Experimental Biotechnology
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Lecture - 39
Polymerase Chain Reaction (Part - 2)

Hello everybody, this is Dr. Vishal Trivedi from department of biosciences and bioengineering IIT, Guwahati.

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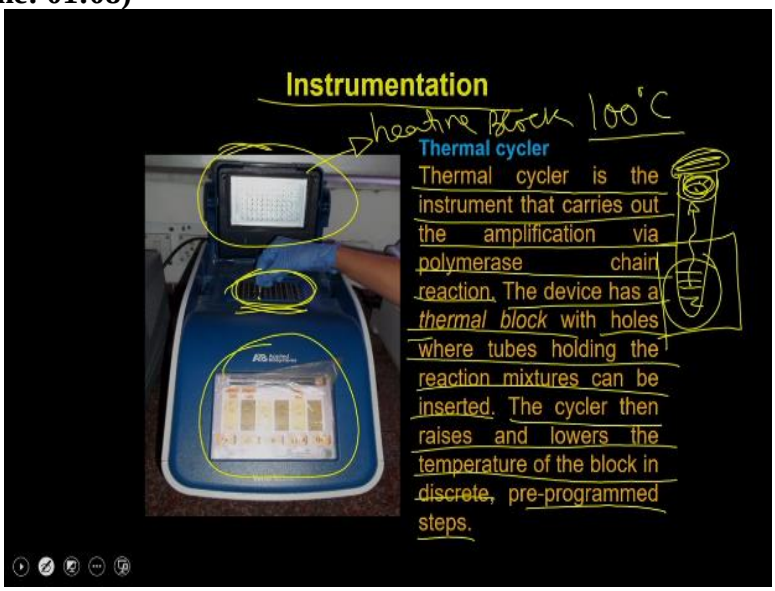

So, this is a, typical thermal cyclers so in a thermal cycler is the instrument that carries out the amplifications via the polymerase chain reactions the device has a thermal block. So you what you see is that you have a thermal block and these thermal blocks are actually having the holes in which you can be able to keep your eppendorf or the PCR tubes and this is the control unit where you can be able to set up these PCR reactions and you can be able to set up the initial denaturation.

Then denaturation, elongations, annealing and all that and you can change all these parameters, this is heating block which is actually been used once you are going to put the sample here then you can going close this led and your top heating block is actually going to be at 100 degrees celsius. So that it is actually going to stop the evaporation of the sample because what will happen is that once the tubes are going to be inserted into the sample block, it is actually going to be heat up.

Because 76 degrees celsius or 94 degrees celsius or 95 degrees celsius so during that period, the liquid what is present in the eppendorf is going to evaporate and if that liquid evaporate it will reach to the cap of that particular eppendorf. So if you do not have any mechanism, so, that it should not do that all your reaction mixture will not going to be present into this particular block it will actually be present onto the top of the cap.

Because if you see eppendorf you have the eppendorf and this is the cap actually so, if you keep the reaction mixture here what will happen is, it will going to evaporate and eventually all the reaction mixture is actually going to accumulate here instead of here because this is the place what you are keeping inside the block. So, if that happens then your eppendorf are going to be empty as far as the building block is concerned.

So whatever the temperature you keep does not matter and that is why people are actually the machines are having a heating block so that it maintains a temperature of 100 degrees but any chance the water will not going to come onto the top of the tube and it will going to condense because that is actually going to stop that the actions. So, this device has a thermal block which, actually holds where tube holding the reaction mixture can be inserted.

The cycler then raises and lower the temperature of the block in a discrete pre-programmed steps. So, once you set up the things it is actually going to increase the temperature as well as lower down the temperature.

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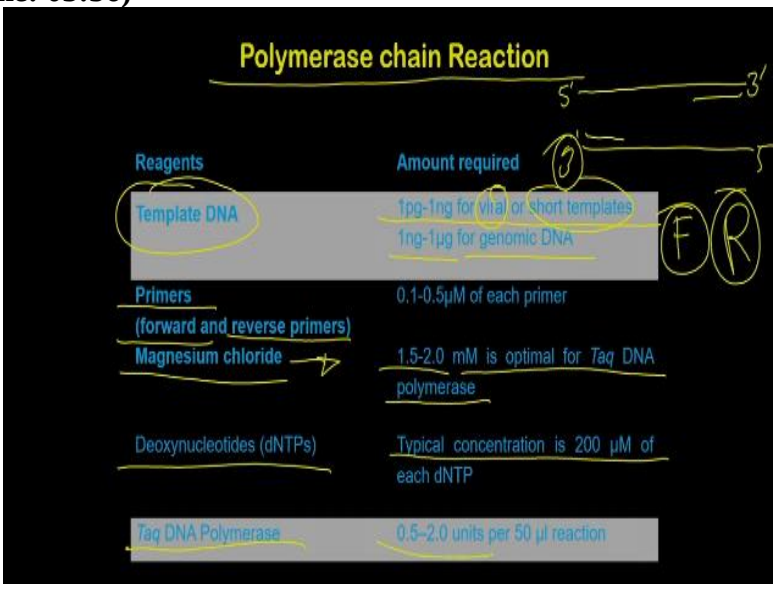

