Essentials of Biomolecules : Nucleic Acids, Peptides and Carbohydrates

Dr.Lal Mohan Kundu
Department of Chemistry
Indian Institute of Technology, Guwahati

Lecture-11
DNA Replication, Polymerases, DNA Sequencing and Polymerase Chain Reaction

Hello, everybody, and welcome back. Today we will talk about polymerase chain reaction in short PCR. So this is our technique that will allow us to make more copies of DNA. In other words, this is a technique of amplification of DNA. So we have seen how the DNA replication happens inside the cells in eukaryotic cells. That it involves so many numbers of enzymes. And it is the stepwise reactions quite complicated, many enzymes are involved.

Along with that, some are some ingredients are also needed. So, polymerase chain reaction is a method that will allow us to do the DNA amplification in laboratory in vitro. So, when something happens inside the living organisms, living cells, we call it in vivo. Outside the living cells, for example, in the laboratory that you are may making is called in vitro. So, we will see today how you can do the amplification of DNA how you can make more copies of DNA in your laboratory.

And this is absolutely essential thing because the amount of DNA that you can isolate from different sources during your studies, for example, diseased cells, for example, microorganisms, you have isolated DNA from the microorganisms from diseased cells and other sources, blood cells and so on, and you want to study the various properties of those genes, then the crucial thing is that the amount that you isolate is actually very small.

And with that, you cannot do your laboratory steps; you cannot do many experiments with them. So, it is absolutely essential that we need more quantity of those genes or more quantity of those DNA. Let us say somebody you have seen, we collect the people, or the scientists or even for the forensic teams, they collect hair sample or the blood samples or skin samples from diseased persons, if there are multi many casualties, many some mishap happens and there are many casualties you are not identified those persons.

So, many times what people do nowadays the forensic team they collect even the hair samples and then isolate the DNA and then they find out the identity of the person. So, you can imagine the amount of DNA that you can isolate from a hair sample is so tiny, so small that with that you cannot carry out proper investigations in the laboratory. So, you really need a large more quantity of material, more quantity of DNA to carry out your needs to carry out your experiment in the laboratory.

So it is absolutely essential to increase the quantity of the DNA and this is the technique that will allow you to make more quantities of the gene or the DNA that you have isolated. That is the beauty of this method and that is where comes the importance of this method. So, until PCR technique, the biochemistry or the biochemical techniques, even some chemistry tools were stuck.

So, after the invention of PCR, it has given a huge boost to the biochemist people to the biotechnologists and even to the chemist that has really increased the level of the research. So the key person who has figured out all the real steps for the PCR polymerase chain reaction is Kary Mullis.

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Module 3

Polymerase Chain Reaction (PCR)

PCR is a technique to amplify genomic DNA, extracted from organisms. It's one of the most important discoveries that has revolutionized biochemical/biological research.

Applications: Identifying cDNAs or any genomic DNA, detection of genomic mutations, diagnosis of genetic diseases.

Principles: Complementary to DNA replication, requires thermal cycles.

The basic process of PCR requires mainly 5 components:

Kary Mullis
PCR → 1983
Nobel prize → 1993
in chemistry

Kary Mullis is the person who has developed the whole systems of the PCR. The PCR technique was actually fully designed or fully developed back in 1983, quite recently, actually not very long. And he received Nobel Prize in 1993 and this is one of the really biggest inventions in the

world or in the research of biochemistry, biotechnology and chemistry. You know, Kary Mullis has actually received the Nobel Prize in chemistry, not in physiology and medicine.

Because as we will see later, very soon, all the methods that you are going to use in the laboratory, they involve chemical processes, chemical reactions. So that is what is the background of the polymerase chain reactions. As the name suggests, of course you can understand it needs the polymerase. And it is a process or estimated that is based on the enzyme polymerase. So, PCR is a technique to amplify genomic DNA that we have extracted from organisms.

