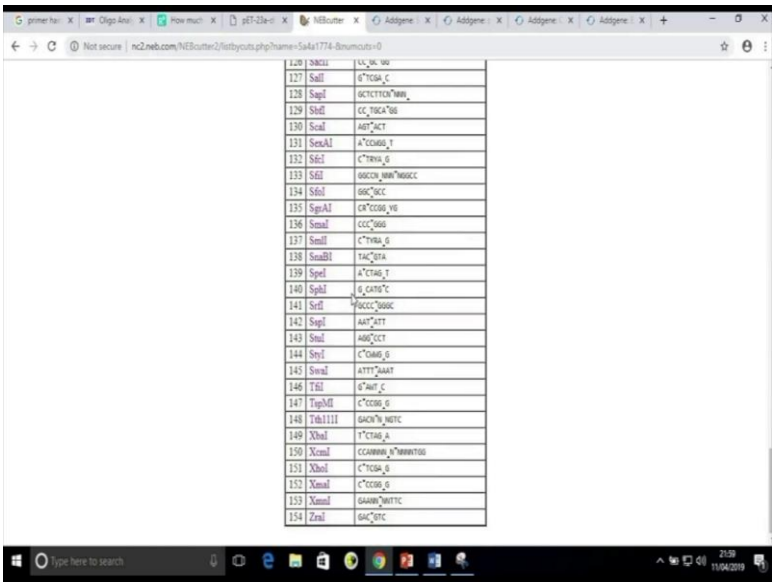

So, that means, you can see here non cutters. So, just click here it will give a number of enzymes which will non not cut inside the sequence. So, once getting this list we have to identify in which vector you want to integrate your amplified region.

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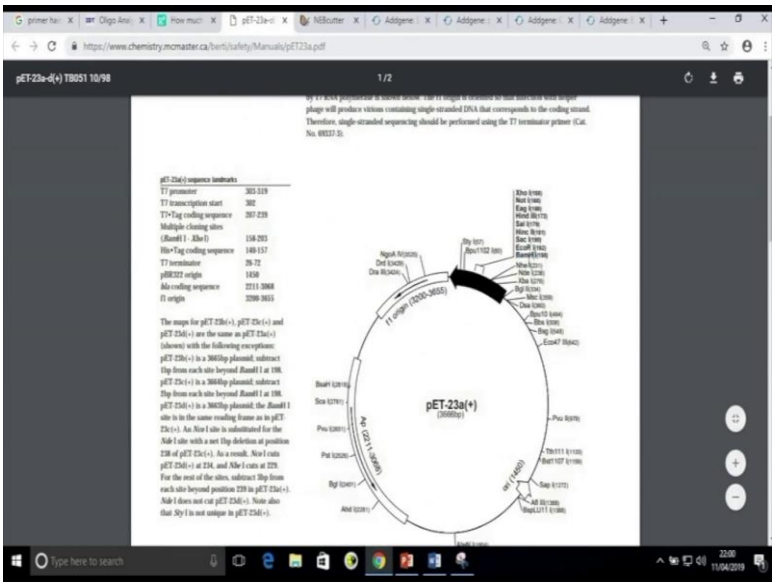

So, for that purpose, so, I have selected for easy of understanding I have selected pET-23a vector. So, you can see this is the vector map. So, this is the 5 prime side, this is the 3 prime side, N terminal and this is the C terminal side. N terminal means forward primer; C terminal means reverse primer.

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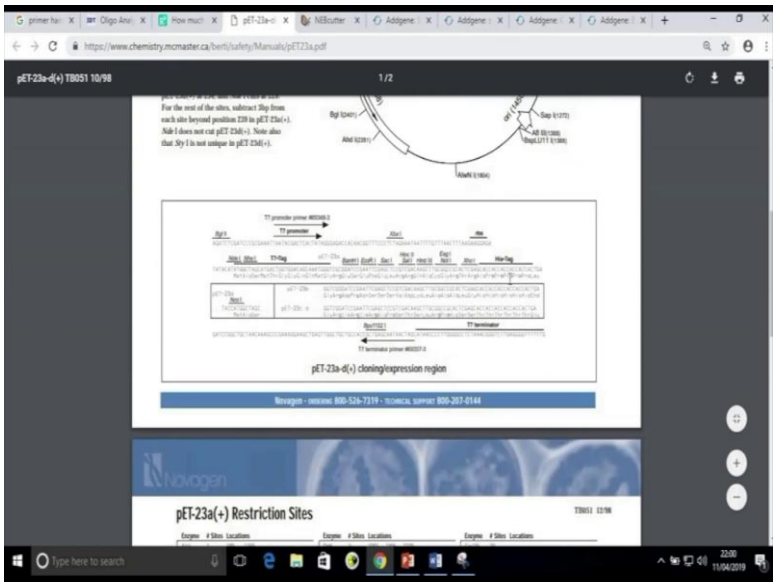

So, I can use BamH1 in forward primer and Xho1 in reverse primer. This is the detailed map.

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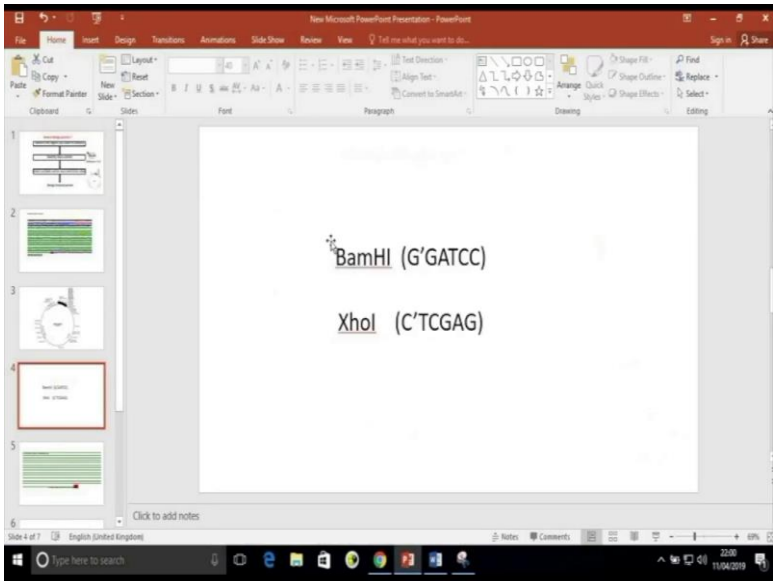

So, I have identified two restriction enzymes that is BamH1 and Xho1. So, I can use these enzymes in forward primer and reverse primer. So, after identifying restriction enzymes and the vector will go for designing forward primer.