So, what are the things you require for polymerase chain reactions you require the template DNA so you have to have a template DNA of 1 picogram to 1 nanograms, if it is a viral or the short DNA. If it is a 1 nanogram to 1 microgram, if it is a genomic DNA then you required the primers you require the 2 primers one is forward primer and the reverse primer because as you have seen that you have a strand so it is going to be 5 prime to 3 prime and 3 prime to 5 prime so you require the 2 primers.

So, this is the strand which is actually going to bind to the 3 prime end of the your target DNA is going to be called as the forward primer and the strand what is going to be bind to your other strand is called as the reverse strand. Then you require the magnesium chloride, so magnesium chloride is going to make a complex with the ATP and that will be in the 1.5 to 2 millimolar for Taq DNA polymerase.

Then you require the dNTPs or deoxy nucleotides which is actually in the range of 200 micromoles and then you require the Taq DNA polymerase which is in the 0.5 to 2 units per 50 microliters.

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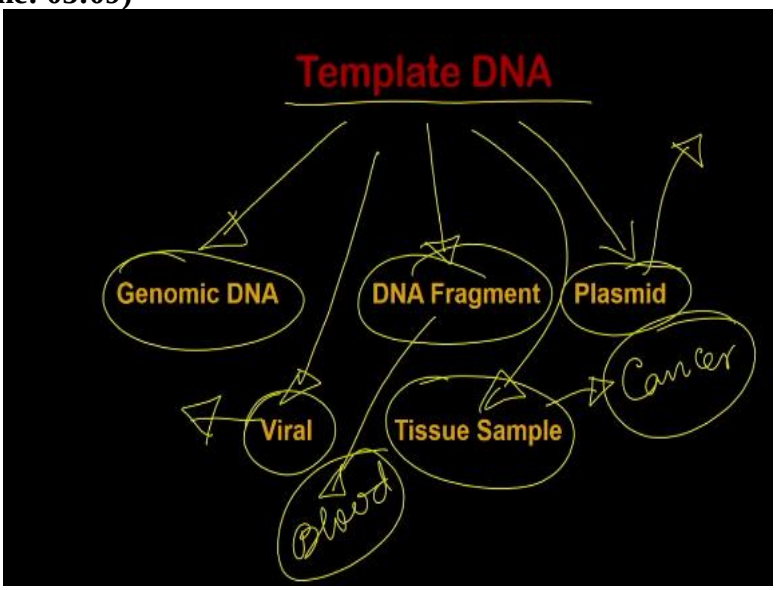

So as far as the template DNA, you have the multiple sources of template DNA for example, you have the genomic DNA. You have the DNA fragments you require you have the plasmids, you have the viral DNA and then you also have the tissue sample because the ultimate goal of the PCR is to amplify a particular DNA and that is how you can have the multiple reactions.

If you remember when we were discussing about the electrophoresis in one of the problem, what we said is that the people have discovered the old rock sample and they were actually got very small amount of DNA from a dinosaur and they were actually interested to deduce all other kinds of information but that was not possible from a small stretch of DNA and that is how they have done the PCR to amplify. So that is why it actually uses the multiple sources either you can use the genomic DNA.

You can use the DNA fragments like for example these DNA fragments could be present in the bloods like for example, if you if people have discovered or you know isolated a blood from a crime site and they want to identify the blood the person whom the blood is being contaminated or the person whom the blood is belonging. Then they can what they can do is they can just isolate the DNA from this particular blood.

And they can do the PCR to identify whether this particular DNA is belonging to that particular person or not same is true for the plasmids. For example, in some cases if you have generated recombinant plasmids you can be able to verify with the help of the primers PCR. Then you can use a tissue sample for example, if a person has the for example if you have discovered a tissue sample from a cancer patients or suppose some tissue sample is been present from the hepatitis.