It is one of the most important discoveries that has revolutionized by chemical or biological research. It is very, true actually. What are the applications identifying the cDNA means the chromosomal DNA or any genomic DNA detection of genomic mutations? That is one of the key reasons that we need to study the genes because if you isolate a gene from a diseased cells, we need to know whether there are mutations inside it or whether the same they are the same gene as the normal or what kind of abnormality has happened to those genes.

So, genomic mutations, diagnosis of genetic diseases. So there are many changes, alterations that happen in the genome, because of that the disease like Parkinson's disease, like diabetes disease like cancers happen. So this is for disease diagnosis, this is an absolutely important tool. The principal will shortly see it is a complementary to DNA replication. Now, if you recall, the DNA replication process that has been happening inside the cell, so we have seen it goes through multiple steps.

Now the question is, if you want to do the same shorter of steps, can you do the same process in your laboratory? Can you do DNA amplification, or the DNA replication that we have seen in a test tube? So let us see that and then assess whether the same process can be mimicked in your laboratory and if not, what are the problems and how can you solve them? So first, if you remember for DNA replication process, what are the ingredients that have been that were needed?

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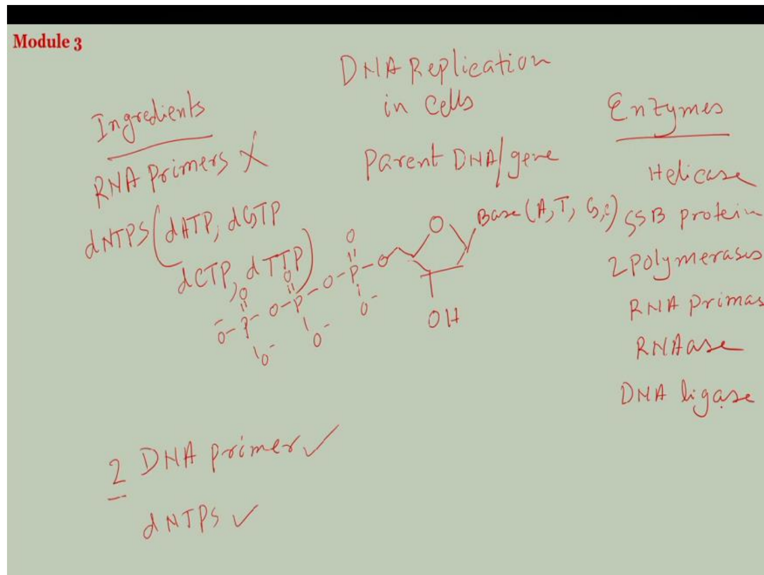

DNA replication that is in cell in living organisms. Ingredients and here I will write the enzymes. What are the reagents that were required? And what are the enzymes that were required? What are the reagents or the ingredients that were required? Of course, in the middle of it, you needed the sample DNA. The DNA that you want to replicate or your parent DNA or gene, that is what you require first, because that is what you want to make more copies off second what you need you needed RNA primers, if I remember correctly.

So first, you needed actually multiple number of RNA primers, because if you remember the leading strand required 1 RNA primer well the lagging strand required many number of RNA primers because you have were starting from the middle of the strand from somewhere in between region of the strands. So, every time the strand was opening up, you had to start with a new RNA primer. So many number of RNA primers were required.

What else did you need you needed the dNTPs the free nucleic acids 4 dNTPs were required. Of course, for example, dATP, adenosine triphosphate, you needed dGTP guanosine triphosphate, you needed dCTP cited in triphosphate and then you needed dTTP thymidine triphosphate. So, general formula being this, is the base A, T, G, C the structure was this 3 prime hydroxyl free O and the phosphate, triphosphate in the 5 prime end this is what you needed. Did you need anything else or any primers dNTPs.

Mostly those ingredients were needed and you needed a lot of enzymes. The first enzyme that you required was helicase. Then you needed single stranded binding protein after that game the polymerases you needed 2 polymerases actually for 2 strands if you remember before that you of course needed a RNA primase that synthesized the RNA primers so RNA primase was needed. After that you needed RNAase to destroy all the RNA primers then you needed DNA ligase to stitch all the fragmented DNA is that have formed.