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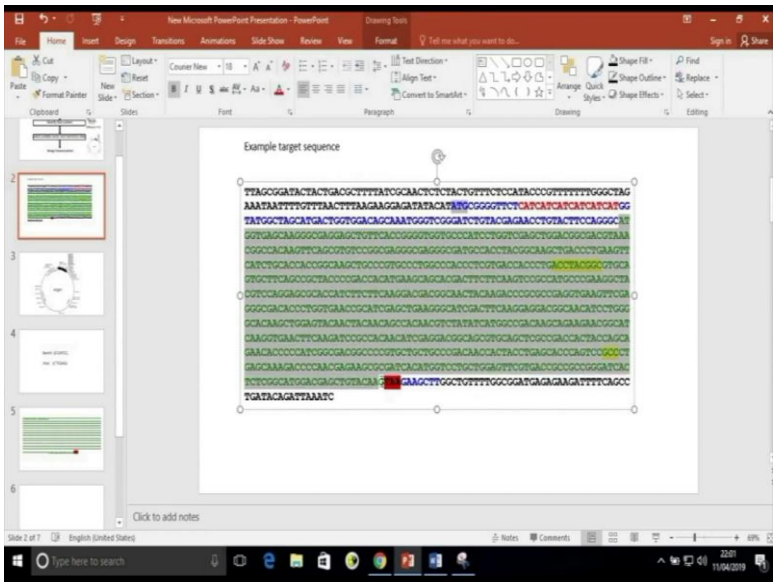

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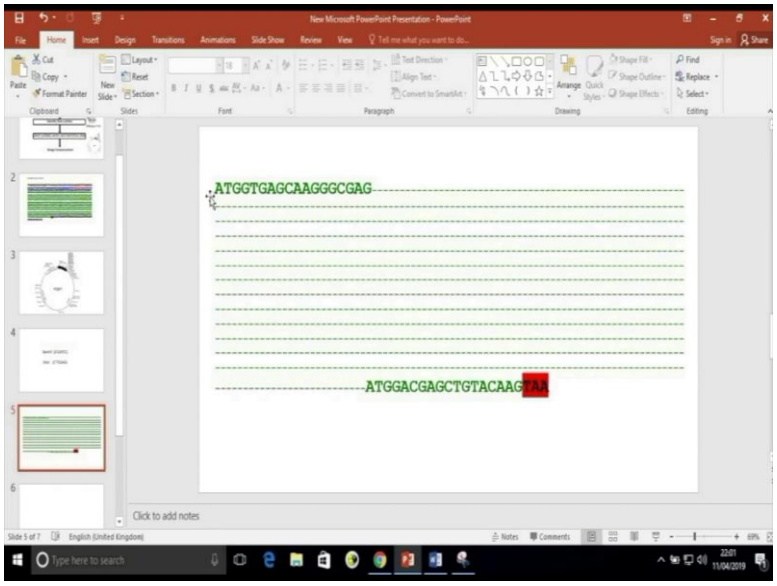

So, I will take this sequence. I want to amplify from here to here. So, I will copy this sequence here. So, for designing forward primer it is very easy. You have to take the sequence whatever you are getting up to 15 to 20 bases you can take as it is.

So, if you want to insert a restriction enzyme, suppose I want to insert a restriction enzyme. This is the sequence as it is given from this whole sequence. So, I want to insert a restriction enzyme that is BamH1. So, this is the sequence for BamH1, here it cuts.

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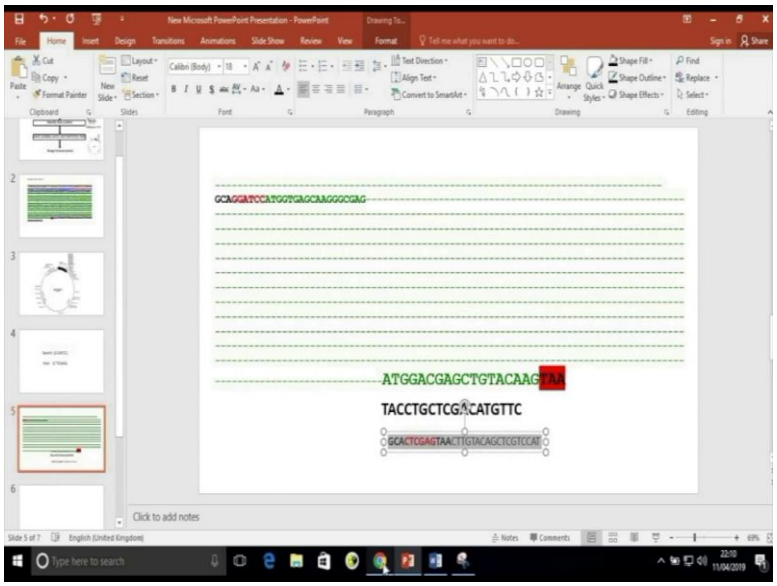

So, I can use this sequence here. So, this is the this is our restriction enzyme here it will cut. So, we cannot simply queue like this. So, there should be some more bases extra bases, we have to add in 5 prime side. So, I will use. So, this sequence I will use. So, now, this is 5 prime to 3 prime side. So, this is our forward primer is ready. So, after designing this forward primer, we have to analyze this sequence. So, this primer.

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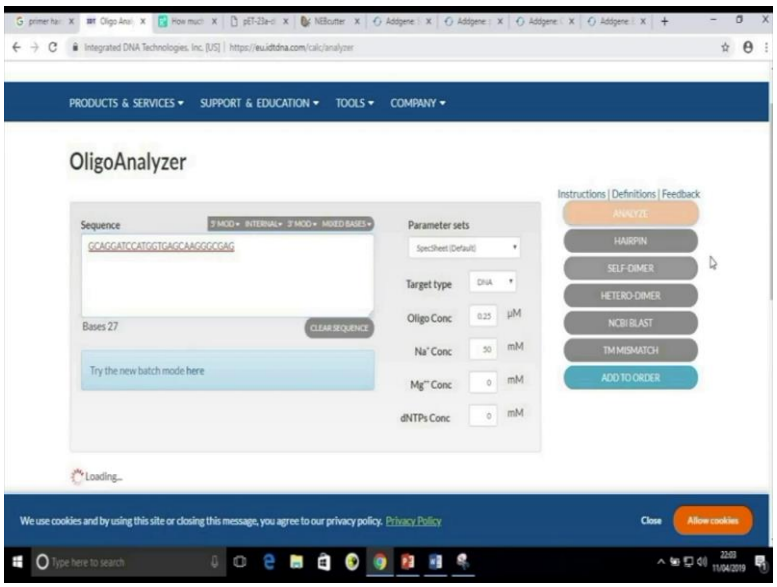

So, what I will do is I just copy this sequence and I will use OligoAnalyzer software which is specially designed for this purpose only. I will paste the sequence. Just ask ANALYZE.

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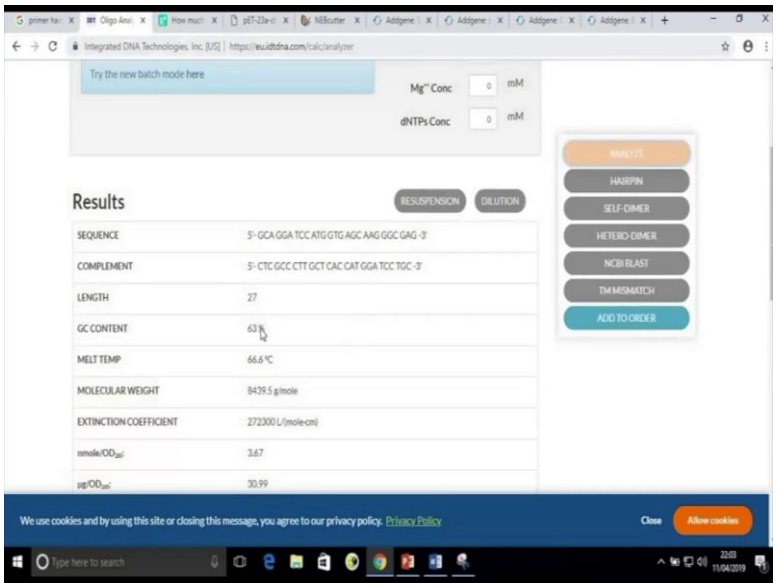

So, here also you can see there are so many options are there, like you can ANALYZE, HAIRPIN loop, SELF-DIMER, HETERO-DIMER.

