**Genetic Engineering: Theory and Applications**
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**Module 3: Basics of Cloning (Part I)**
**Lect 09: Isolation of Gene Fragment (Part III)**

Hello everybody, this is Doctor Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And what we were discussing? We were discussing about the isolation of the gene of interest from the three different sources and in this particular module, what we were discussing? We were discussing about how to isolate the gene from the, applying different approaches. So what we have discussed? We have discussed about the two approaches.

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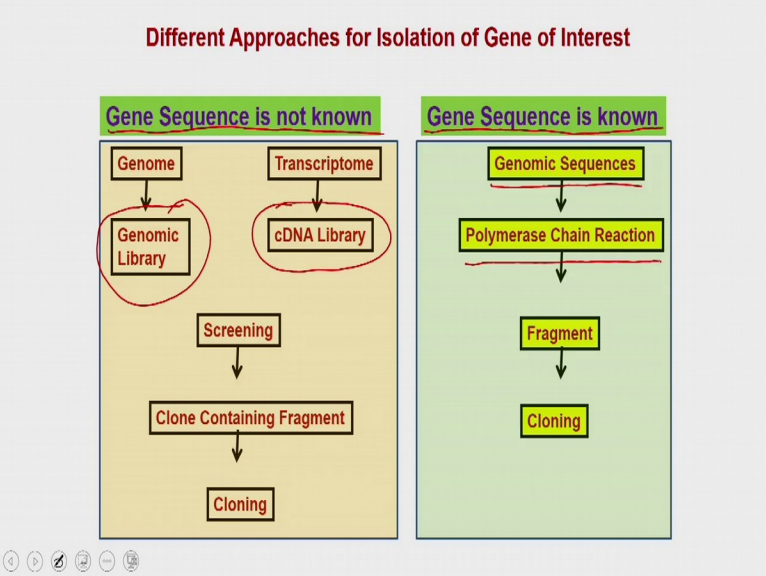


When you have the gene sequence not known, which means that the, you don't have the genomic sequences of that particular organism known then you can actually use either genomic library approach or the cDNA library approach. But if the gene sequences is known then the isolation of a particular gene fragment or gene of your interest is easy because then you can use the genomic sequences, you can perform the polymerase chain reactions and that actually will give you a fragment of DNA of your interest.

So in the today's chapter what we are going to discuss, we are going to discuss about the polymerase chain reactions and before getting into the integrate mechanism as well as the

technical details of how to perform the polymerase chain reactions, let us go back and see how and why there is a DNA synthesis required in the eukaryotic or the prokaryotic cell.

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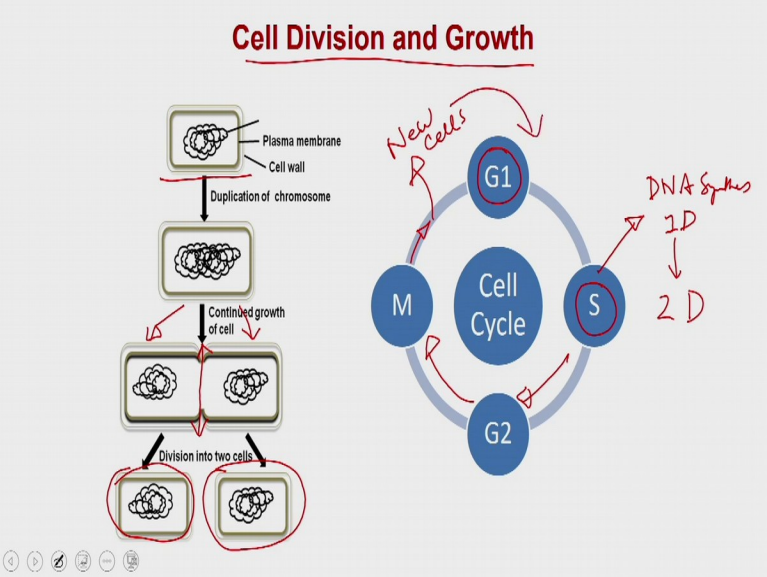


If you remember while we were discussing about the cell division or the bacterial replications we have discussed that in the binary fission or budding what happened is the genomic DNA is getting synthesized and duplicated and once it is get duplicated, the genomic DNA get moved to the two different parts of the mother cell.

And then ultimately these mother cell is going to be, go for the division where the cell is going to be divide from the half and that is how you are going to have the two different cells containing the genomic DNA, okay. So during the cell division and the growth of the prokaryotic organism the genomic DNA is being synthesized. You are making the two copies of the genomic DNA and then the individual genomic DNA copy is being distributed into the daughter cells.

Similarly in the case of prokaryotic cells the prokaryotic cell is going through different phases within the life cycle or within the cell cycle in which you have the G1 phase followed by the S phase. So S phase is the phase where you are actually going to do the DNA synthesis and because of that the one DNA will get converted into two DNA molecules which means the genomic DNA is going to be duplicated into the S phase.

And then it will enter into the G phase and then from the G phase it will enter into the mitotic phase and in the mitotic phase the cell is going to go through with the different steps is being distributed between the two cells and then after this you are going to have the cytokinesis and because of which the new cell is going to be formed.

And this new cell will again perform the similar cell cycle reactions. So what you can see is that if the cell division or the growth is required to be performed or to be continued, one of the crucial step is to perform the DNA synthesis, whether it is in the case of the prokaryote or the eukaryotes.

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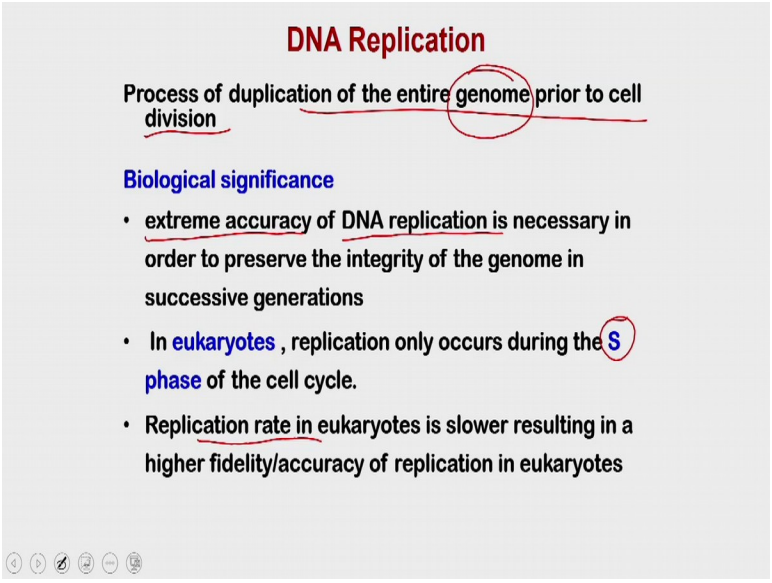


Now the DNA synthesis is a process which will allow you to the duplication of entire genome prior to the cell division. So this is important that you duplicate or you make the double copies of the entire genome prior to the cell will prepare for the division.

Then what is the biological significance? The biological significance is that this replication, because you are amplifying the genome and the one genome copy from the another genome copy should not be different. That is why the DNA replication in the case of cell, whether it is prokaryotic in origin or the eukaryotic in origin, should be done with the extreme accuracy.

As we discussed in the previous slide that the replication only occurs during the S phase which is called as the synthesis phase of the cell cycle in the eukaryotes. As the eukaryotic cells are slow-growing and their growth rate or duplication rate is lesser the DNA synthesis is

also slower in the eukaryotic cells compared to the prokaryotic cell. DNA replication is semi-conservative.