So at least these are the number of enzymes that you required and these are the ingredients chemical structures that you required for the replication of DNA inside the cell. Now, the question is, if you take all of these together in a test tube are going to be successful is it going to work in doing the replication.

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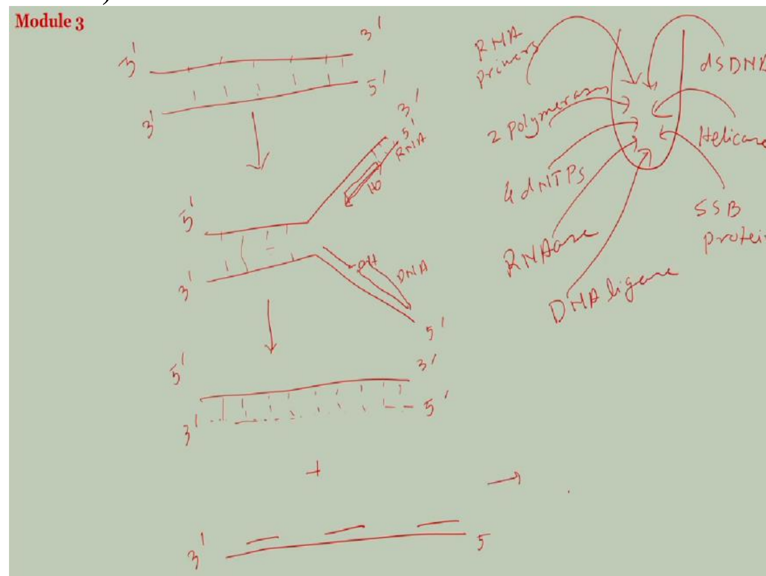

So if we see, let us say, this is your test tube, and you put first your double stranded DNA target DNA, this is your double stranded DNA, 5 prime to 3 prime, 3 prime to 5 prime all the hydrogen mornings are there now, the first thing was you had to open the double helicase and you needed the helicase. So we had helicase here that is going to open up make a replication fork and then that had to be stabilized by SSB protein. So far so good.

Now what you need, now you need the RNA primers, at the RNA primers maybe with the help of the primase enzyme and that will start the replication. So this is your RNA that was 5 Prime you have 3 prime hydroxyl group free. Here the primer would be this with the 3 prime hydroxyl

group free good enough. Now, what you need you need to replicate you need the extension you need to synthesize the complimentary strand that is done by polymerase. So, you add the 2 polymerases for 2 different strands.

So, the polymerase is going to bind here and then you need the all the 4 dNTPs. So suitably according to what you have on the opposite strand that the complimentary triphosphate would be taken up by the polymerase and it will go on continue the synthesis same thing will happen here. So, you will get the so this is RNA, and you will synthesize the DNA on it. So, this is your DNA. This is your DNA so, your half portion is finished and then you can open up more and can go back.

That is how you will get the leading strand. We have seen 5 prime to 3 prime that is the leading strand and you can synthesize continuously. The complimentary new strand that is 5 prime to 3 prime, this here constant plus this is your lagging strand variant, 3 prime to 5 prime and you will synthesize the DNA in fragments. All the gap were originally occupied by the RNA primers that you have digested that you have to cleave by the enzyme RNAase. And then you have used ligase DNA ligase to get the full complimentary strand, 3 prime to 5 prime, 5 prime to 3 prime for this.

So, from one DNA you can synthesize in the first cycle to DNAase fine. Now, this thing should go on and on in a cycle because you have added all the enzymes, they are not going to stop there. And you need more quantities of DNA. So now think of the next cycle is it going to work is this and this going to be replicated further under your condition in your test tube, I do not think so. I do not think it is going to work in the next cycle anymore. Once you have added all the ingredients in the same test tube, let us see whether it can go on.