And you want to know whether the hepatitis virus is present or not so in those cases what you can do is you can just simply isolate the DNA from those hepatitis samples and then you can do the PCR for the particular virus. Similarly you can do a viral sample itself for example if you have a viral sample and you want to identify the virus. Then you can do the PCR and so depending on the template you have to take the amount for example if it is a viral or the small DNA you can take the up to the nanogram level but if it is a larger DNA then you have to take it into a microgram level

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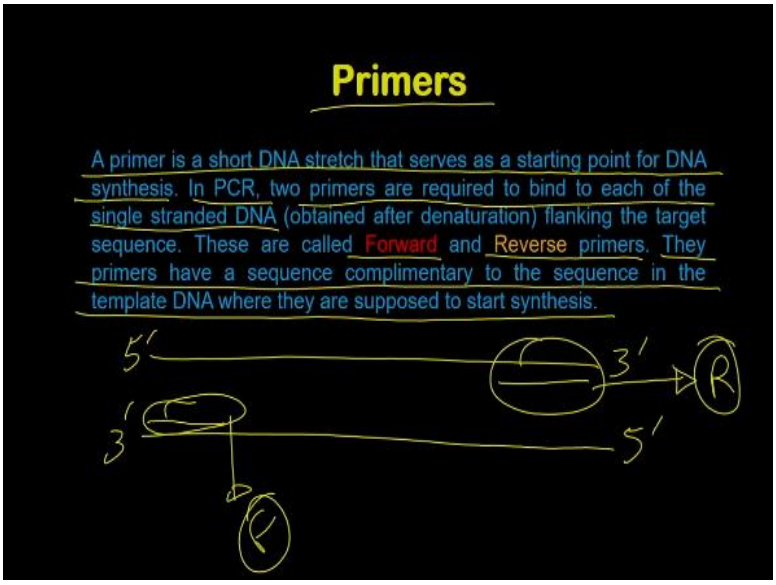

Then you require the primers so primer is a short DNA, strand that serve as a starting point for the DNA synthesis in PCR. You required the 2 primers which are actually going to bind to the single stranded DNA for required a forward primer as well as a reverse primers. So they primer have a sequence which is complementary to the sequence in the template DNA where they are supposed to start the synthesis. So the primers designing as well as primer analysis of how to design the good primers anyway we are going to discuss later on.

So the primers are like the complementary strands and what you can see is that a 5 prime to 3 prime if this is the template DNA. So what you can do is you can simply take the small stretch of this DNA and that is actually going to serve as the forward primer and if you take this stretch, it is actually going to give you the reverse primers. So, reverse primers actually goes into the reverse directions of the chain of synthesis, whereas a, forward primer goes into the same directions.

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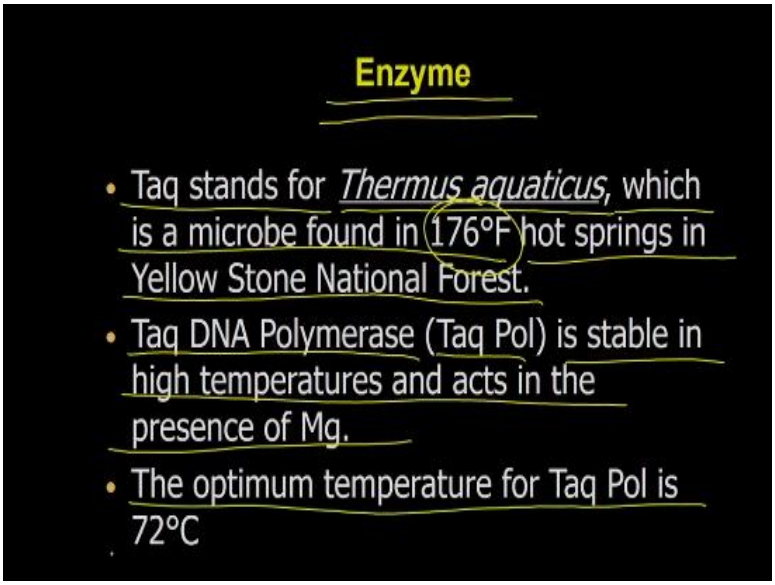

Then you require the enzymes so, you can have the different types of enzyme people have normally started with the Taq DNA polymerase. So the Taq DNA polymerase is been isolated from the Thermus aquaticus, which is microbe found in the 170 degrees celsius Fahrenheit hot springs in the Yellow Stone National Forest. So the Taq DNA polymerase or the Taq DNA is a stable DNA polymerase in a high temperature and it acts in the presence of magnesium. The optimal temperature for the Taq DNA polymerase is 72 degrees celsius.