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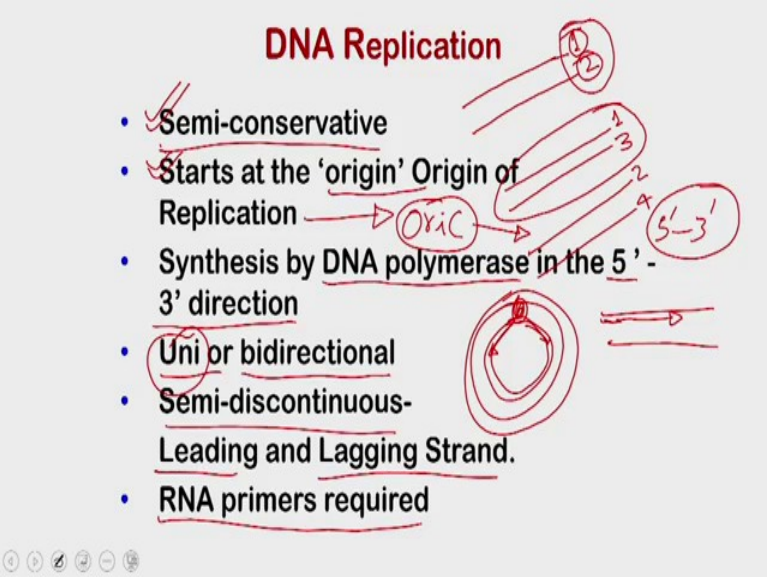


What is mean by the semi-conservative means that suppose this is your parent DNA. So if I label the parent DNA as 1 and 2, the parent DNA, the DNA number 1 or DNA number 2 will going to give the separate chains. In the duplication what will happen is the parent DNA 1 will, and parent DNA number 2 is going to split and it will get divided into the two halves.

So, and the new synthesis is going to happen which is the strand number 3 or the strand number 4, which means that the DNA which is present inside the parent is going to be divided into the half and that is how it is called as the semi-conservative, which means that the DNA which is present in the original cell is not going to be conserved.

Then most of these replications or the DNA replication starts at the origin. These are called as the origin of replications. If you remember we have discussed about the Ori C which is actually in the prokaryotic system and we have also discussed about the origin of replications in the eukaryotic system.

Then the DNA polymerase, the enzyme which is synthesizing the DNA is synthesizing in the direction of 5 prime to 3 prime. So this is what we are going to discuss in the future, in the later lectures. So don't worry about when it says the 5 prime to 3 prime, this is actually a

direction of, in which the DNA is been arranged and the synthesis occurs in the 5 prime to 3 prime directions.

DNA synthesis or DNA replication could be unidirectional or the bidirectional. So it depends on the type of template what you have and what you are using for the DNA synthesis. If it is a circular DNA, for example in the case of prokaryotic system what will happen is that you are going to have the origin of replication in the center and then in that case, 1 DNA polymerase will move on to the this direction, the other DNA polymerase will going to move on this direction.

And that is how they will going to do a synthesis of new strands, one for this one and the other one is for the outer one and that is how this is going to be a bidirectional DNA replications whereas in the case of unidirectional, unidirectional will be in this case, you don't have the circular template but you have the open template. Well in that case, you have the synthesis in this direction.

So the DNA synthesis or DNA replication could be unidirectional or the bidirectional, which means the 2 DNA polymerase can sit on to the same DNA and can run in two different directions. Then the semi-discontinuous which means that if you see the eukaryotic replications what you will see is that you have one of the strands which is called as the leading strand, the other one is called as the lagging strand. All this we are going to discuss in the subsequent slides.

So in the case of leading or the lagging strands what will happen is one of the strand on which the synthesis is continuous and it is running along with the direction of the fork whereas in the other case when it is a lagging strand it is actually synthesizing by lagging from the opening of the DNA stretch, okay.

So because these are the normal characteristics of the DNA replication in the prokaryotic or the eukaryotic system let us recall what we have is, the DNA is semi-conservative which means the parent DNA is getting divided and it will be distributed with the daughter cells, so the parent DNA will not going to keep its own copies with them but it will going to be shared with the daughter cells. Then the DNA synthesis cannot start at any place.

It has to be start at a particular place which is called as the origin of replication or Ori C. In the Ori C is a classical example of one of the origins of replication in the prokaryotic system. Then the DNA polymerase is an enzyme which actually catalyzes the DNA synthesis or the DNA replications in the eukaryotic or the prokaryotic system, so DNA synthesis occurs into the direction of 5 prime to 3 prime. Then the DNA synthesis could be, or DNA replication could be unidirectional in which it will utilize the Ori C and it will continue for, into the single direction or it can go from the both directions and in that case the DNA replication will be the bidirectional.

And then at the end the DNA replication is going to be semi-discontinuous which means one of the strand is going to be the leading strand which actually will go along with the fork, running into the fork direction, the other one is going to be the lagging strand which will lag in terms of the DNA synthesis compared to the leading strand. And at the end, this is what is the most important thing is that the DNA synthesis or the DNA application by the DNA polymerase requires a RNA primer which is being synthesized by another enzyme.

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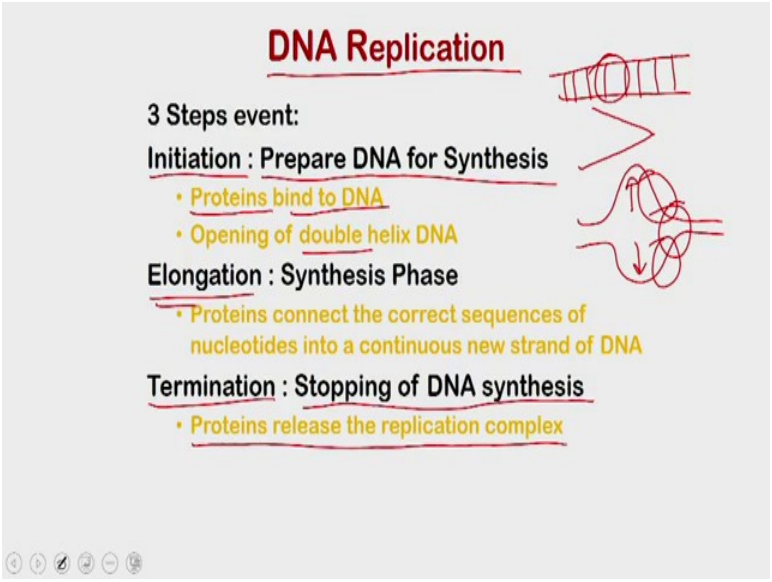


There are three steps in the DNA replications. These are called initiation, elongations and the terminations. So in the initiation step what you have is the initiation step is going to prepare the template DNA for the DNA synthesis, what it require is that the proteins which, the (machine) machinery which will go and bind to the double stranded DNA and the, if you have seen the double stranded DNA is helical in nature and it is been very bound to each

other by the hydrogen bonding between the bases. So these hydrogen bonding has to be broken down so that you are going to see the opening of this DNA.

So that is what is that, these proteins actually go and bind to the DNA and in the next step what they do is that they open this DNA in such a way so that the, it will accessible, the DNA sequence is going to be accessible for the synthesis. So you can imagine that you have the DNA and now it is pulling. So this protein will go and bind to the DNA and then they are pulling this DNA apart from each other and that is how this particular stretch is going to be available for the synthesis.

Then in the elongation step, the DNA polymerase will go and sit into onto this DNA and it will start adding the nucleotides on to the template, on to the primer strands and that is how the, it will continue the DNA synthesis. Once the DNA synthesis is getting over, then it is going to stop the DNA synthesis and there are proteins which are required to release the replication complexes or from the template DNA. And that is how the DNA replication is going to be complete within the eukaryotic or the prokaryotic system. So let us see what are the different steps what you have? So the first step is that you have a double stranded DNA.