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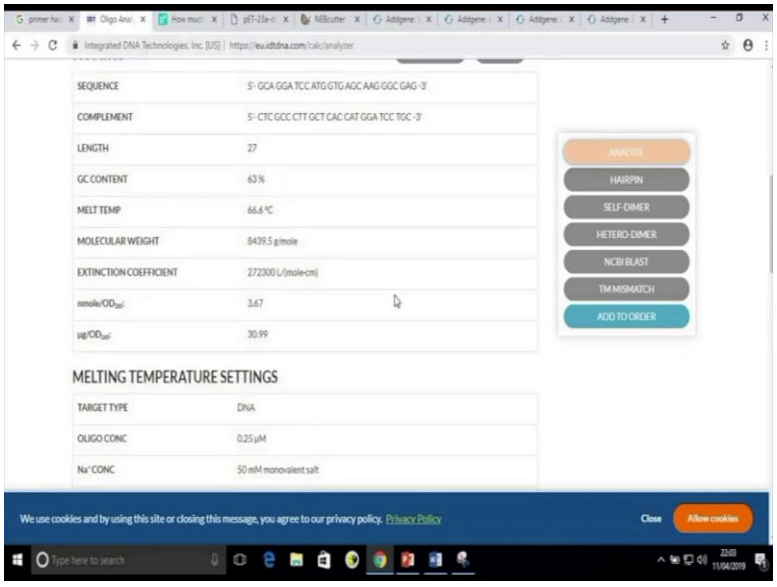

So, these are the general details – what is the length and GC content, melting temperature. molecular weight. So, these are normal details. I will go for hairpin loop, is there any hairpin loops.

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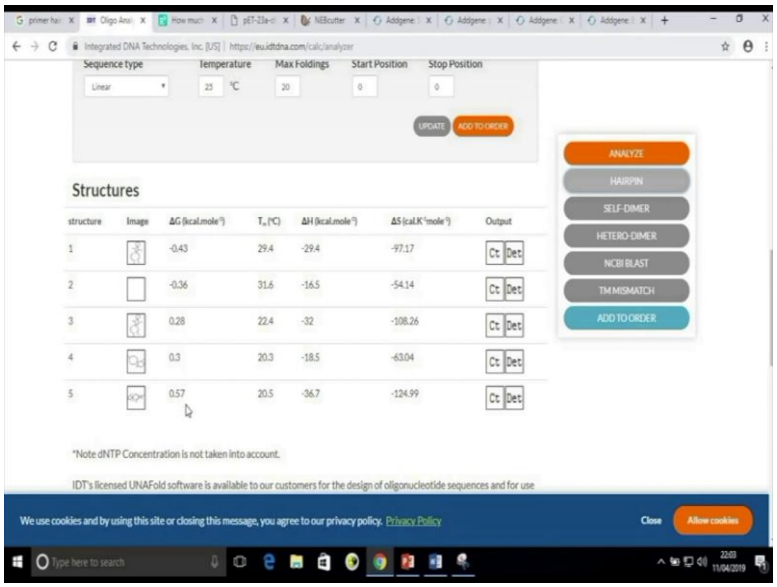

So, we can see there are number of hairpin loops, we can see different different structures predicted by the software.

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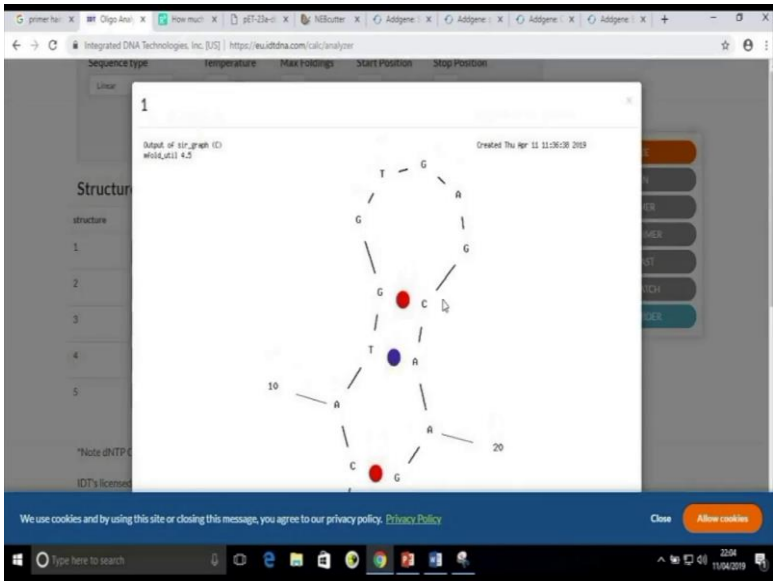

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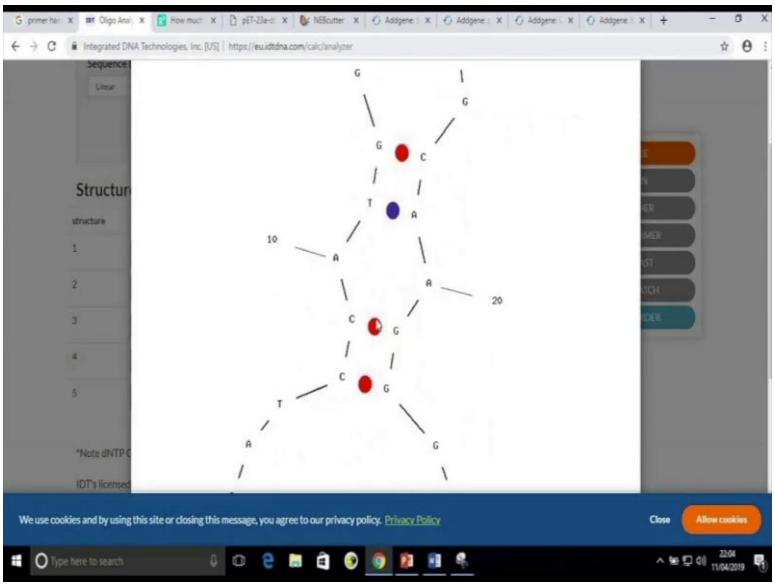

So, if you want to explore this thing you can explore only two bases two bases it is forming and the delta G value is minus 0.43 kilo calorie per mole. So, this is fine. Up to minus 10 kilo calorie per mod is fine. Those hairpin loops broken during the during the amplification process, but above that above minus 10 kilo calorie for mole cannot be broken.

So, in that case what we will do? Either we redesign the primers or we will add 5 percent one percentage B tan or 5 percentage DMSO. These are these chemicals disrupt the these loops so that the amplification will be fine.

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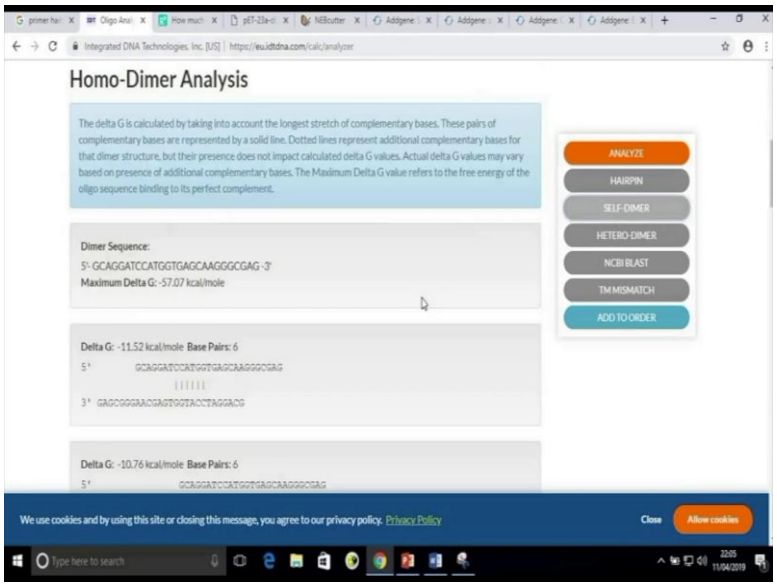

So, next I will analyze for self-dimer.