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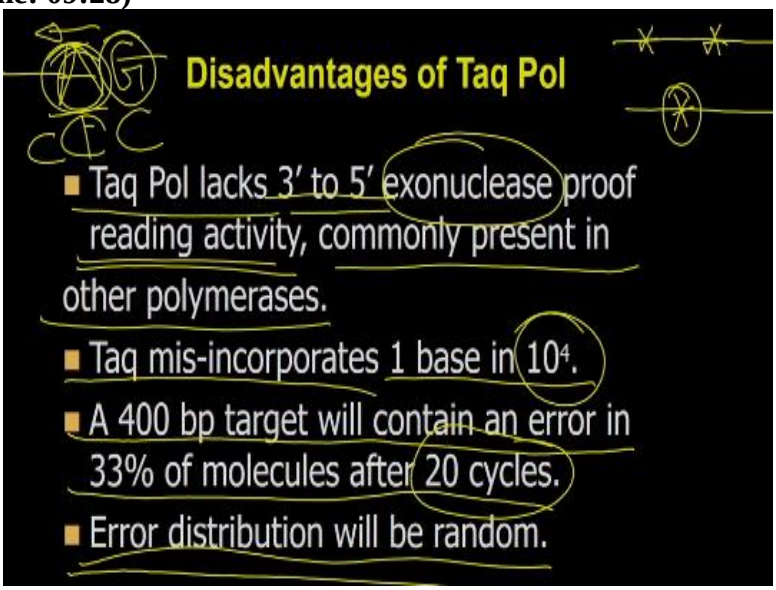

The Taq DNA polymerase lacks the 3 prime to 5 prime exonuclease proofreading activity and that is why it is actually commonly found in other polymerases. So what happened is that when you have the 3 prime to 5 prime exonuclease activity, it actually is a proofreading activity. For example when you are reading a sentence you can actually do a proofreading you can actually ask someone else to do a proofreading for example if you are writing a manuscript and you can ask someone to do a proofreading.

So, what the other guy will do is it is actually going to read your articles similarly the many enzymes have the proofreading activities. So, in this case it is a 3 prime to 5 prime proofreading activity which means the enzyme actually goes back and check whether I have added the correct nucleotides or not for example, if you have a strand if you have a A in the template and then the enzyme is actually going to add the T because that is the complementary nucleotides.

But what will happen is suppose you have the G after that so it is going to add the C but before it will go to the G it will actually go back 1 nucleotide back and then it will check again whether the T is being attached against to A or not and that is what it actually keep doing and that is what is called as a proofreading activity. For example by chance if it added the C for example instead of T it added the C.

Then it what is the enzyme is going to do is if it goes back and check that it is not T it is C actually then the enzyme is first going to correct the mistake and then only it will keep going. So that is why the proofreading activity is very important if you want to synthesize a DNA with no errors in that because of that the Taq mis-incorporate 1 base is in every 10 to power 4 bases which means if you have a 400 base pair target.

It is actually going to have an error 33% of the molecule after the 20 cycle which means if you have a 400 base pair the target DNA 33% of the molecules what is been going to synthesize after 20 cycles will actually going to have some errors if you are using the Taq DNA polymerase which means at that error is going to be distributed throughout the sequence because it is going to be random.

So in some sequence the error is being incorporates at a 10 nucleotide the 10th positions in some places it is the 100 positions in some. So what is mean is that if you have a strand like this and you are actually started generating the you know the mutations because wherever you have the A and if you put the C actually that is actually going to be changing the sequence because the your daughter sequence is now being changed.

So once the new sequence is going to be generated, the DNA is going to add the G instead of C actually because this is going to be a template later on. So that is actually going to generate

the erroneous DNA which is actually going to be not the identical copy because the ultimate goal of the PCR is to provide you the identical copy but it is going to give you the DNA with mutations which is actually going to be alteration of the bases.

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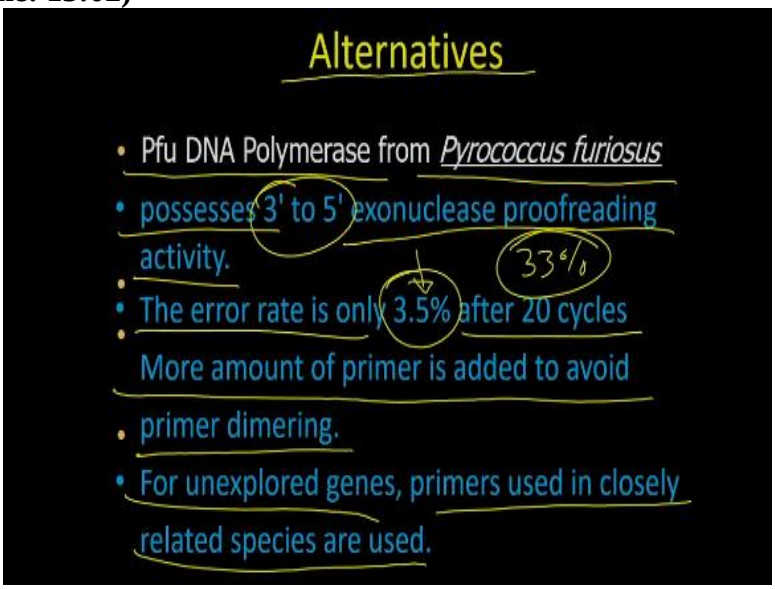

So what is the remedy for that? The remedy is that you can use the some new enzymes and one of the new enzyme what you can use is a Pfu DNA polymerase, which is from the Pyrococcus furiosus and that actually possesses the 3 prime to 5 prime exonuclease proofreading activity which means this enzyme if you use it is actually going to go back and check whether it has added the right nucleotide or not.