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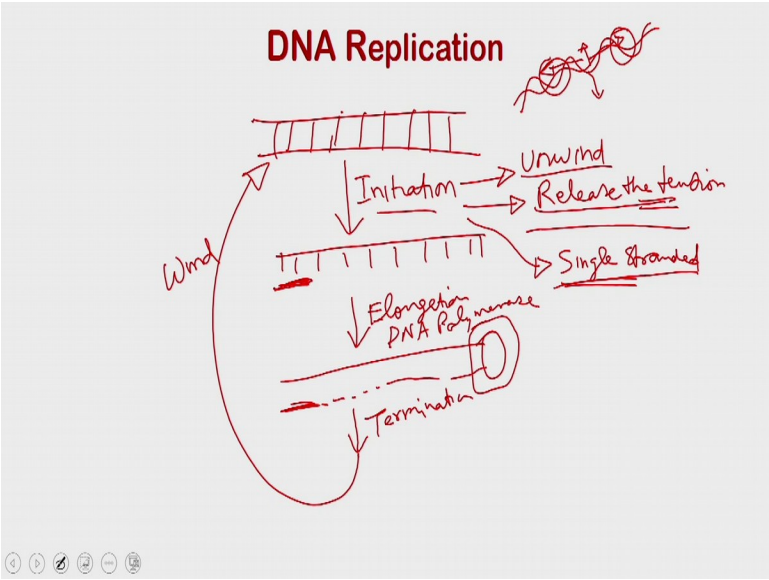


So the double stranded DNA is, in the first step what will happen is the double stranded DNA is getting converted into the single strands, so you are going to have the two strands, Ok. So that is the initiation step. So in the initiation step what will happen is, and if you know that the DNA is helical in nature, so it is actually going to, once you break the DNA, it is actually going, and it is attached on to the corner.

So that is how, because of that the DNA has to be, the DNA will create a tension or it is not going to possible that you actually unwind the complete DNA simply by protein is binding and breaking the interaction between the nucleotide. Because what will happen is once this helical DNA is going to be break, the, it will actually create a tension on to the corners and these tension on to the corner has to be released. Let me show you how this actually happens.

So imagine that if you take a electrical wire and, have you seen the electric wire? Electric wire is like this, right where you have two wires which are attached to each other. So these electric wires are nothing but the complete mimicking of the DNA. So if you see a electric wire and try to pull one of the strand, like if you try to pull from, in the center of the, this electric wire, what will happen is to some extent you will be able to unwind this DNA but once you try to do it slightly more, it actually going to create the tension on to the two corner of the electric wires.

So if you see this is what we have, a electric wire and if you try to open this, what will happen is that if you open like this, it actually causes a tension on to these two corners and this, the tension on these two corners has to be released so that it will be able to, it will be able to allow the machinery to move on.

So if you can imagine that the DNA will be keep unwinding like this but as you see, the wire, the corner wires are actually moving or the twisting actually. And that is possible because the other corner and other part, other corner of this particular wire is open. But if you, if I tie this up and try to do this like this, then it is not going to possible to unwind this whole wire without releasing the tension on to the corner.

So you actually need the cellular machinery or the protein machinery which will bind to the DNA in the initial stage and do the two jobs, one it will going to unwind the DNA so that you are going to have the single stranded DNA and then it is also going to release the tension so that the tension what the wire is experiencing on the corner should be released. And once that is done, then you have to ensure that the template what you are producing is remain as single stranded, okay.

So the third step is to keep the strand as single stranded. And the fourth step is that you need a enzyme which will come and design the primer on to the template strand and then you will enter into the next phase which is called as the elongation phase and when you enter into the elongation phase, this particular template with the help of the primer is going to synthesize the other strand. And that is how you are going to get, you will continue into the elongation phase and once you enter on to the corner of these DNA template then you will enter into the termination codon, you will enter into the termination step and then this cellular machinery or the machinery what you are using for DNA synthesis will be dislodged from the DNA.

And once this is done then you have to reverse the events what you have done in the initiation which means DNA has to be wind, which means you are going to create the double stranded DNA, you have to remove the proteins which are releasing the tensions and you also have to remove the proteins which are maintaining the single stranded DNA of the templates. And that is how ultimately what will happen is that once you, after the terminations, these proteins, once the, you will achieve the template DNA or the double stranded DNA where one of the strand is going to be from the original strand, the other strand is going to be the synthesized strands.

So as you can see, the DNA replication is a very, very complicated step or complicated process where you need to unwind the DNA, you need to release the tension. You have to ensure that the template will remain as the single stranded and you also need to have the elongation step where the DNA polymerase is required, and you also need a termination step; so all these steps are being performed by the specific proteins within the prokaryotic or the eukaryotic cells.

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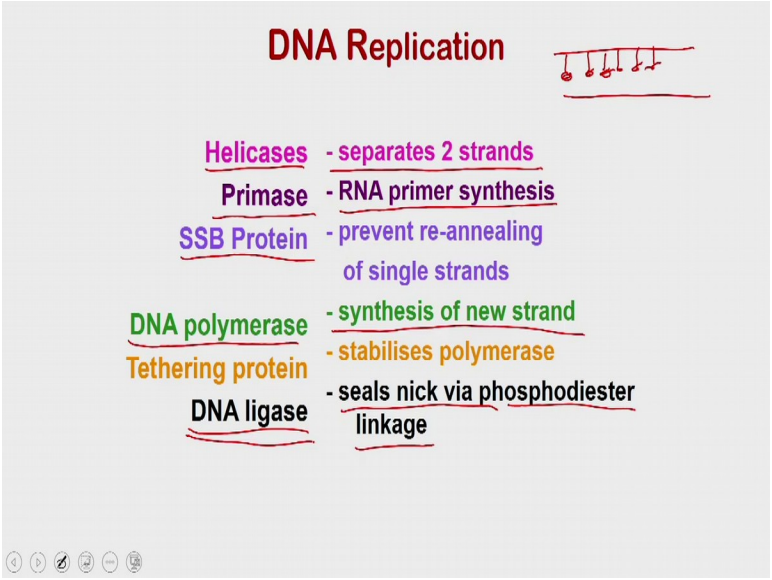


These are the proteins, so you have the helicases. The helicases job is to remove the two strands; it means it is going to unbind the DNA. Then what you have is, you have an enzyme which is called as primase. Primase is a RNA polymerase which utilizes the DNA as the template to synthesize the RNA primers. Then you have the single stranded DNA binding protein and this single stranded DNA binding protein will go and bind on to the nucleotides which are there on to the template strands.

So they will go and bind and because of that the template is going to be remained as a single strands. Then you have the DNA polymerase. DNA polymerase, the job is that it will synthesize the new strands utilizing the information what you are providing in the template strands and then you have some of tethering proteins which actually stabilizes the DNA polymerase.

And then you have the DNA ligase which actually seals the nicks, because when you are doing synthesis you have the leading strand as well as the lagging strand. So on lagging strands you are actually creating the multiple nicks and these nicks are ultimately going to be sealed simply by the enzyme which is called the DNA ligase.

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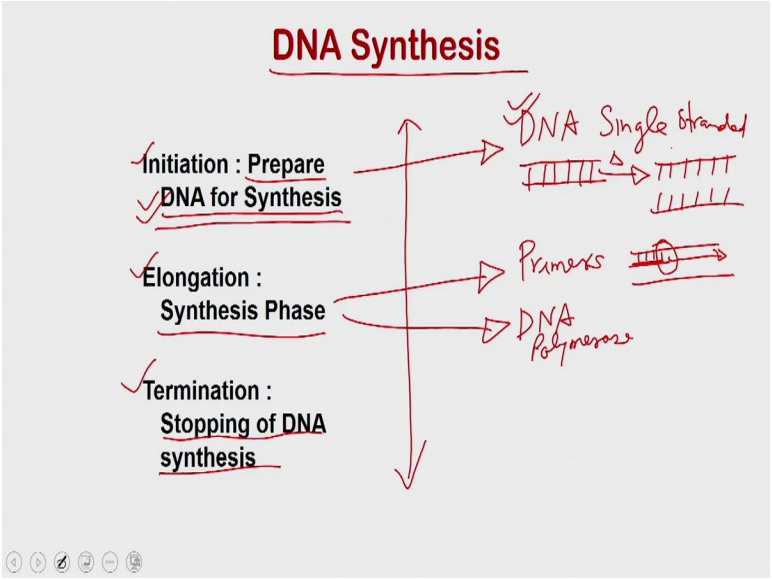


Now if I tell you that, by doing the DNA replication you are doing nothing but the DNA synthesis. And for this particular type of DNA synthesis in the cell, you require multiple steps like initiation, you need the elongation and then you need a termination. And for all these steps which are actually, so what you are doing is, in the initiation step you are preparing the DNA for the synthesis.

In the elongation step you are using an enzyme which is actually synthesizing the DNA with help of the nucleotide being attached on to the primer and the termination which is actually stopping the DNA synthesis and DNA synthesis stops when the machinery reaches on to the corners, or on to the end of the template strands.