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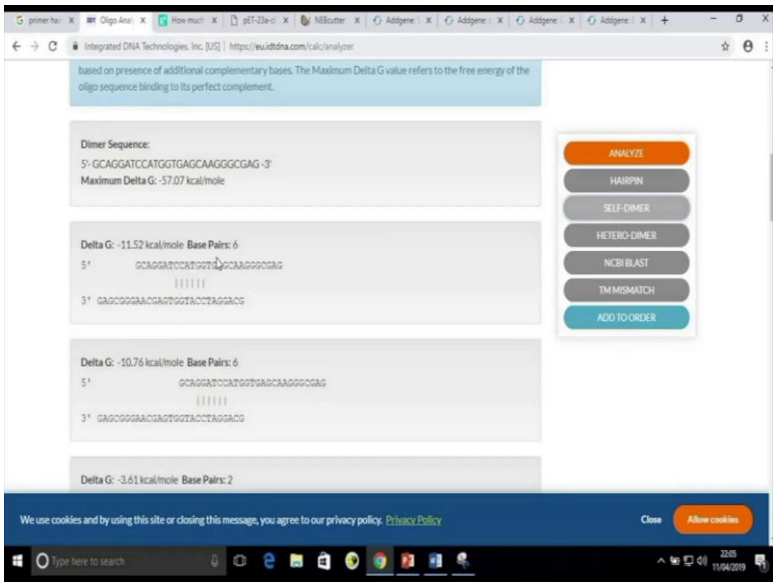

Is there any self dimers and what is the maximum delta G. So, this is forming continuously five bases. It is because of the restriction sites. So, those are restriction sites those.

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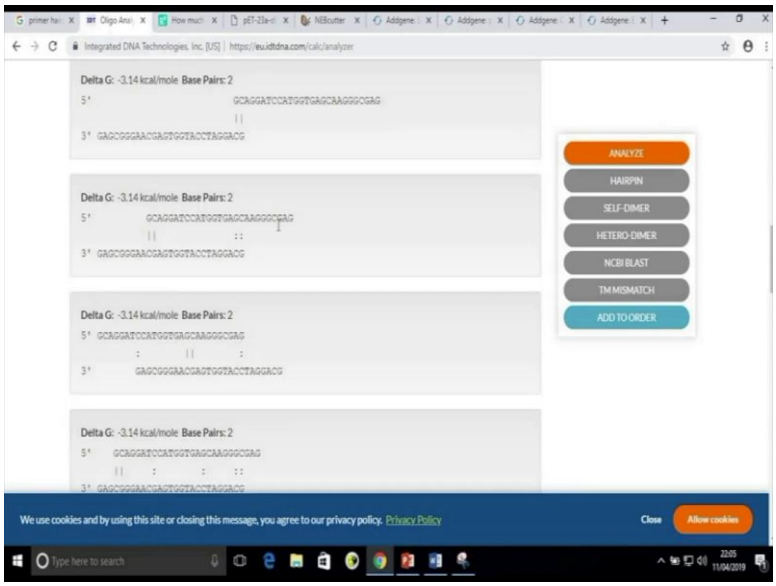

Homo-dimers forming due to restriction site can be broken, there is no issue. But, other than that this is also because of restriction site, but other than that we have to look carefully. So, is there any continuously 4 or 5 bases forming this homo-dimer then it is very difficult. These interactions can be broken easily.

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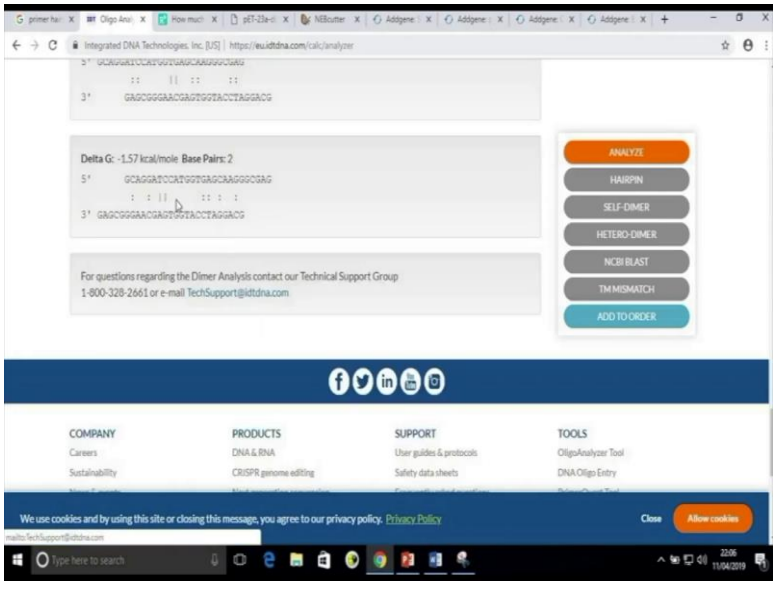

So, here some of the consecutive base pairs are there, these are very weak interactions. So, they can be broken. So, other than that there is no significant self-dimers. So, this sequence can be used. And, for hetero-dimer predicting, hetero dimer you need a

complementary sequence with reverse prime like reverse primer you need. So, that we will discuss later on. So, we got our forward primer here.

So, it is very easy to generate forward primer, but in case of reverse primer it is somewhat difficult because not in terms of predicting things it is somewhat tricky. So, what I am saying is here we have sequence. So, in case of forward primer we just taken as it is sequence 15 to 20 bases as it is from sequence itself, but here we have to take complementary sequence not 3 prime to 5 or 5 prime to 3 prime sequence we have to take complementary to this one.

Say, this is the sequence we got from here, so, what is the complementary to this one? So, just I will add here. So, this is the complementary to this particular sequence. So, as you can see this is we have to keep from this direction 5 prime to 3 prime. So, I will take like this. So, what we have to do is, we want to insert a restriction site here. So, we can insert a restriction site here directly.

So, in reverse primer we wanted to insert Xho1 site. So, this is the restriction site, as usual we can use we have to insert T here. So, this is the restriction site we added. We can add flanking regions in between flanking bases before this restriction site. So, now we got our reverse primer.

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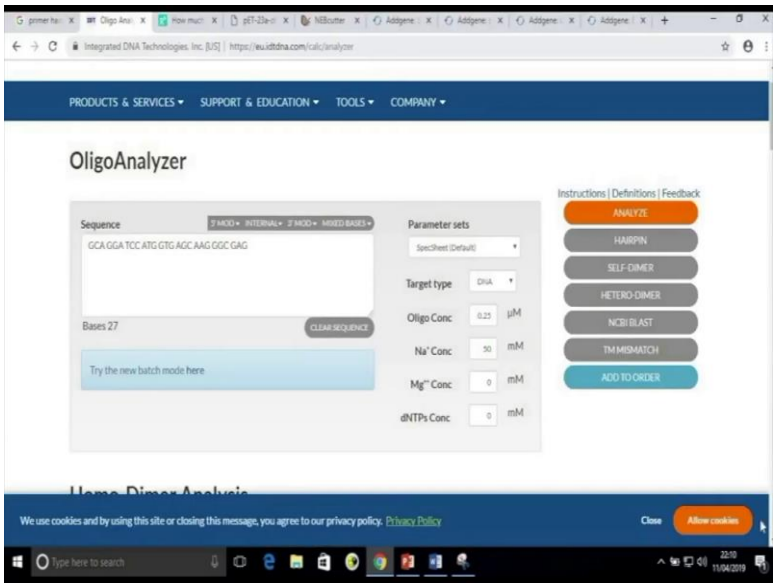

So, we have to go through same procedure like what I have shown in case of forward primer. So, just I will copy paste here and analyze the reverse primer.