First thing is that you have added RNAase there in your test tube. So for the second cycle also, you need RNA primers for this strand and this strand all strands basically you need RNA primers. So, even if you add second cycle.

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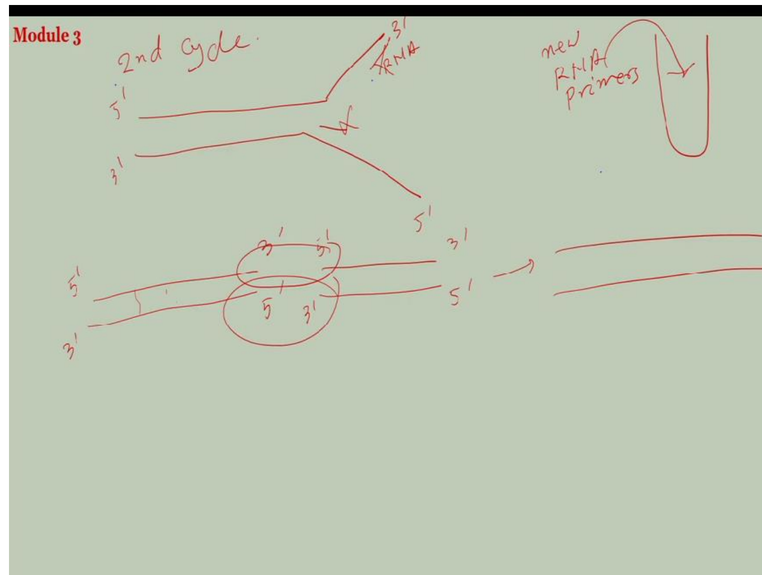

So 5 prime 3 prime, you had opened the second replication fork for one strand, 3 prime, 5 prime, you need the RNA prime on here, you need that RNA prime on there. This is your test tube that contents all of them already. But you had RNAase there. So RNAase has actually cleaved off or destroyed all the RNA that you have used before. So even if you add new RNA primers is it going to are they going to work? No because you already have the RNAase present in your test tube, the moment you add the RNA primers, they will be destroyed.

So, all the RNA that would be destroyed. And if the RNAase are not there, the extensions cannot happen. Polymerase cannot react. That is problem number 1. Second problem is, you see, you have synthesized 1 new strand, these 2 new strands there. So, you have some copies of DNA already in your solution. You can write it this way that you have a double stranded DNA, 5 prime to 3 prime,3 prime to 5 prime and here you have again some copies may be the same.

So 5 prime, 3 prime, 3 prime, 5 prime some copies are floating around in your solution and you have DNA ligase there so, the very first thing, what will happen is DNA ligase will stitch together whatever DNA you have in the test tube. So these 2 would be stitched together these 2 would be stitched together and instead of getting your real DNA, you will get now longer version of DNA.

So your originality is lost. Moreover, if you think that even if it goes instead, without RNAase even it can synthesize the next strands, you are going to synthesize the fragmented DNAase and those fragmented DNAase before they are synthesized. I mean, right after they are synthesized right after they have been made, they can and nonspecifically connect to each other because you have ligase present already in your solution.

So several problems in other words, if you want to do Using the same set of enzymes, using the same set of ingredients, that happens in a biological cell, you are not going to get your amplified DNA that process of DNA replication cannot be performed smoothly in a test tube without the living cells. So that is the problem of mimicking the polymerase mimicking the DNA replication in the lab.

So, what is the way out? How can you change this? How can you make it work? So, one thing you have to remember is that you are in a laboratory and you are doing it in a test tube. So, you have some freedom that you do not have in a living cell. So, we have to think that way and think what we can do to develop or to find out a new method to so that the process may work.