Now if I want to do all these reactions under the artificial situation or the in-vitro system what I have to do is, so if you see in the initial stage what we are doing, we are doing, we are making the single stranded DNA, okay. We are making a single stranded template, okay. Since in the biological system what you do is you prepare the DNA for synthesis simply by adding the enzymes as well as the factors which actually ensure that the template is going to be broken down into the single stranded and then it remains single stranded.

And that is very, very complicated because that is kind of a reaction you cannot mimic under the in vitro conditions. That is why when you have the double stranded DNA, what you can do actually is you can just heat this DNA to such an extent and that heating is actually going

to create the single stranded DNA. And as long as you keep that temperature high, you are going to maintain the single stranded DNA.

Now in the second step what you will do and while it is in the high temperature state, you will add the primer, okay, just like as it is being added into the initiation factor, initiation stage of the DNA replications. Then you add the primers and you allow these primers to bind to your template DNA.

And then what you do is you add the DNA polymerase, the enzyme which is responsible for synthesizing the DNA and what will happen is DNA polymerase will sit on to this and will complete the synthesis. The termination part is anyway, will be done eventually once the template, once the machinery will reach on to the end of the template strands.

So because these are possible simply by changing the environmental conditions or the reaction conditions, people have thought of doing this kind of DNA replications under the in-vitro conditions and that is how they have evolved and developed the polymerase chain reactions or PCR. So let us see what are the different stages through which the technique is being evolved.

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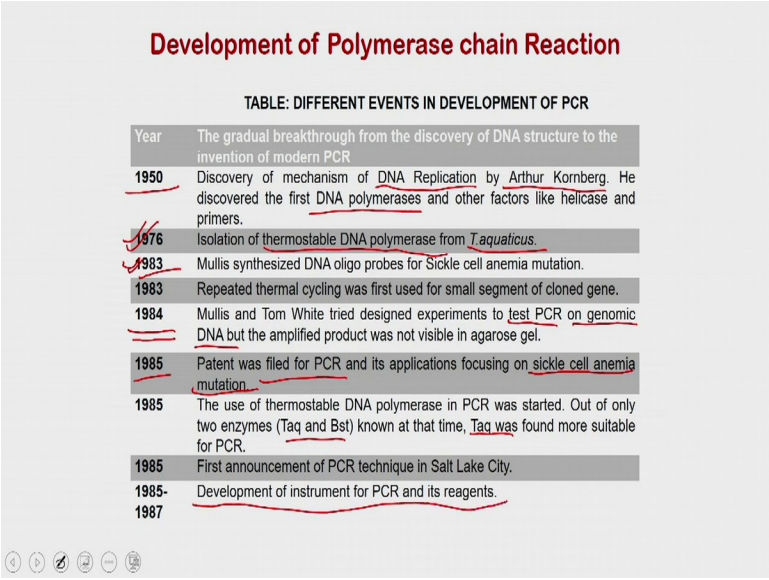


So it started very early on, when in the 1950s, the Arthur Kornberg, the Nobel Laureate has discovered the mechanism of DNA replication and Arthur Kornberg has also discovered the DNA polymerases, the enzyme which is responsible for the synthesis of DNA. Then in the

age of 1976, the people have discovered the first thermostable DNA polymerases from the thermus aquaticus.

And then in the year of 1983, the Kary Mullis synthesized the first DNA oligo probe for detecting the sickle cell anaemia and that was actually the ground breaking discovery to see that you can actually do the in-vitro reactions to synthesize the small stretches of DNA without going through or without utilizing the cellular machineries.Then the subsequently in the, in the year of 1984 the Kary Mullis and other people have discovered or performed the PCR reactions on the genomic DNA and then, in the 1985, they have applied for a patent so that PCR reaction can be used to detect the sickle cell anaemia mutations.

And in the, subsequently in the 1985 or so on, the PCR is being developed completely and now what you use is, you always use the thermostable DNA polymerases or the Taq DNA polymerases and that is more suitable for the PCR reactions and in the 1987, first PCR instrument for performing the PCR is being developed which is called as the thermal cyclers.

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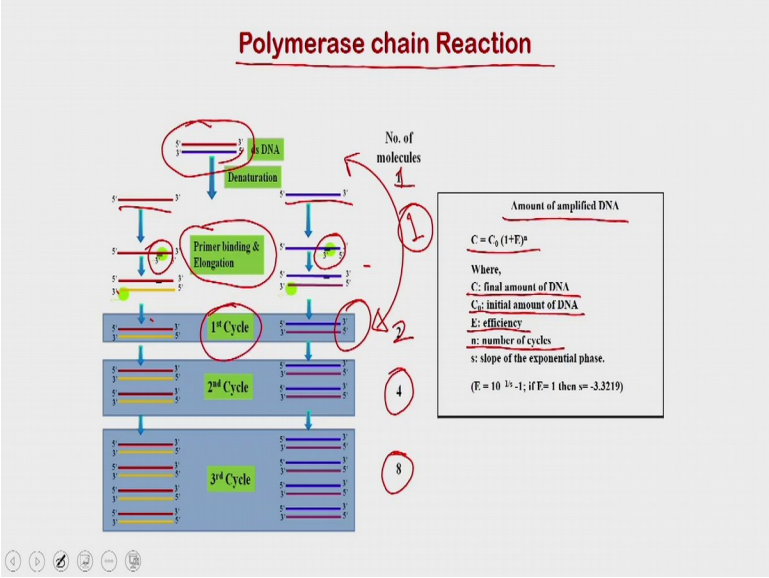


So this is what is happening when you are doing a PCR chain reactions. What you have is you have the double stranded DNA. The red strand is in the direction of 5 prime to 3 prime. The blue one is in the 3 prime to 5 prime. As you know that the DNA is complimentary to each other, so what will happen is you, first step what you will do is, you will do a denaturation. So in the first step of denaturation the red strand is, and the blue strands are

going to be get separated. Then what will happen is you will use the primers. So you will use the two primer, one for the red strands, the other one for the blue strands.

And what will happen is the primer will go and bind to the blue strand as well as for the red strands. And then it is going to start the synthesis part. So after the first cycle, since you started with the one molecule, after the first cycle you are going to have the two molecules. Then, again in the second cycle these two molecules are again going to work as a template and as a result, what you are going to get?

You are going to get the four molecules, eight molecules so on. So what you see is that, at every reactions it is getting the double which means the, if you want to calculate the how much amount of amplified DNA what you are going to get, the formula is that C is equal to C 0 1 plus E to the whole power n, where the C is the final amount of DNA.

So suppose you started with the 10 nanogram of DNA, then you can put the C as, and you can calculate what is the amount of DNA you are going to get? So initial volume, the C 0 is the initial amount of DNA what you have used. E is the efficiency, the efficiency of the PCR reactions and the n is the number of cycle what you are going to perform.

So what you can see is the, this actually constitutes, so once you start from here and end up in having the two molecules the event what you see is called, considered to be a one cycle. So what you see is, after the first cycle if you started with one molecule, it is going to be two molecules so that is what is the number of cycles? So as you increase the number of cycle then amount of final product is going to be increased.

And then you also have to use the slope of this DNA synthesis and by doing, or by using this particular kind of formula you could be able to estimate the amount of DNA which is going to be produced. So for doing a polymerase chain reaction, you need...So this is what you need to suppose to do. In the polymerase chain reaction what we are doing is we are doing the denaturation, primer binding and elongations then we are doing the annealing and all those kind of events.