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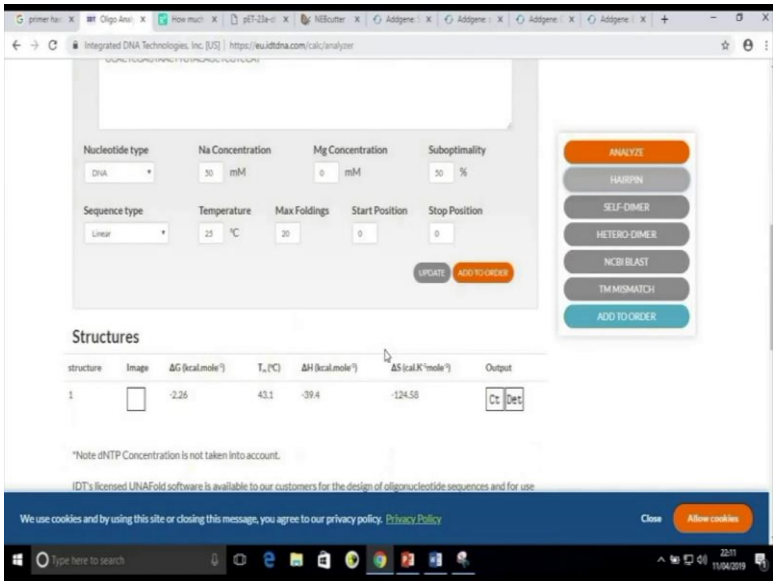

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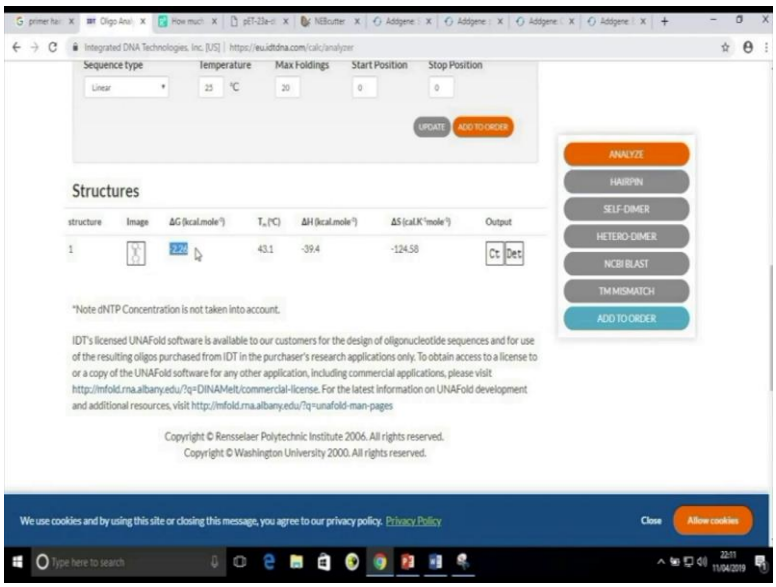

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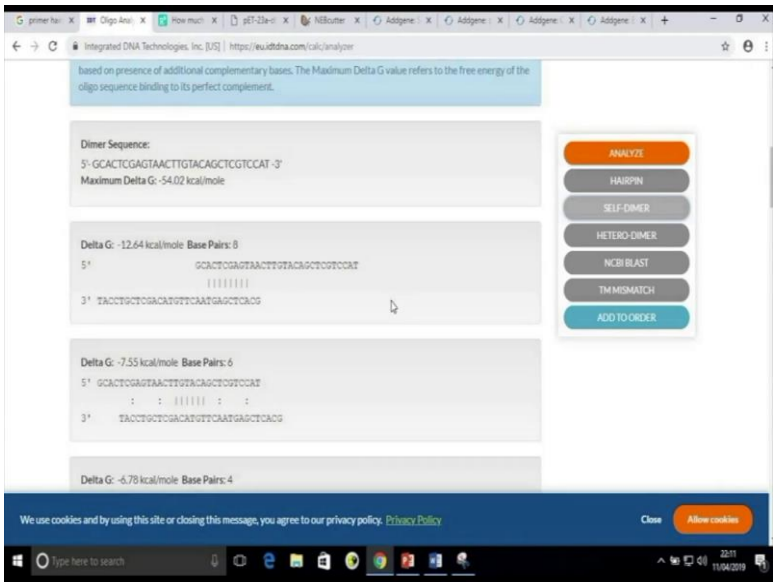

So, is there any hair pin loops? Only one hair pin loop that is within the range of delta g. So, there is no issue and self-dimer? So, we can see here continuously four bases are forming. In this case we have to either change the sequence or remove the some of the bases. We can ignore those restriction those dimers forming through restriction site.

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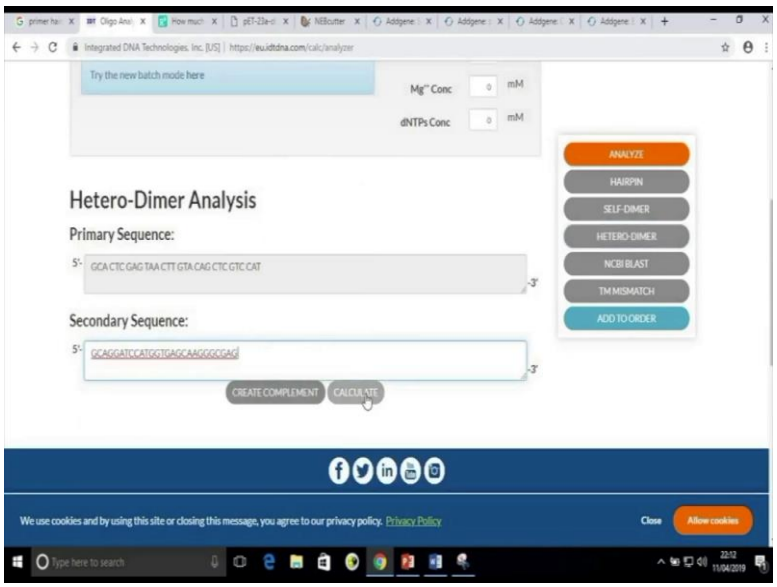

So, next hetero-dimer we have to analyze.