The error rate is only 3.5% if you remember the error rate was 33% in the case of Taq DNA polymerase but here the error rate is only 3.5% after the 20 cycles. The only problem is that it requires the more amounts of primers because and if you do so it is actually going to start producing the primer dimers so for unexplored genes, the primer used in closely related species are used.

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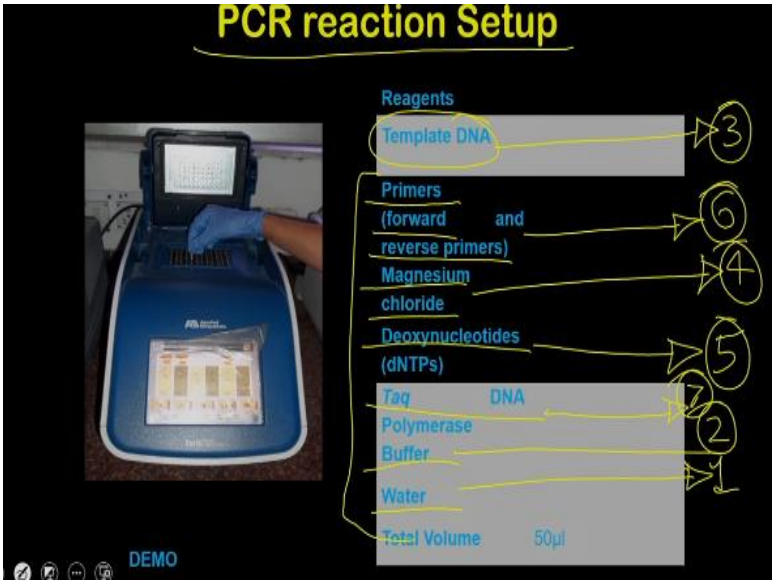

Now how to set up the PCR reactions, so what you require you require the template DNA as per the requirement if it is a genomic DNA it should be in the microgram range. If it is a short DNA like plasmids or virus then you have to take the primers like forward primers as well as the reverse primer then you require the magnesium chloride. You require the dNTPs you require the Taq DNA polymerase buffers and water, the sequence in which you are going to add all these is very simple.

You have to first take the water then you have to add the buffer and then you are going to add the template then you are going to add the magnesium chloride then you are going to add the dNTPs. Then you are actually going to add the primers and then lastly you are actually going to add the enzymes in some cases what people also do is they actually make a master mix utilizing all these and then they add the different templates.

So if suppose you have different templates and you want to use then what you can do is just exclude the primers as well as the template make the master mix aliquot it and then you add the template as well as the primers and then you just put it into the PCR machines and that is how it is actually going to do the PCR.

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In this video we will be demonstrating how to set up a PCR reaction and analyze the results using agarose gel electrophoresis. PCR or polymerase chain reaction is a widely used in molecular biology techniques to amplify a particular segment of DNA. It is also implied in biomedical research and forensic medicine. The main application of this polymerase chain

reaction is cloning to set up a PCR reaction we need template DNA, site specific primers, dNTPs mix, nucleus free water.

And Taq polymerase for a 50 microliters reaction in a typical concentration of 10 to 100 nanograms of template DNA use and 5 picomoles of each primer will be used. This is an earlier version of thermal cycler which contains display unit where we can observe the parameters and change the parameters. This is the hot shield this is sample holder and inside there is a peltier system.

Which can maintain the temperature fluctuations for setting up a PCR reaction in initial denaturation at 95 degrees, celsius 3 minutes and this steps, we will use 30 repeats where initial denaturation will be 30 seconds and annealing it. Extensions time should be given 1 minute per kb and here final extension should be given 10 minutes and hold it 4 degrees Celsius 10 minutes.