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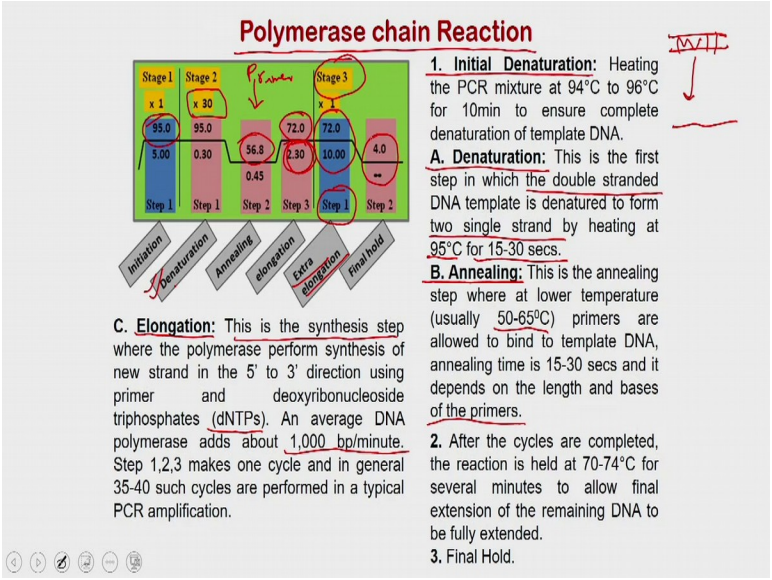


So these are the different events what you have to do when you set up the PCR chain reactions. What you do is, you would first do the initial denaturations. So in the initial denaturation what you will do is, you will heat up the sample at 95 degree Celsius for 5 minutes and that actually will convert the double stranded DNA into the single stranded DNA, Okay. So now your single stranded template is going to be ready.

Then what you will do is, you will do a first event of denaturation. The initial denaturation what you are using is going to destroy the double helical structure of the complete DNA. In the second one is actually a, when you do for cyclic reactions, so the initial denaturation what you will do is to double stranded DNA is going to be denatured to form the two single stranded DNA by heating enzyme for 15 to 30 seconds.

Then what you do is you allow, you go to the annealing step. In the annealing step what you are trying to do is you are trying to let the primer to sit and attach to the template. So what you will do is you will lower down the temperature. So once you lower down the temperature you are actually allowing the binding of the nucleotide sequences to each other, or you are trying to make the hybridization of the primer to the template strands.

So usually you can lower down the temperature to 50 to 60, although the temperature, exact temperature what you will use is depends on the many factors, for example the T m what you have or the melting temperature of the primer what you have, what you got into the, while

you were designing the primers or the annealing temperature is decided based on the quality of those kind of thing.

Then the third is the elongation step. So then you will make the steps slightly, you again, you will increase, again increase the temperature and that actually will allow the DNA polymerase to synthesize the new strands and you will keep the time for which the elongation steps will continue. During the elongation steps the dNTPs what you are going to provide into the reactions is going to be incorporated into the primer sequences and that is how the, it will use the sequence information from the template and it will synthesize the other strands.

How much time you should keep for elongation depends on the processivity or the speed of that particular DNA polymerase. So according to an average the DNA polymerase added 100 base pair per minute. So suppose you have a gene of 2.5 Kb, then you can give roughly around 3 minutes because you can give slightly more so that you should not have the problem of the incomplete amplifications or the elongations.

Once this elongation is over, then again you will continue into this stage 2, and you will again put the reactions for denaturation, annealing and elongation. So this will continue as many reactions you want in this particular figure what we have shown is that you are doing this for 30 cycles. So that is what is called as the 30 cycles. Once these 30 cycles are over, then you enter into the stage 3 where you put in the reactions for the final extension or the extra elongations.

During the extra elongation what happens is whatever the molecule which are being half complete or incomplete elongation synthesis occurs that will be done in the last elongation steps and ultimately since you have to terminate the reactions what you do is you lower down the temperature to 4 degree and then you store this reaction at 4 degree. So for doing the PCR these are the reagents you require.

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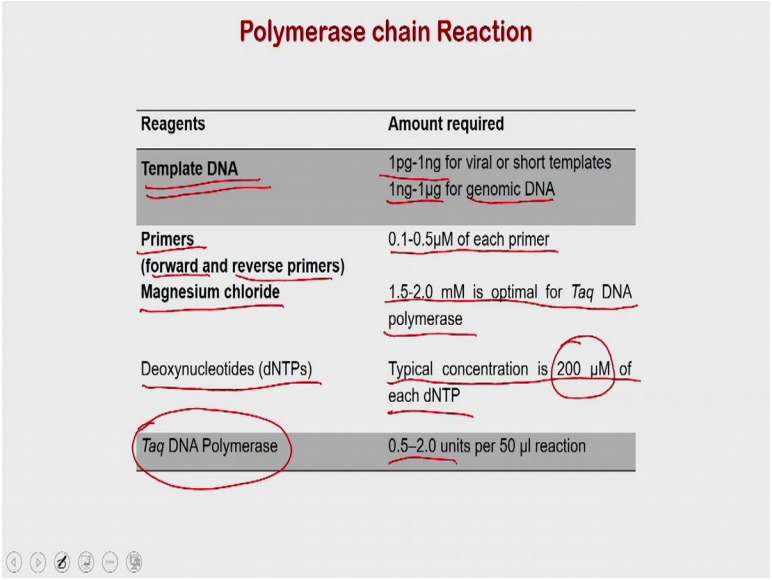


So you need a template DNA, this template DNA could be from different sources. What you can do is, you can use the 1 pico gram to 1 nano gram for viral or the short templates. and 1 nano gram to 1 microgram for the genomic DNA. So the template DNA is depending on the source of DNA what you are using for amplification, if it is a bigger DNA, then you have to use into the microgram range. If it is a smaller DNA then you can take it the smaller version. Then you need the primer, you need the two different primers, the forward primer as well as the reverse primers.

Then the DNA polymerase is utilizing the magnesium chloride as a co-factor, so that is also required. In some cases you might have to optimize the concentration of magnesium chloride to achieve the amplifications. Primers you have to use in the range of 0.1 to 0.5 micro molar and then you also have to provide the dNTPs, so what you have to do is you have to add the ATP, GTP, TTP and CTP, all these 4 dNTPs you have to provide in the concentration of 200 micro molar, and at the end you also have to provide the Taq DNA polymerase. Normally we use the 0.5 to 2 units for every 50 micro liter reactions.

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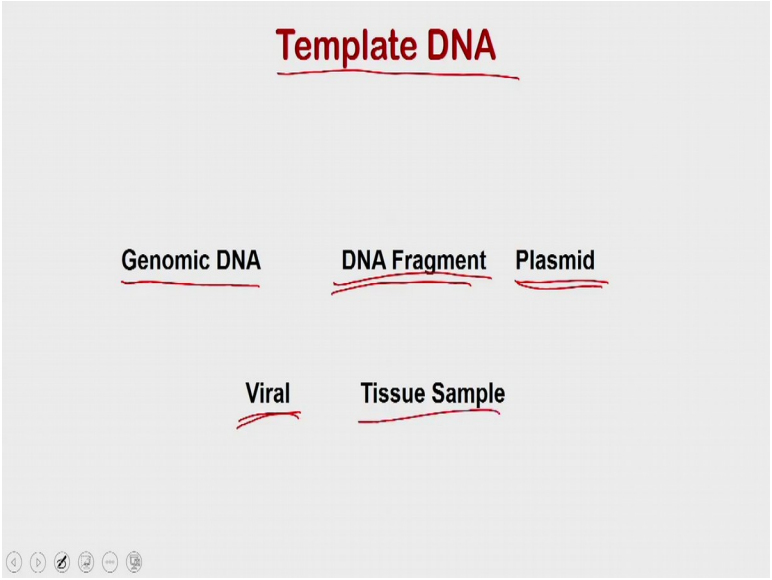


So what you have is you have the template DNA. So template DNA could be the genomic DNA could be the DNA fragments, suppose you are isolating a particular fragment from a particular site. For example if you are doing the, some kind of investigation, you got some DNA samples; in those cases you may have the fragments instead of the complete genomic DNA. So those can be also used for PCR. Then you can use the plasmids where your particular gene or fragment is already been cloned or present.

So you can use the plasmid where the DNA is already been cloned. Then you can use the viruses which are already harboring that particular gene or you can use the tissue samples. For example if you have some tissue samples from which you want to do the PCR then you can use the DNA from the tissue sample and that can also be used. So this is about the template. Apart from template you also have to design or you also need the primers as I, as we discussed we need two different types of primers. One is called as the forward primer, the other one is called as the reverse primer.