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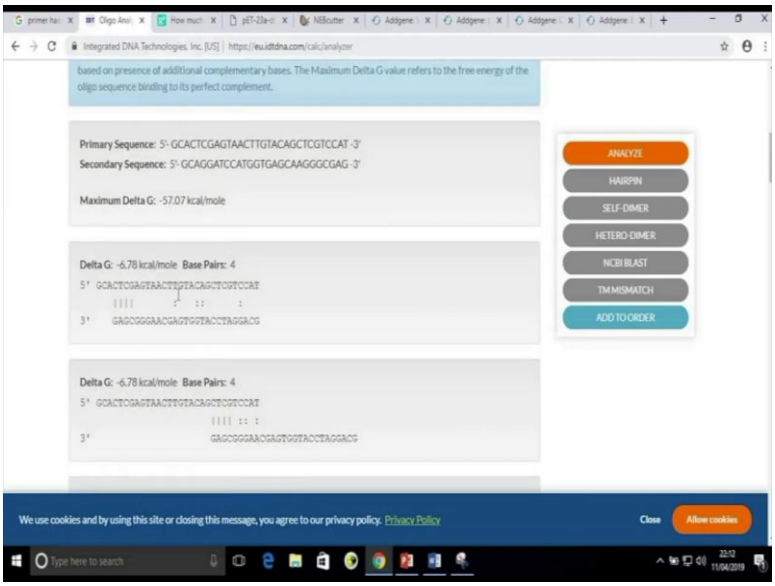

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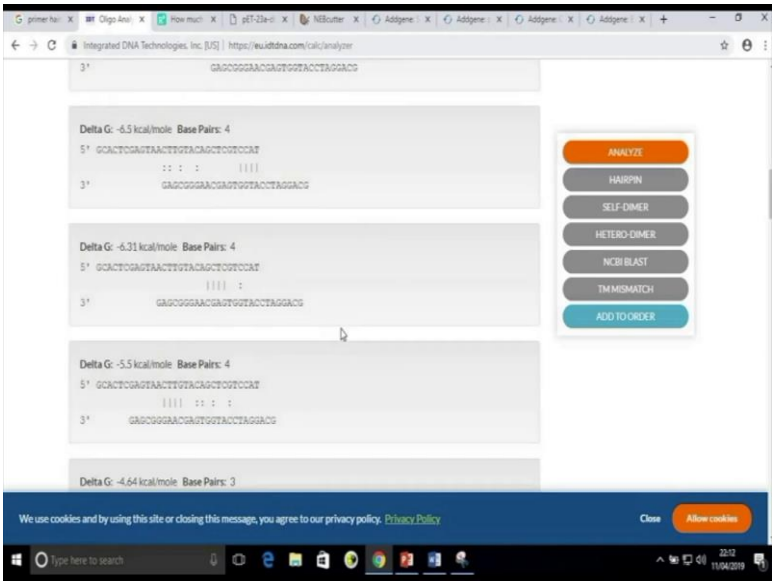

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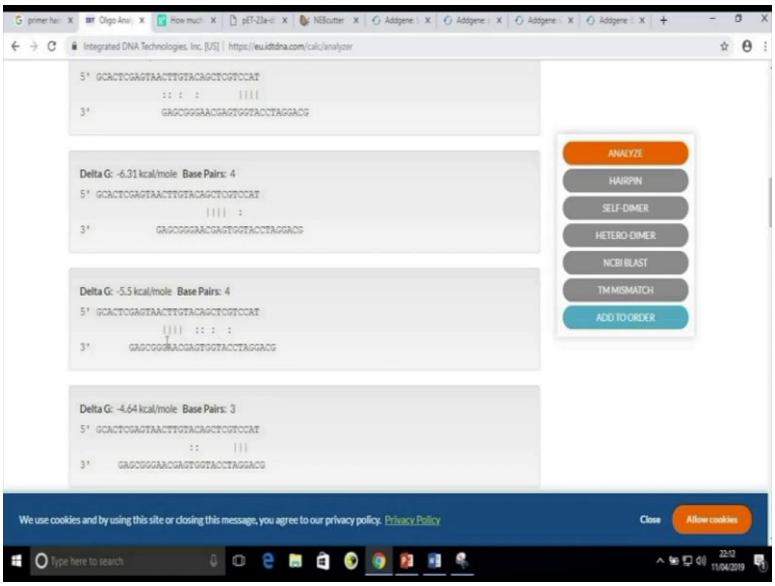

For hetero hetero dimer we need forward primer just Copy, Paste here and CALCULATE. It will give is there any hetero-dimers. This is because of restriction sit, this is also because of restriction site, this can be broken. Those which are at the end of the sequence they can be broken, but which is in middle if you those bases are middle it is very hard to disrupt those interactions and our amplification will be not good.

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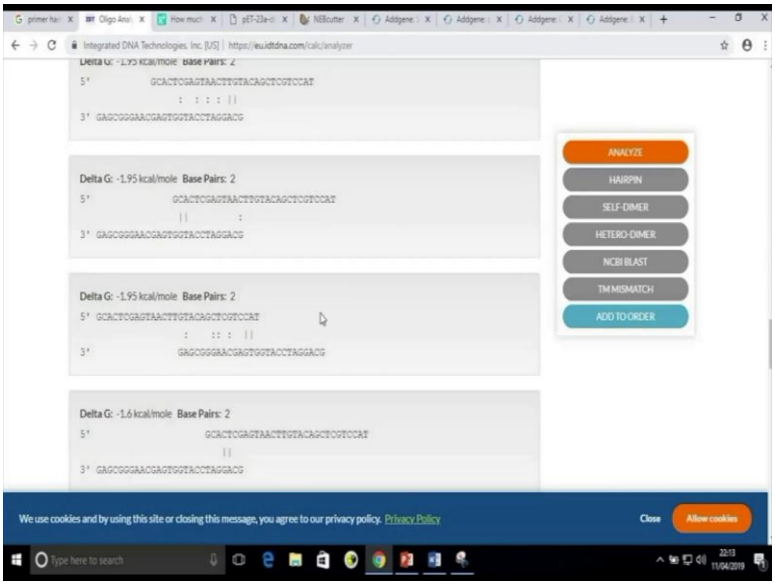

So, there is no amplification literally. Other kind of interactions will be broken easily. These are quick interactions. So, this is how we can prepare design the primers and

analyze the primers. We have done all these processes for designing forward and reverse primers.

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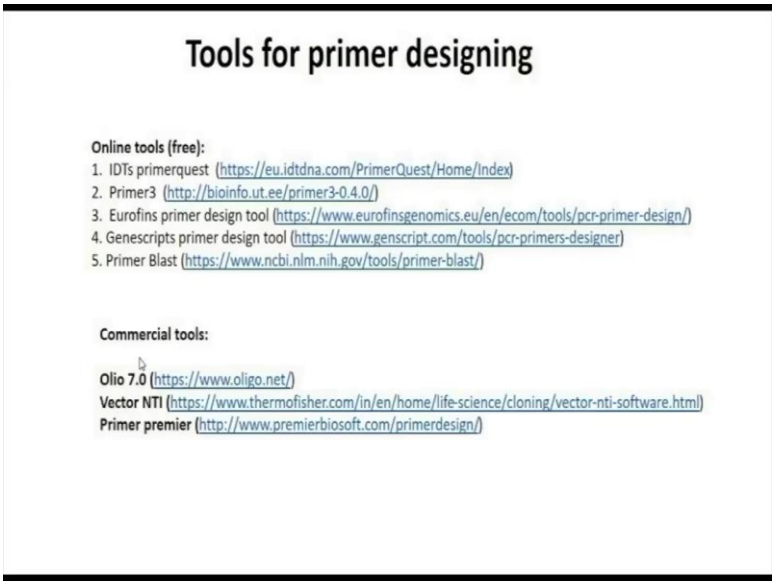

But, instead of doing manually, we can do it online. We just have to submit the sequence and it will return the forward and reverse primers. These are some of the tools available online for freely, but there are commercial tools also available like Olio 7, Vector NTI, Primer premier. So, if you interested in these softwares or you can just go through these sites and submit your sequence you will get your primers.

So, primer designing is a very very crucial event because it actually provides not only the specific product, but it also very very crucial for the getting the desired amplified products.