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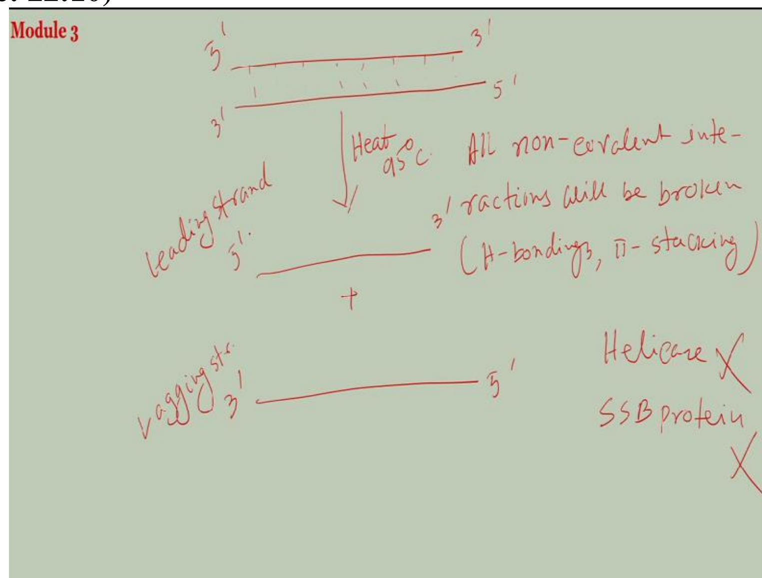

So, if you look, you have initially your target DNA double stranded DNA. What was the first criteria? The first criteria was that you need to unfold the DNA at least partially. That is why you needed the enzyme helicase and that is why you needed the enzyme, single stranded binding protein. Now, think of it can you do this thing in your laboratory? Can you unfold your DNA or

the gene in your laboratory or what should be the condition if you want to open them up. You remember we have talked about DNA, DNA denaturation.

DNA denaturation means that to a double stranded DNA would be split into 2 single standard DNA, that is DNA denaturation, So now think of it way, how can you do our DNA denaturation basically means that you have to break all the noncovalent interactions that is present within your DNA, all the hydrogen bonds, all the pie stacking interactions should be broken down. And in your laboratory, what is the tool you have to do it? Very simple, by heat, if you simply heat it up to a little bit higher temperature, we usually do it at 95 degrees Celsius.

You do not go to 100 degrees Celsius because in that temperature your solution which usually in water, DNA soluble, very much soluble in water. So most of the work of DNA we do in aqueous medium, there are other buffers in it, but the medium is aqueous. So it will start boiling at close to 100 degrees Celsius temperature. And so we do not want that. That is why that temperature is kept a little bit less close to 95 degrees Celsius. If you heat your DNA at 95 degrees Celsius, what is going to happened?

It is going to break all your non covalent interactions. So at this stage, will be broken which includes hydrogen bonding pi stacking and all. So, in other words, your base pairs would be destroyed and you can very much have 2 single stranded DNA free from each other no interaction at all. So, by simple trick of heat, you can separate the DNA into 2 single stranded DNA. Now, of course, you can understand the same condition obviously cannot be used in cellular system.

Because 95 degrees Celsius temperature inside our cells if you want to increase that temperature. We will all die so, that is what the freedom we have in the laboratory that you can use heat to make the double stranded DNA into the single stranded DNA. What is the other advantage in cell you have opened the DNA or you have opened the gene partially and that is where all the problems were in the lagging strand?

Here, you see once you put heat, they are completely separated whole of the double strand is completely separated not partial separation, complete separation of the double strand into 2 individual single strands. So, that gives you full freedom of the single strands and now, you do not have the replication fork, they are all open and as we will see you can understand that it makes the process so simple now.

Because of the simple treatment because of this very simple ingredient that is the heat in this case so, since you have opened with the heat, you do not need helicase it is not needed your SSB protein not needed. Now you move on to the next step what else is required? So let us take this strand first. I still call it leading strand and lagging strand which was originally being called in the cell. But they are no longer actually leading stand they are no longer lagging stand they will behave as equal. Now we will see. So if you take this 5 prime to 3 prime