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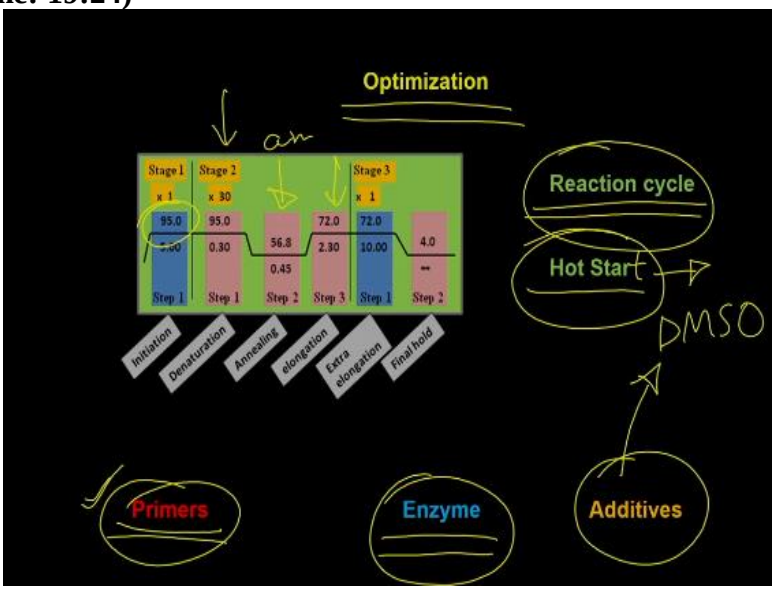

And then you have to do the setup of the reactions optimization also you have to do so there are multiple places where you can do the optimization for example in the within the reaction cycles. In the stage 2 you can be able to optimize simply by looking at the annealing temperature as well as the extension temperature because the annealing temperature is actually going to allow the annealing of the primer to the templates.

So that step is very crucial if you take if you keep the annealing temperature too low it is actually going to generate a nonspecific DNAs if you keep the annealing temperature too

high. It is actually not going to bind to the template and so it is actually going to give you no products apart from that, in some cases people also do hot start like for example if you remember what we were discussing that we actually you know going to do all this and then you are going to keep it into reactions.

But what happened is even during that you know room temperature when we are doing all these reactions and even if you are doing it in the 4 degree. Some initial products are being formed and once the initial products are being formed it actually going to interfere into your amplifications. So, if, there will be any nonspecific products, which are being produced and then it is actually going to affect your overall productivity.

Because, ultimately it is a PCR or polymerase chain reaction; where, the quantity of your final product as well as the accuracy of that sequence is very important. So if you actually going to add the reactions and you are going to keep it into the block if the block normally takes some time for it to heat up but during that process, some of the nonspecific you know product is going to be formed.

And that is actually going to start eating up your primers enzymes and all that machinery and because of that the overall production is going to be less and on the other hand it is actually going to give you the nonspecific product. So, what people do in hot start is that they keep the cycle running and then they let the machine to reach to a 100 degrees celsius temperature or 95 degrees celsius once the machine reach to that particular temperature.

Then they keep the reactions into the machine then they actually put the reaction that is how actually called the hot start because you are starting the reaction or you are incubating the reactions only when it is in the hot conditions. Apart from that you can also play a lot with the primers because primers are the crust or you know the major bottleneck if you are not getting the amplified product it could be because of the you know primers.

Because the primer what you are synthesizing may have multiple problems. So, all that anyway we are going to discuss then enzymes anyway we have already discussed that if you are getting the mutations, then you can use Pfu DNA polymerase instead of the Taq DNA polymerase. And then you also have the some of the additives like you can use the DMSO

you can use the betaine and all those kind of additives so that it can actually break the secondary structures.

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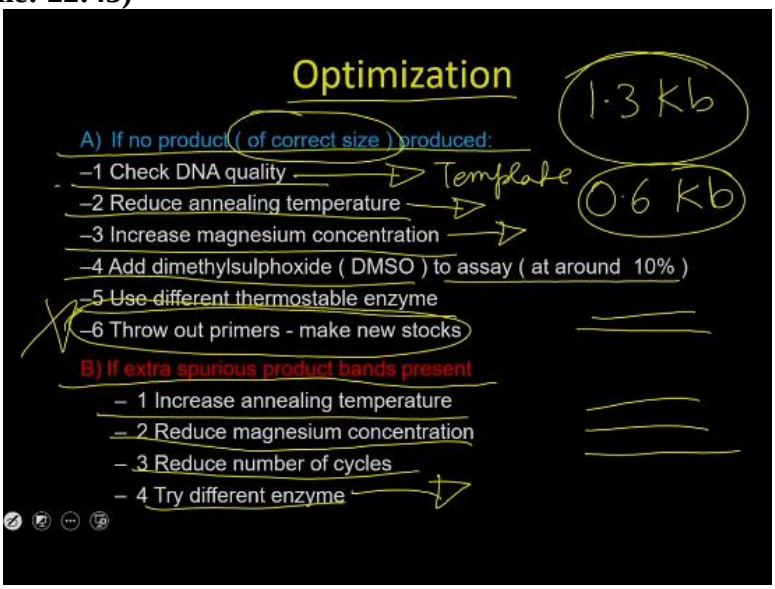

Now you can do optimizations so you what are the optimization if you suppose see no products or if you do not see a product of your correct size. So when you start a PCR you normally know that what product I should expect for example, if I am expecting a 1.3 kB product if I am getting 0.6 kb amplification that means I am not getting the right product. So, what you have to do is in that case, first you have to do is you should check the quality of the template DNA which means your template DNA you reduce the annealing temperature.