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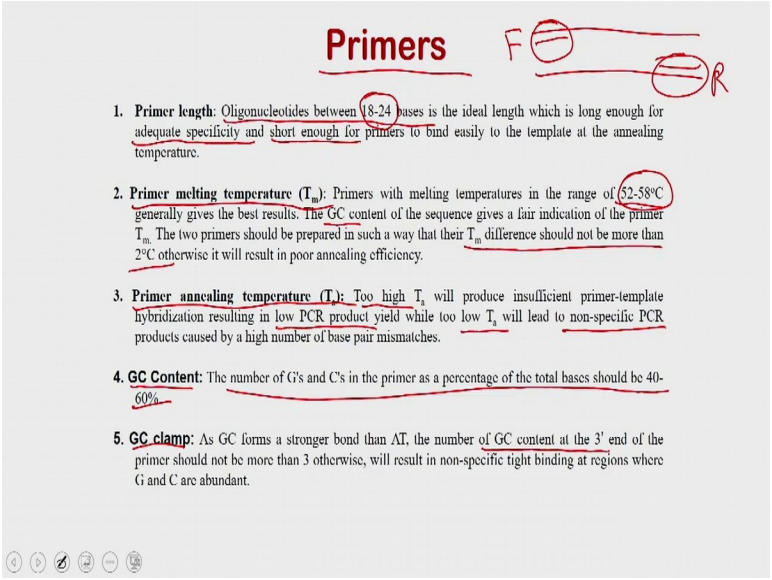


So there is no difference, there is a terminology difference only but the forward primer or the reverse primer is that this is called as the forward primer and this is called as the reverse primer. Because the forward primer synthesizes the DNA for the template strands whereas the reverse primer synthesizes for the template, for the DNA of the other strands. So the first thing what you have to do is you have to see the length.

So irrespective of whether you are synthesizing the forward primer or the reverse primer, the length could be somewhere around 18 to 25 base pair. And if you go lower to this, you are actually going to compromise on to the specificity or that particular kind of primer is going to bind to the different regions and that is how you are going to have the non-specific amplifications. If you go beyond this length then you are also going to create other kind of problems like primer will not going to, will going to show the secondary structures.

So that is why it is advisable that you use a oligonucleotide which is in the range of 18 to 24. That actually will provide you the adequate specificity and shorter to this length or longer to this length is going to create trouble in terms of giving you the desirable products. Then once you design the primer, you also have to see what is the melting temperatures of these primers.

So melting temperature of these primers should be into the range of 50 to 58 degrees Celsius. You can actually modulate the melting temperatures simply by playing with the GC content of the sequence or you can take the other region where the GC content is optimal for that you can get this particular type of melting temperature.

Melting temperature below to this is going to create the formation of the non-specific binding and the Tm differences between the two primer what you are going to synthesize, as I said you know you are going to have the forward primer and you are going to have the reverse primer should be 2 degree. Which means once you set up the Tm, the Tm between the two primer, forward primer and reverse primer ideally should be have a difference of 2 degree Celsius. Then you also need a primer annealing temperature.

The primer annealing temperature is being calculated simply by taking the primer melting temperature. If you have the too high annealing temperature that will actually going to compromise on to the low product yield. If you have too low annealing temperature that actually is going to create the non-specific primers and you can actually vary the primer melting temperature as well as the primer annealing temperature simply by modulating the sequences or incorporating the GC content into the sequences. Ideally the GC content should be in the range of 40 to 60 percent and it is always been advisable that you also should have a GC clamp.

What is meant by a GC clamp is that it actually, if you, if you take the GC at the end of the primers, that actually will allow the primer to hold on to the template more strongly compared to if you take the AT sequences. So you know that the G C is forming the 3 Base Pair or GC is forming the 3 hydrogen bonding. So the hydrogen bonding between a G and C is 3, compared to a hydrogen bonding between A and 2 is 2. So that is why it is advisable that that you take a GC clamp on to the corner of the primer so that once it attached to the template that binding should be very, very firm.

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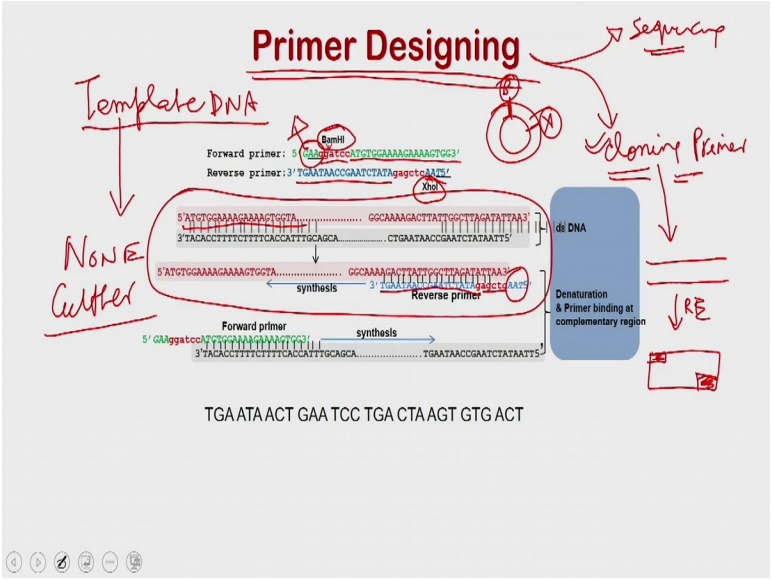


Now let us see how to design the primer. Before you design the primer, the primer are being designed for two purposes. One, which is called as the sequencing primer and the other one is called as the cloning primer. Sequencing primers are easy to design because sequencing primers are the primers which actually will amplify your region and then you can actually get that amplified PCR product and that you can use. Whereas the cloning primers are little bit more complicated to design because of the simple reason that the cloning primer is required a very specific set of things, okay.

So before you design the cloning primers, what you have is you have your template DNA, though, for template DNA what you have to do is, because cloning primer means you are going to take this particular fragment and clone it into the particular type of vector or plasmid or some other transforming agents so that you can produce a recombinant DNA. So for that what you have to do is, first what you do is first what you do is you take the template DNA, the DNA from where you want to extract your gene or the gene of interest and then you calculate the non-cutters, which means you actually have to find out the enzymes or restriction enzymes which are not going to cut your gene because these are your restriction enzyme which you can use for cloning purposes.

All these we are going to discuss in our subsequent lectures. So what you need is, you need a non-cutter so that it you will be going to use those enzymes for cutting your gene and then putting these fragment into the vector of your interest. So for example in this case, we have taken this gene which is actually the gene, small fragment and we have given you an example

how to design the forward primer or the reverse primer. So what we have done, we have taken this particular sequence.

First we have put into a software. So there are multiple softwares which you can use to calculate or to know that which are the enzymes are non-cutting to your gene fragment. So once you know that these are the gene, these are the enzymes which you can use for cloning purposes, for example in this case, we know that we have the two enzyme, one is called as the BamH1, the other one is called the Xho 1.

So we have decided that we will put on the BamH1 in the forward primer and the Xho 1 in the reverse primer. This, what enzyme you use for the forward primer and what enzyme you should use the reverse primer depends on the sequence in which these enzymes are being placed on to the plasmids or to the vector, okay.

So suppose in a vector, the bacterial plasmid, you have first the BamH1 followed by the X ho, then you have no option but to take the BamH1 in the forward primer and Xho 1 in the reverse primer. All this we are going to discuss when we will discuss about the plasmids. So for now what we have done is we have taken the BamH1 in the forward primer, okay.

So for the forward primer what we have done is we have just simply taken this particular sequence, okay, this is what you see here, okay and then in front of this particular sequence we have added the BamH 1 or the restriction sites what is corresponding to the BamH 1 so which in this case, it is GGATTC and because the enzyme requires the, the nucleotides on to the other side we have added three more nucleotide GAA.

So G is added on to the forward primer simply by ensuring that there is a GC clamp, so that, this, once you put this forward primer on to the template it will go and stick to the template. Then the same is, we have done for the reverse primer but you have to little careful with the reverse primer, what you will see is that for the reverse primer what we have done is, the gene whatever we were having, we have synthesized the complimentary sequences.