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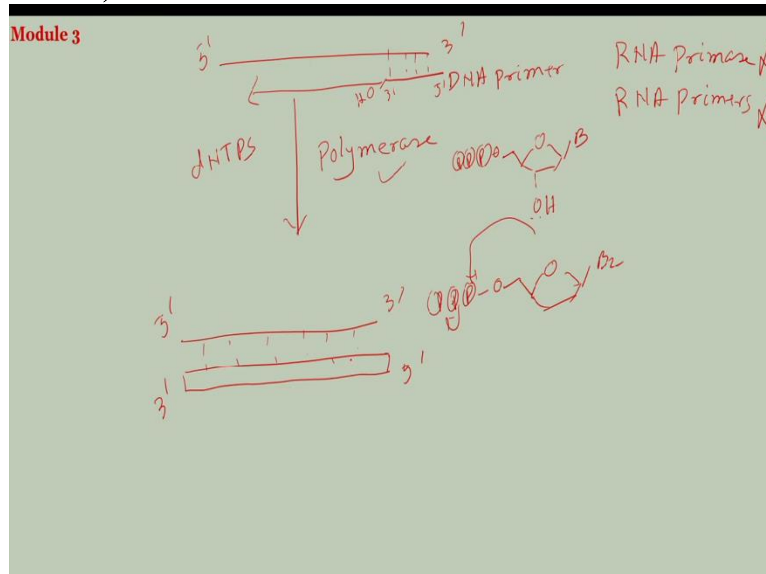

Now if you want to make the complimentary strand, what do you need? For cell, you needed the RNA primers that were synthesized from the RNA primase. Now in laboratory, you do not need RNA primers, because we have already learned to synthesize oligonucleotides. So we will use DNA primers instead of the RNA primers. So now we will use DNA primer in this case this is only one.

So DNA primer, RNA primer is not required for the laboratory technique, because that DNA primers do not exist in our body does short sequences short oligonucleotide sequences of DNA

do not present in our body RNA oligonucleotides are present to some extent. So, since you do not require the RNA primers, therefore, RNA primase that enzyme is also not required. Therefore, RNA primers are also not required. So, you see how the process becomes simplified one by one.

So, use a DNA primer and now, for the extension of course, you need a polymerase that is needed because the reaction that I have shown that if I am not writing the whole thing again here O P P P triphosphate, triphosphate these 3 prime hydroxyl which is here . So, this is 5 prime, this is 3 prime will react with B 2 O P P P this reaction can be catalyzed or this reaction can be done only by this enzyme polymerase there is no other goal.

So, we need the enzyme polymerase and you need all the individual dNTPs that is also required. So, since DNA primary is their DNA polymerase is going there bound and suitably the complimentary DNTP will be picked up and your strand would be synthesized in this direction. 5 prime to 3 prime so, this is the new strand and that you are synthesizing that you have synthesized 5 prime to 3 prime continuous without a break or without any interruption.

Now, since you did not use RNA primer here all DNA, so, you need not go any other steps your amplification for this strand is complete. Now, look at the other strand.

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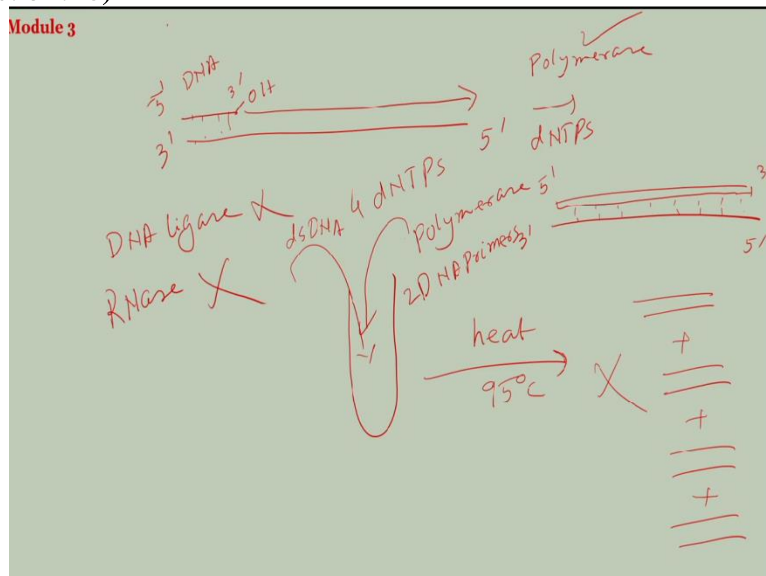