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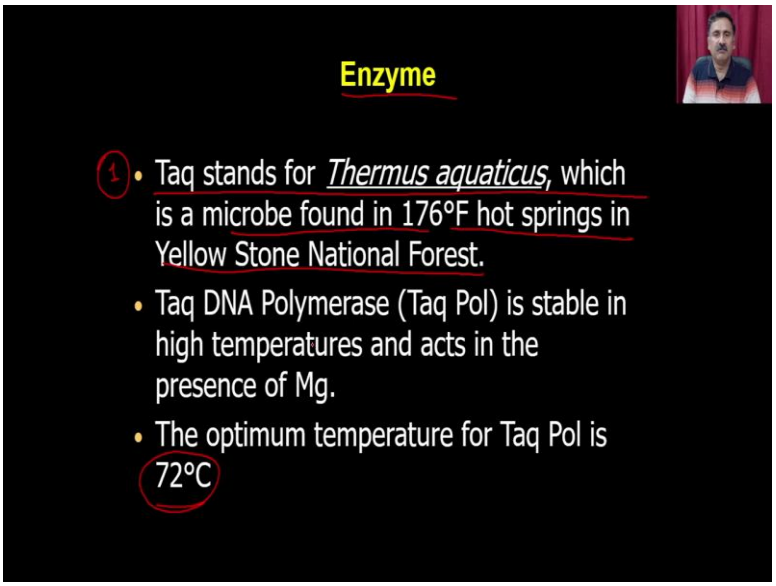

So, after the primer you also require the enzymes. So, you can have the multiple enzymes available, earlier there were only one enzyme the Taq DNA polymerase was available, but now there are multiple enzyme which are available. So, you can actually be able to use the Taq DNA polymerase.

So, Taq DNA polymerase which is a microbe found in which is from a microbe which is found in the 100 and 70 degree Fahrenheit in the Yellow Stone National Forest. Taq DNA polymerase is stable in high temperature and act in the presence of magnesium. The optimal temperature for Taq DNA polymerase is 72 degree Celsius.

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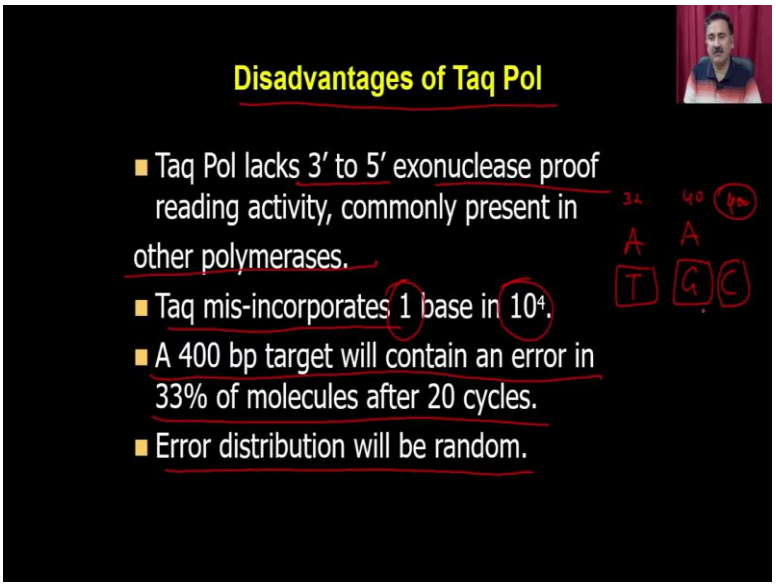
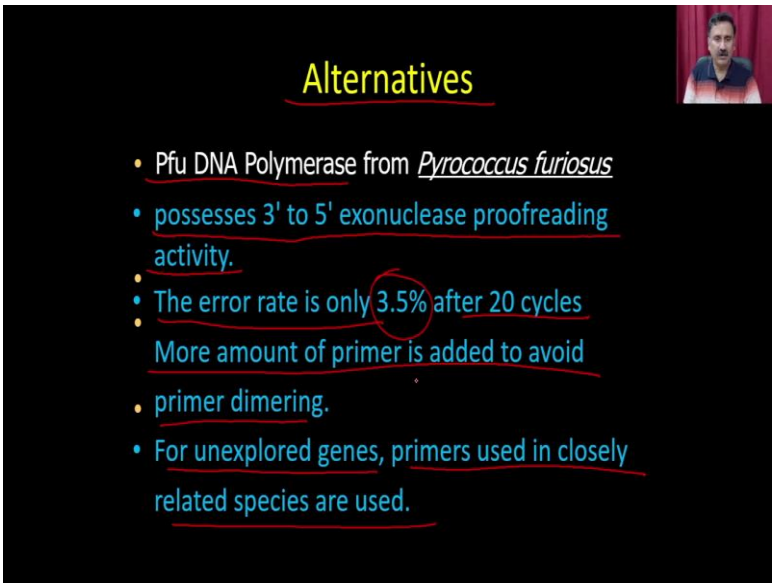


So, Taq DNA polymerase is one of the stranded enzyme what people use, but Taq DNA polymerase comes up with the following disadvantages. You can actually be able to get you know Taq DNA polymerase lacks 3 prime to 5 prime exonuclease proof reading activity, commonly present in other proteases other polymerases. Taq DNA polymerase mis-incorporate 1 base in 10 to power 4 bases.

This means it is actually going to incorporate wrong nucleotides. So, instead of A it is it should incorporate D, but instead of in front of A it actually going to incorporate G or C. So, in that cases it is actually going to lead to the formation of the mutated genes.

A 400 base pair target will contain an error in the 23 percent of the molecule after 20 cycle. Error distribution will be random which means sometime it may actually do the mutations at 32 positions, sometime it will do a position at 40 400 like that. So, it is. So, it is not like the error is going to be at a very specific point, it can be anywhere.(Refer Slide Time: 63:20)



So, to avoid this we have the alternatives you can actually be able to use the Pfu DNA polymerase from pyrococcus furiosus. And, it possesses the 3 prime to 5 prime exonuclease proofreading activity. So, because of that it actually even if it incorporates a mutated nucleotides it will actually go and correct that. The error rate is only 3.5 percent after 20 cycles.

So, more amount of primer is added to avoid the primer dimering formation. And, for unexplored genes, the primers used in closely related species are used, ok.

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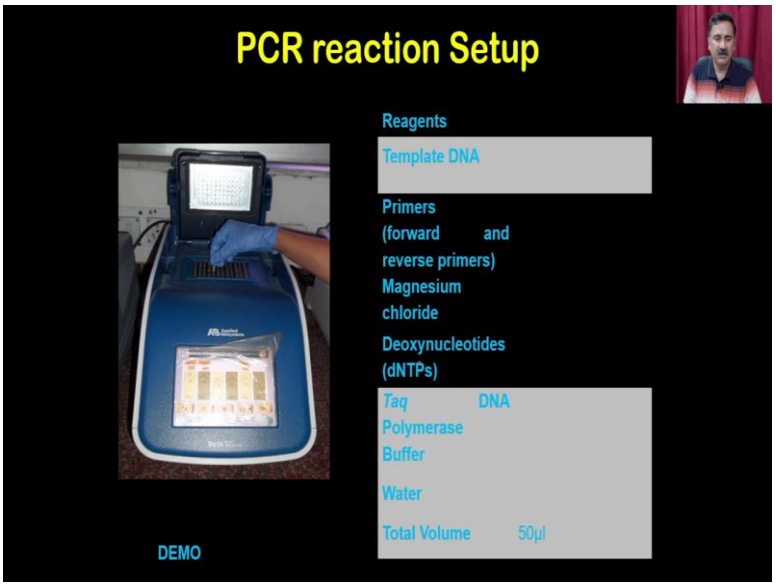

So, this is all about the PCR.