And then what we have done is, we have taken this complimentary sequence, then we have added the restriction site and instead of adding on to the 5 prime site, what we have added is

we have added the three nucleotides so that you are going to have the inner space for the enzyme to sit and cut this particular fragment.

Because ultimately, once you have this particular kind of gene fragment and you will do a restriction enzyme analysis what will happen is that this is going to create the sticky ends on both the sides. And these sticky ends allow this particular fragment to be sticked to the particular plasmids or the vector.

So once you synthesize these forward primer as well as the reverse primer all these forward or the reverse primers has to be analyzed to see whether they are satisfying your quality parameters or not. So we have discussed in the past what are the quality parameter it should have. It should have the Tm, it should have annealing temperature, it should have all those kind of things. So let us see how, in the analysis part how you are going to analyze this? And what are the problems you are going to encounter?

Okay so for the analysis, we have the couple of softwares which you can use to analyze these sequences, to calculate different type of parameters, for example the Tm, annealing temperatures, GC clamps looking for the all other kind of problems. So when you design a primer you are going to encounter many problems such as the formation of the secondary structures. So what are the secondary structures are being synthesized?

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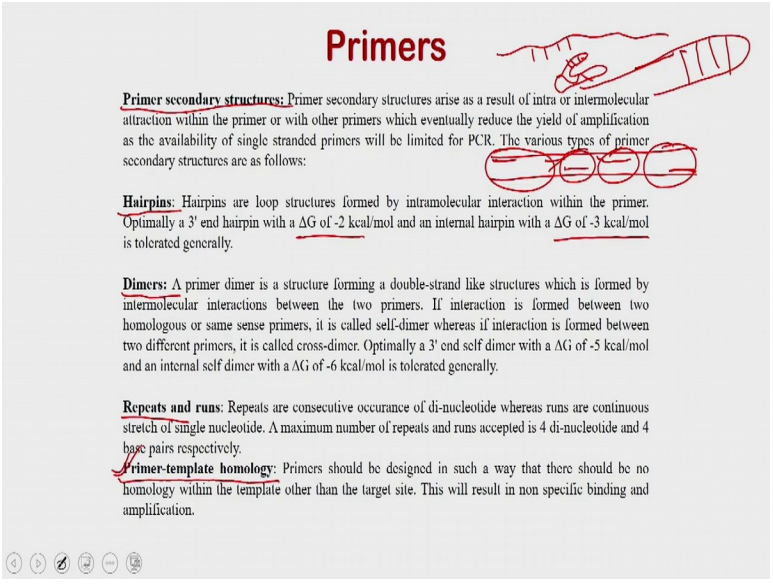


So primer you know, that primer is single stranded DNA, okay or oligonucleotide. These single stranded oligonucleotides, they can form the different types of secondary structures

because of the complementarity between the different types of nucleotides what you are using to synthesize this oligonucleotide. And these complementarities will give you different types of secondary structures.

One of the classical secondary structure what you will see is called as the hairpin. So hairpin is like a loop kind of structures where the one of the strand will flip and it will give you the intra-molecular interactions within the primer or within the one primer or between the two primers and the internal hairpin is going to be not a problem if you have the delta G of minus 2 kilo calorie or minus 3 kilo calorie.

Then you are going to have the dimers. So you have the forward primer, you have the reverse primers. Though both of these primers actually can have the sequence complementarity to each other and that is how they can actually, once you lower down the temperature for annealing, instead of annealing to the template what will happen is they will anneal to each other and that is how they are going to have the dimers.

And these dimer structures are not good because they will, first of all they will not allow the primers to go and bind to the template. The second is they are going to interfere into the DNA synthesis part and they are going to disturb the DNA polymerase mediated DNA synthesis. Then you also have the repeats. For example you may have the consecutive occurrence of AA GG CC like that, okay. So these kind of sequences are going to create the loops as well as other kind of secondary structures and that actually will going to create trouble.

The second is, once you design the primer you also should do a in situ PCR to know that your primer is only binding to the desired sequences, not to the other part of the same template. For example if you have a gene of this length, Okay you will want that your primer should bind to the corners. But it should not be the case that some part of the primer is binding to all over your sequences.

In that case what will happen is you are going to get the synthesis of a small stretches of this DNA instead of the total length and that is also, has to be ensured when you design a primer. So these are the secondary structures. So as you can see we have designed the two primers, one this primer and other one is this primer, okay.

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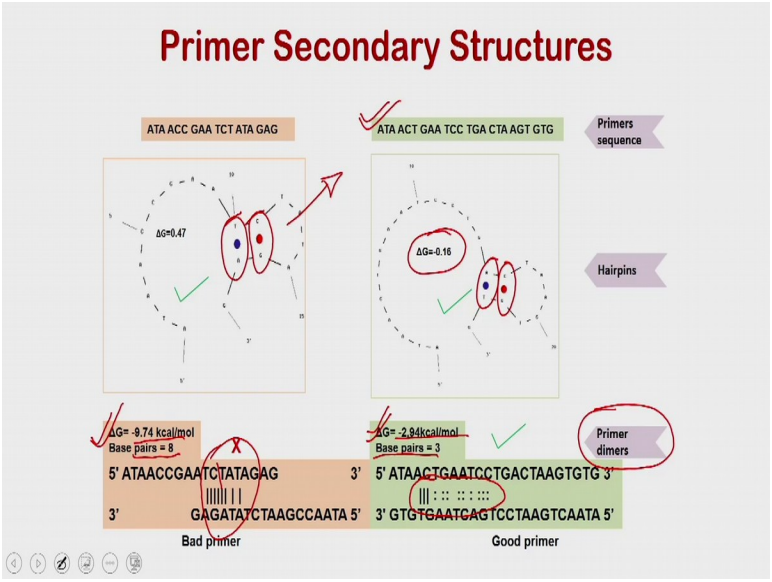


So this is what is we are talking about the hairpins. So in that case what you will see is the one particular primer sequence is getting looped and you will see that the GNC or ANT are actually binding to each other and that is how they are creating the hairpins. These hairpins, if they are strong, then they are going to disturb running of the DNA polymerase and that is how they are going to disrupt the DNA synthesis whereas if these strands are very, very weak, for example if these interactions are weak enough then what will happen is once you bring them to the annealing temperature, all these secondary structures are being broken down and that is why this particular primer what we are designing is good in terms of the hairpins as well as in terms of the primer dimer.

What is the difference between this primer and this primer is that what you see is, this primer is making a hairpin loop which is of, which require, which will be more stable under the annealing temperature. Also it is also forming the very, very strong, the primer dimers, okay. And what is the difference between this sequence versus this sequence is that the primer dimer what is forming is very, very strong compared to that it is giving the hydrogen bonding between the complimentary sequences, whereas, and it is very, very; it is within the small stretch. So it is very, very strong compared to this one, the interaction is dispersed throughout the sequence and because of that this particular kind of primer dimer is easy to break when this, when you bring this to the annealing temperature because as you can see delta G is lower in this case whereas the delta G values are very high.

So, and the number of base pair what you see in this case, is 8 base pair and the two consecutive base pairs are being involved into the base, formation of primer dimer whereas in this case, what you have is only the three base pairs which are actually been involved into the formation of primer dimer. And that is why this particular primer is not considered to be good because this primer is going to make the primer dimer even when you put it for the annealing temperature and as, because of that it will not going to anneal to the template DNA, instead the primer is going to make the dimer on its own.