Other strand was 5, so, this is 5prime to 3 but this is 3 prime to 5 prime all right here, so, that it will be more clear, 3 prime to 5 prime that is this strand, lagging strand 3 prime to 5 prime. Now, that is also free fully. So, in lagging strand in DNA replication in cell you have seen that RNA primer came was bound in the middle of somewhere because you were half open there or partially open there. In this case, your strand is fully open.

So, and your reaction is always starting from the 5 prime to 3 prime direction, what is the 5 prime to 3 prime direction here? 5 prime to 3 prime. So you are not starting at the middle of somewhere, you are starting at the very end, just like the leading strand. So, you are again, you can use another DNA primer, suitable DNA primer that is suitable for this strand. And then of course, use the same enzyme. Now you need a polymer is but do you require a different polymer is no.

Here is the same thing 5 prime to 3 prime 3 prime hydroxyl is free for lagging strand, since your synthesis was happening in discreet manner in the middle part or somewhere in the portion of the gene, you had required a different polymerase in this case is the same reaction is happening exactly same as here. So, the same polymer is will do it you do not need a different polymerase so, the same polymer is that you have used in the earlier stage will also work here.

And the same dNTPs that you have in your solution will be used and you can have a continuous synthesis without any interruption. So, this is your parents strand, 3 prime to 5 prime and this is what the new strand that you are synthesizing, 5 prime to 3 prime. So, you see both leading strand and the lagging strand are behaving as equal in a test tube. And since this is happening, you are not creating the Okazaki fragments.

Therefore, you do not need the DNA ligase to ligate them, that is not required and since you do not have the RNA primers, you do not need to destroy them. So, the enzyme RNAase is also not required. So, what you do need here is if I now go back and check what I had written initially here and there, you do not need for the laboratory one you do not need the RNA primers. Instead, you need only DNA primer and you need 2 of them, 1 for each strand RNA primers were needed in multiple numbers dNTPs yes these 2 are needed.

Enzymes helicase no need single standard binding protein, no need polymerase, you need 1 RNA primers, no need RNAase no need DNA ligase you do not need. So you have come down to a single enzyme that is polymerase for the whole process and a single trick of heat and the ingredient, one extra ingredient that is the DNA primer which you can synthesize. So, that is the beauty of doing this replication process in the test tube. Now basically what you can have you can have your test tube you can take all the dNTPs for you can use a single polymerase.

And you need the DNA primers 2 DNA primers and you will have your amplification as you move on. So, that is the brilliant development of this method that you have simplified such a complicated process into a really doable thing. Now, what another requirement you have you have to use heat for here right and then only you will get your amplifier DNA, more number of DNAase number of copies of DNA you are going to get there is still a problem.

Do you see the problem? If you use all the 4 dNTPs if you use the DNA primers if you use the polymerase and you have your target DNA, double stranded DNA. Actually, still you are not going to get your amplified stuffs. Why? There is a key problem. The problem is you are using heat close to 95 degrees Celsius. And in this temperature, obviously, we are breaking the DNA. We are denaturing the DNA at the same time, you will denature the enzyme