Because that may be the reason that the primer is not binding to the annealing temperature the primer is not binding to the target DNA, you increase the magnesium chloride so, that it will actually going to provide the energy into the system and therefore, it is actually asking the Taq DNA polymerase to work more efficiently you can add the DMSO as well as the betaine which actually is going to affect the secondary structure of the primers and that actually also going to you know help in terms of getting the synthesis.

You can use them other in thermostable enzymes or ultimately if nothing works then you can actually throw your existing primers and you can actually synthesis the new primer because what happen is sometime when the primers are being supplied and you are setting up the reactions primers are single stranded DNA. So, these single stranded DNA are very susceptible for DNases.

So if you have any kind of degradation of those DNA, it is actually going to affect the overall amplifications. In second condition is that if you have the extra products like if you are expecting 1.3 kb but instead of that you are also getting few more products in that case. You increase the annealing temperatures you reduce the magnesium chloride and you reduce the number of cycles.

And you try another enzyme you try the enzyme which is actually having the high fidelity which means the enzyme which is having going to provide more specificity. So, in that cases the enzyme is going to be specific for your specific target sequence compared to the nonspecific target sequence and that is, how you might be able eliminate these nonspecific bands.

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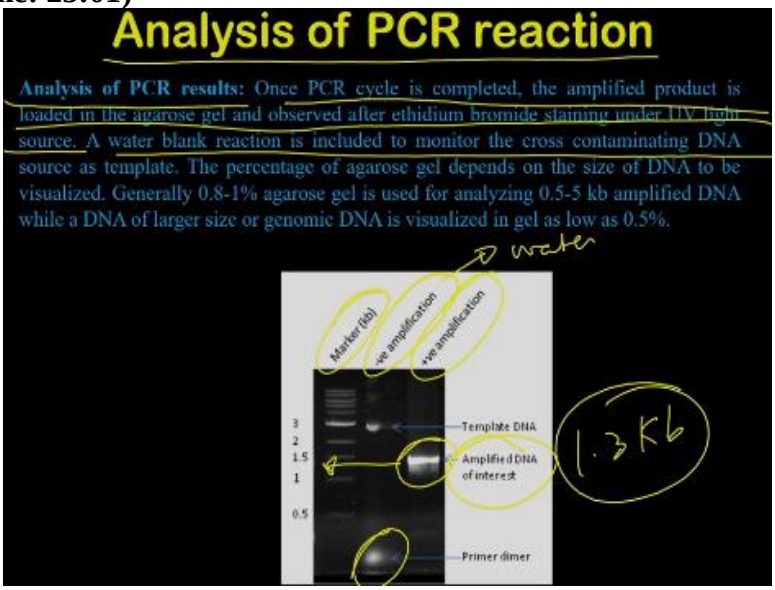

Then you have to analyze the PCR so you have to do analysis of PCR reaction so once a PCR cycle is complete, the amplified product is loaded into agarose gel and observed after the ethidium bromide with the UV light source a water blank reaction is also been added. So, that you can be able to monitor if there will be a nonspecific question. So instead of adding a template you can also add the water.

So, that you will know what kind of amplification you will get if there will be only water present and ideally what you do is you run with the 1 kb marker. So, this is the DNA marker it is a negative amplifications, where you have added the water and this is the positive applications where you are actually going to get the single band. So, this is the amplified

DNA of your interest and then you can be able to calculate whether I am getting the desired size or not.

So for example in this case I am getting a band of 1.3 kb so that is a desirable band and what you will see is that you are getting a primers dimmer because the primers are not being used in the negative reaction, so that is why they are being presented into the reactions. So, this is all about the PCR and how to set up the PCR and we have prepared a very small demo to show you how to set up the PCR reactions how to analysis the mixtures and how to analysis the products and in this particular demo the students have discussed different steps related to the PCR.