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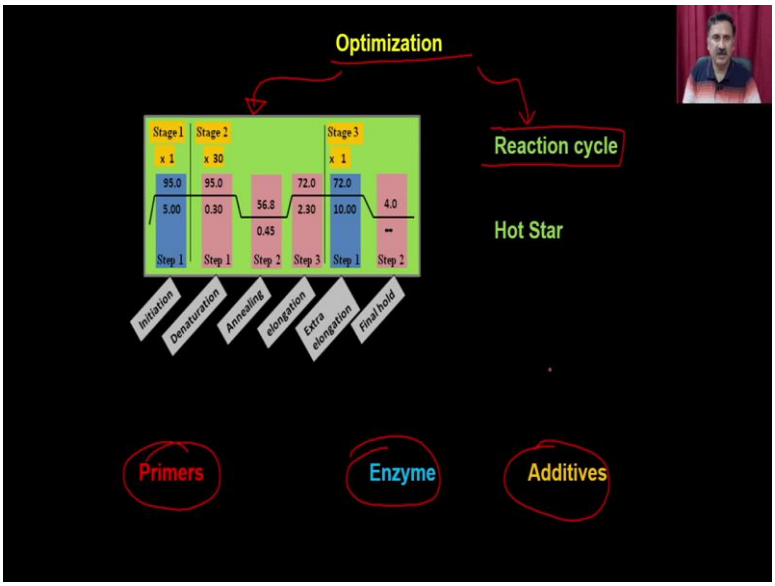

And, we have also discussed about the optimizations and there are many way in which you can be able to do the optimization of the PCR. So, when you perform the PCR you are actually going to face many problems like non-specific amplifications, no amplification and so on.

So, you actually have to adopt multiple optimization techniques to overcome that problem and optimization could be at the stage of setting up the reactions or the

optimization could be even at the reaction cycle also, ok. And, sometime the optimization can be done for primer sequences, enzymes and the additives. So, you can actually be able to use many of these things.

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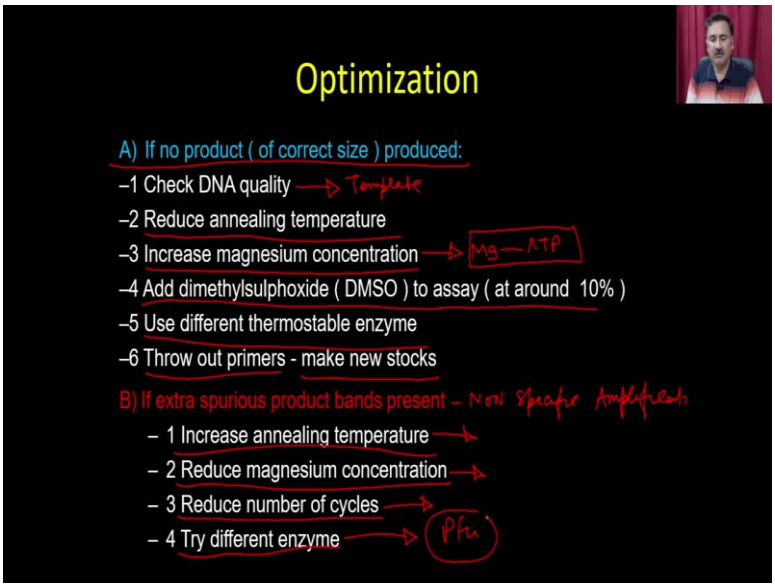

So, in one condition if no product can be produced, what are the things you can actually be able to use for you know to optimize? You can actually be able to check the DNA quality which means you can be able to use the template the quality of the template. You can actually be able to reduce the annealing temperature.

You can increase the magnesium concentration because magnesium is a co-factor. So, magnesium is forming a complex with ATP and that is how it is actually very very essential for the Taq DNA polymerase to perform the polymerization reactions. So, you can actually be able to vary the magnesium concentrations. Then you can also add the DMSO and if you add the DMSO it is also going to confer the specificity in the system.

You can use the different thermostable enzymes and that also is going to sometime work to provide you the product. And, if any of these things does not work then you can actually be able to design the new primers and it may actually be able to work. If the second condition is that in case you are getting the non specific amplification, so, if you are getting the non specific amplifications then you have to increase the annealing temperature.

If you increase the annealing temperature only at the point where you have the specific interactions only those are the points it is actually going to work. You can reduce the magnesium concentration. So, you can if you reduce the magnesium concentration, the Taq DNA polymerase is not going to work optimally. And, that is how it is actually going to work with those templates only where the you know the interaction is very strong.

You can reduce the number of cycles. Sometime what happen is if you are running it for 30 cycle, 40 cycle the enzymes actually you know becomes you know tired or it becomes more it becomes started making the non-specific products. So, in those cases you can actually reduce the cycles.

And, you can also start using the different enzyme like the PFU, so that the there could be more specificity in the system and that is why it is actually going to give you the better amplified product.

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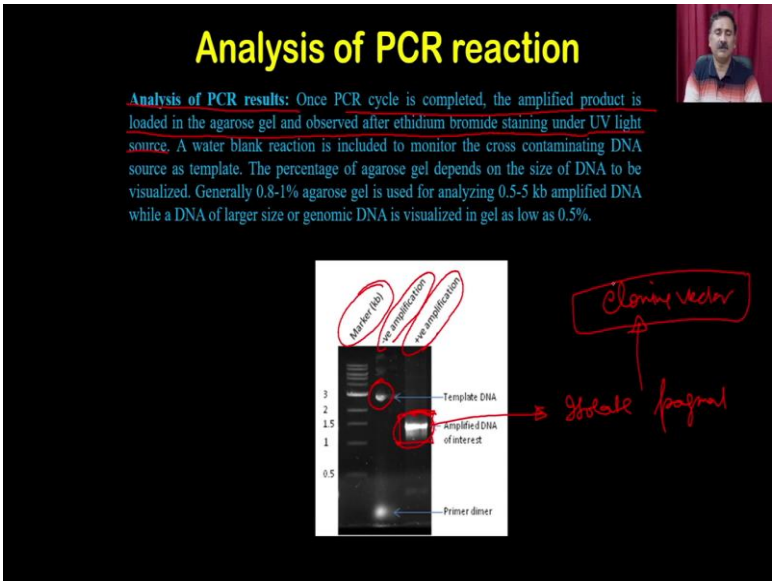

Once you have done the PCR, you are going to analyze that PCR onto a agarose gel. So, analysis of the PCR, once the PCR cycle is completed the amplified product is loaded onto the agarose gel and observed after the ethidium bromide staining under the UV light.

And, what you see here is this is the 1 kb ladder. This is the negatively amplified product which means it is only going to show you the template DNA and this is the positively amplified product which is actually going to give you the amplified product. So, this amplified product is completely absent in the negative amplifications.

So, this is all about the PCR reactions and which you can actually be able to use to amplify the gene of your interest. And, once you got the gene of interest for example, here you got the gene of interest then you can actually be able to cut this agarose block and you can be able to isolate the fragment, and once you isolate the fragment you can be able to put that into the cloning reactions.

Or you can actually be able to put into the cloning vector and that is how you can be able to use for that for the protein production. So, this is all about the PCR and how you can be able to set up the PCR, how you can be able to design the primers and so on. So, with this I would like to conclude my lecture here.

Thank you.