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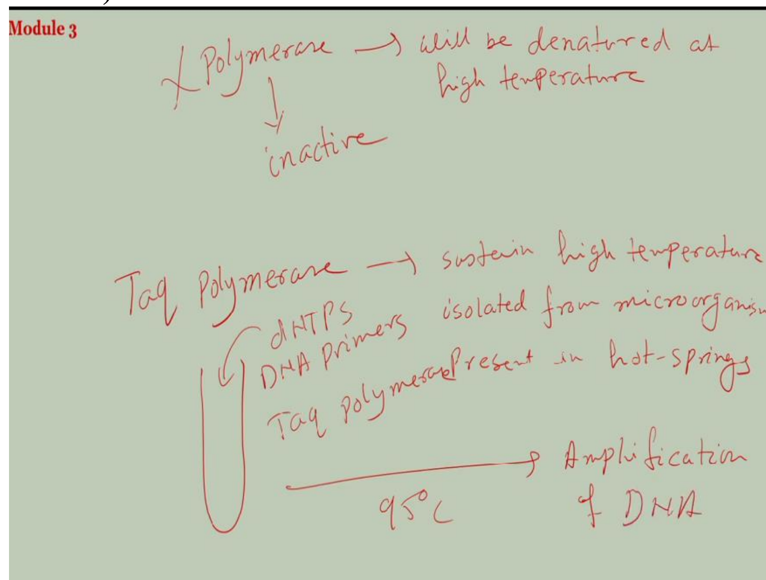

Also the polymerase we denatured at high temperature so polymerase is the protein and the protein functions when it is in tertiary structures coiled form 3 dimensional form. I will show you

the structure of polymerase briefly in a just very soon. So, but any protein when it functions enzymes they are all in the coil form folded confirmation and those confirmations are formed along with some covalent interactions, mostly the non covalent interactions, hydrogen bonding electrostatic interactions, hydrophobic interactions, at high temperature.

Those will all be gone and your protein will be dead polymerase will be as good as inactive. So, at that temperature, your polymer is cannot work and that was a key problem that had been sustained for a long time and people were searching to find a solution, what can be done? Can this be achieved at all? So, finally, and this is again another very important discovery is that they have isolated one kind of polymerase is that is known as Taq polymerase.

This is a polymerase that can sustain high temperature because this polymerase was isolated from the microorganisms present in hot springs so hot springs have high temperature close to boiling temperature. But still, there are many microorganisms living there thermophilic bacteria we call them or thermostatic microorganisms, thermophilic microorganisms, there are life still in the hot springs. So what they have done, they have and of course, since there is a life, they were having their cell divisions as well to the must have polymerase.

We have isolated those polymerases and that is known as Taq polymerase. I will show you why Taq, so the Taq polymerase can sustain high temperature and now your problem is solved. So, if you now use these in your laboratory with the dNTPs. With the DNA primers and the newly found polymerase high temperature you are going to get your amplification of DNA. So finding out Taq polymerase was a crucial part of this whole method development. So now I will show you how is it done.

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Module 3

The whole setup and PCR Mixture

1. DNA Sample (template)
2. Taq polymerase
3. oligonucleotides (two DNA primers)
4. dNTPs (dATP, dGTP, dCTP, dTTP)
5. $MgCl_2$ buffer solution.

Figure 1: PCR Machine

So the whole of the PCR is done inside a machine since very small machine bench top. You can put it on your table, small machine which can be programmed. So, you can see the buttons here, it can be fully programmed all the steps it can be preprogrammed, so that it will go on automatically. Here is a sample chamber where the things are done. And typically use a very small quantity of volume less than 200 microliter most of the times less than 200 microliter, so the test tubes.

I was talking about are actually the PCR tubes in this model of sizes were quite small size. So everything is done here. And the key steps are the key things that you need here is of course, you need the DNA sample that you have isolated and that is going to act as your template. second is you need the polymerase Taq polymerase number 3, you need the oligonucleotides which we call primers, so 2 DNA primers 4 is all the 4 dNTPs dATP, dGTP, dCTP and dTTP these are the 4 dNTPs unit.

And lastly, you remember when we make a DNA hybridization we always had to use salt because phosphates that are present in the backbone will rebuild each other. So in order to overcome that, we always use either sodium chloride or magnesium chloride. And the second reason you need to use a magnesium chloride is because the polymerase uses as magnesium chloride as its cofactor. So our fifth thing is you need magnesium chloride buffer solution. So next we will see how the steps actually work. Thank you.