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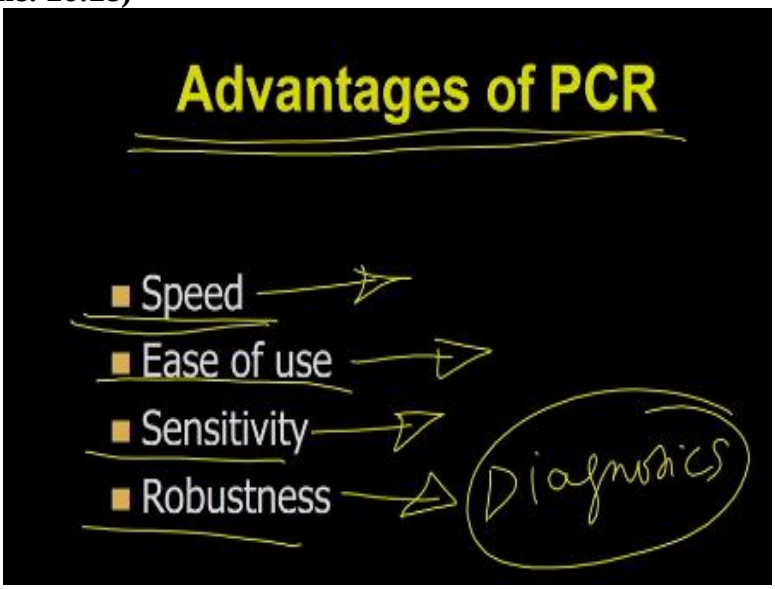

Now what are the advantages of the PCR so the advantages of PCR is that it is actually a fast process is easy to perform which means it is actually very easy you can just take the templates and you mix all these recipes into a you know kind of a particular order and then you just put it into the machine and that actually is going to give you the desired product it is very sensitive which means if you can modulate you are actually going to get the desirable results.

And it is very robust which means it is actually going to give you the reproducible results so if you have the same sequence of primers, you have the same template and you have the same enzyme. It is actually very, very robust it will be keep giving you the results and that is why you can be able to use the primer for many applications like you can use it for diagnostics and all that because it is very robust so it will actually going to give you the desirable results.

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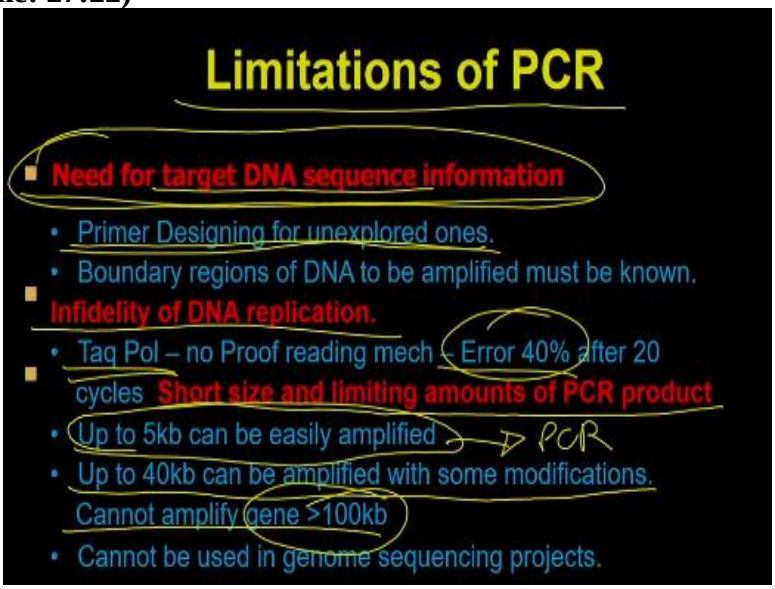

What are the limitations of the PCR? The limitation of the PCR is that it requires the target the need for a sequence of the target information like you have to design a primer. So until you do not have the target sequence, it is very difficult for the primer designing. Then you require the fidelity of the DNA replication so Taq DNA polymerase is a low fidelity DNA. So it is actually not going to give you an enough amplifications and the error is going to be around 40%.

So, it is actually going to it is good for the small stretches of DNA but it is not good for the long stretches of DNA and then the PCR having a limitation in terms of the you know amplification of the different sizes of DNA up to 5 kb DNA it can be easily amplified with the help of the PCR. But if you go beyond the 5 kb like up to 40 kb still the DNA can be amplified with some modifications.

But if you go beyond that it cannot be able to amplify the gene which is of 100 kb which means it is actually having the limitations and that limitation is actually causing a lot of problem because you cannot use the PCR for sequencing the genomes and you cannot use that PCR for that particular application. So that is why the PCR is been always been limited to those applications where you have a very small stretch of DNA and like for example 1 kb, 2kb so that it will actually going to give you the amplified products.

So, this is all about the polymerase chain reactions and we have discussed about the different types of technical aspects, how to set up the reactions and what are the different parameters

you should consider while you are setting up the PCR and at the end we have also going to show you a demo we are actually going to show you all the steps what you are going to do and with this I would like to conclude my lecture here. Thank you.