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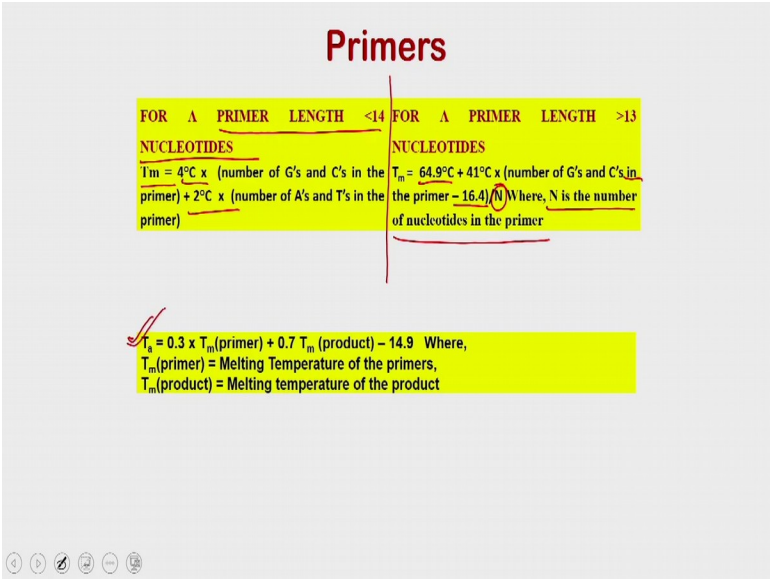


For calculating the Tm values, you have the two formulas; you have the formula when the primer is less than the 14 nucleotide; so for the Tm what you have to do is take 4 into number of G and C in the primer plus 2 into number of A and Ts in the primers and that actually will give you the number of, the Tm values. Similarly if it is more than 13 nucleotides then what you have to do is the Tm is 64.9 plus 41 degrees Celsius into number of GCs in the primer minus 16.4 divided by the N where N is the number of nucleotide which are present in the primers. This is actually the formula for calculating the annealing temperatures. And that is, if you see the annealing temperature is connected directly or indirectly to the melting temperature of that particular primers.

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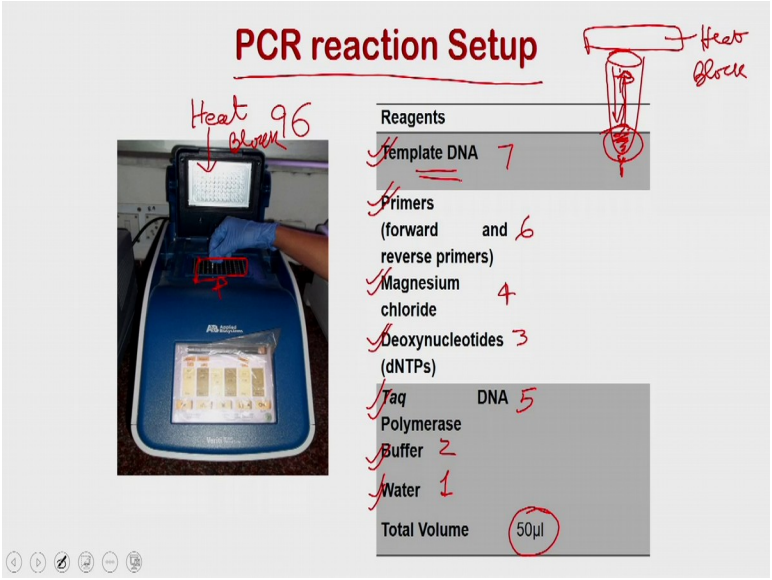


So this is what the PCR reactions, this is the PCR machine, typical PCR machine what you have is the heating block where you are going to setup the reactions. So in this heating block you have the place for keeping the Eppendorf. Normally you have the 96 places for keeping the reactions. And on the top of this, you have a heat block; this heat block is being placed on the top so that when you heat these reactions from the bottom it should not going to evaporate the sample.

So what will happen is you can imagine that you have the sample into the Eppendorf and you have the volume at the bottom and if you heat up from the bottom this particular thing will come into the vapor phase. So because of, to avoid this what will happen is they will putting a heat block from the top. Once you have the heat block whatever vapor is going on to the top is again coming back and that is how you are going to have the volume at the bottom.

Apart from that you also need to provide the template DNA. You need to provide the primers, you need to provide the magnesium chloride, dNTPs, Taq DNA polymerase and then you also have to provide the Taq DNA polymerase buffer and the water. Normally we do PCR reactions in a volume of 50 micro liter.

And the sequence in which you are going to add all these reaction is that first you will add the water, then you will add the buffers, okay. Then you will add the dNTPs. Then you will add your magnesium chloride. Then you will add your tagged DNA polymerase. Then you will add your forward primer. And at the end you are going to add the template DNA.

So this, if you follow this particular kind of scheme or events you will ensure that when you add the template DNA, there will be no pre-run or there will be no pre-synthesis because that actually will going to create the artifacts when you are getting the, from normal PCR reactions.

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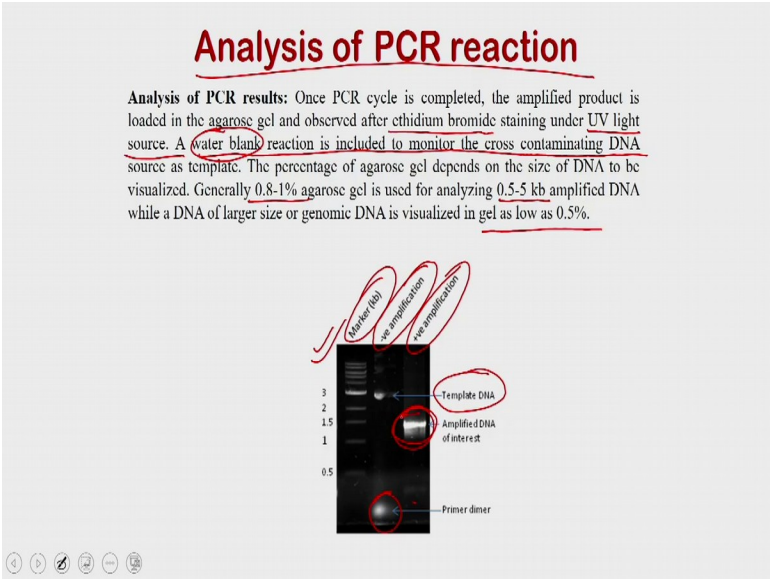


You have to do analysis of this particular PCR reaction. So once the PCR cycle is complete what you have to do is, you have to take out the reactions from the, these heating block and then you have to add the, you have run it into the agarose gel and then you have to visualize that under the UV light.

And the gel is containing the dye which is called as the ethidium bromide and in the presence of this, so ethidium bromide is intercalating dye and it intercalates into the DNA and that is how it actually gives orange color fluorescence. Many of the reactions, many of the time when are doing the PCR for the first time, you always run a water blank, which means when you are setting up the reactions, instead of adding the template you are adding the water so that you will know that there is no cross-contamination into your reactions.

Generally we use 0.8 to 1 percent agarose gel for analyzing the amplified DNA but if you are amplifying, or if you are amplifying a DNA which is larger to the 5 Kb then you can also reduce the concentration of the agarose what you can use, you can use even up to the 0.5 percent.

This is a typical analysis of the PCR products. What you can do is you can run it along with the marker so that you will know that what is the size of your DNA what you are purifying or what you are getting. So you can see that we have run a negative control which means the water control and in that case only the template DNA is been formed. And the primer what we have added is not been incorporated and it is present as the primer dimers. Whereas when we are adding the template we are getting the amplified product and there is no primer dimer because the primer what we are using is been used up in synthesis of this particular DNA.

So there are multiple technological steps what we have discussed. So we started with the very basic that how the DNA synthesis occurs in the prokaryotic or the eukaryotic system and then slowly we moved on and tell you that, what are the technological steps required to set up the PCR reactions and what are the different types of reagents required to perform the PCR reactions. Apart from this, we have some of the technical topics to be discussed. So in the subsequent lecture we are going to discuss about the three aspects related to the PCR.

Number one, what we are going to discuss about how to design the primers, how to analyze the primers and how to verify that the primer what you have designed is good for PCR reactions, number two we are going to discuss about how to set up the reactions, how a typical thermal cycler looks like and what are the precautions and you are going to take while you are setting up the PCR reactions.

And the third is how to prepare agarose gel, how to analyze your PCR reactions and you how you will know that your PCR amplified product is correct or wrong and at the end, we are also going to teach you or we are also going to show you that how you can actually be able to analyze this particular image after the analysis. So with this, I would like to conclude our lecture here and in the subsequent lecture we are going to show you the couple of small clips or the movies to elaborate all these steps. Thank you.