Molecular Biology
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Module - 09
Molecular Techniques (Part 2)
Lecture-37 Polymerase Chain reaction (Part 1)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati. And what we were discussing, we were discussing about the different aspects of the molecules into the course molecular biology. So, for what we have discussed, we have discussed about the cell biology, we have discussed about the cellular structures organelles their role into the cellular metabolisms and then we also discussed about the different types of biomolecules. So, we have discussed about the proteins, lipids, enzymes, DNA and RNA and we have also discussed about the their structure and functions and how they are regulating the different events. And then subsequent to that we have also seen the how these molecules are even regulating the cellular events.

So, we have discussed about the apoptosis, we have discussed about the autophagy and so on. And we also discussed about the cell division and then subsequent to that we have also discussed about the central dogma of molecular biology and we have discussed in detail about the different events what is comprising the central dogma of molecular biology. So, we discussed about the replication, transcription and translations and following that we have also discussed about the different types of techniques. So, when we were talking about the molecular techniques in the previous module, we have discussed about the blotting techniques and purpose of the blotting technique is to detect the particular biomolecules.

So, in the southern blotting we are going to detect the presence of a particular gene fragment into a particular DNA molecule or the genomic DNA. Then we discussed about the northern blotting. So, the northern blotting is going to tell you about the presence of that particular gene product. So, we are going to talk about the transcriptional activity of that particular gene and then we also discuss about the western blotting. So, western blotting is going to talk about the expression of that particular gene.

So, in the previous module we discuss about the blotting techniques. In the current module we are going to discuss about the some of the amplification techniques. So, in the amplification technique one of the most popular amplification technique the polymerase chain reactions. So, what is the polymerase chain reaction? So, polymerase chain reaction is a technique which actually allows you to the production of or the synthesis of a specific sequence from the genomic DNA or the other source of the DNA. Now, before we get into the detail of how to this technique is been developed, how this technique is been evolved, it is important that you should actually be able to understand the process of DNA synthesis.

And for that I would highly recommend that if you have not seen the my previous video of the DNA applications then you should actually should go through because the sole basis of the PCR

is the fundamental steps what is been found when the people have discovered the DNA applications. And more or less the machinery also remains the same except that some of the events are been modified where the protein was required. So, it has been modified with the physical parameters so that you can be able to achieve the similar results. So, it is important that you should be able to understand first the DNA application in detail so that in fact, you should also read about the DNA structures and other things. So, that it will be easy for you to understand the process.

Now, what is PCR or polymerase chain reactions? So, polymerase chain reaction is been used to amplify a double standard DNA molecule from the genomic DNA and it is been required to amplify a double standard DNA with the same size and sequence by the enzymatic method and the cyclic method. So, for example, you can imagine that if this is the original DNA right what is mean by the PCR is that this is the original copy of the DNA you are actually going to generate the multiple copies of this particular DNA. So, for example, I have generated first copy, second copy, third copy, fourth copy all these four copies are of having the same sequence what is present in the original copy. So, this is actually the original DNA what I have used in this case and I want to amplify this DNA. So, what I have done is I have amplified this DNA and I have made the four copies now and with the same size and the same identical sequence and this is been achieved by the process of called as polymerase chain reactions.

Now, when you want to understand the polymerase chain reaction it is as I said you know it is important to study the DNA structures and the DNA applications. So, the process of this polymerase chain reactions or the basic understanding why you can be able to do to you why you how you can be able to do this is actually lies into the DNA structures. So, do you know that the DNA is actually the complementary in nature. So, you are going to have the primary strands. So, this is the first strand and then you also going to have the complementary strand.

What is mean by the complementary strand is that whatever you are actually going to be present here it is mean going to be complementary and complementary is going to follow the rule that A is actually going to have the two base pair or two hydrogen bonding with T and G is actually going to have the three triple bonding with the C. So, wherever you have A into the primary strand it is going to be T in the complementary strand. Similarly, wherever you have G in the primary strand it is going to be the C in the complementary strand. This means if for example, you have G here. So, you have C here, you have G here, you have C here, you have T here.

So, if you have T here in the primary strand then it is going to be A into the complementary strand and so on. So, if you have A in the primary strand then it is going to be T in the complementary strand. So, if I want to amplify this particular sequence what I can do is I can use this DNA replication machinery and I can be able to use this as a template for the leading strand. So, for example, this is going to be a leading strand and then this is going to be a lagging strand. So, I can actually be able to do the synthesis of this into utilizing this as a template and I can use the synthesis of this utilizing this as a template.

And that is how I can actually divide this into two parts. I can divide this into two sequence. So,

you can have this is the 5 prime to 3 prime and the other one is 3 prime to 5 prime. So, this is the first this is the complementary strand, this is the primary strand what you are going to see here. And then what I can do is I can just use this and I can just put the DNA replication machinery and it is actually going to give me this particular sequence.

Similarly, I can put the DNA replication machinery on this side and I can actually be getting this. So, if I if you recall the DNA replications so that DNA replication machinery only can be used for achieving this particular target. So, that you can be able to have the same sequence identical sequence been present as multiple times. So, this can be done by multiple times and that is how you are going to have the amplified DNA. So, for this you actually need to run the DNA replications.

Now, if you see the DNA replication, DNA replication has three steps. You have initiation, you have elongations, you have terminations. So, in the initiation the DNA is going to be you know prepare. So, you are going to prepare the template DNA if I write like this. So, if you are going to have prepare the template DNA, that template DNA is preparation involves the protein binding to the DNA and then you are going to have the opening of the double helix, which means at this stage you are actually going to open and this you know break the hydrogen bonding between the binds.

And that is how this is going to be 3 prime to sorry 5 prime to 3 prime strand and this is going to be 3 prime to 5 prime strand. So, both of these strands are now going to be prepared for the synthesis. Now, in the elongation step you are going to have the synthesis step. So, in the initiation step also remember that the DNA a primer is going to be synthesized. So, in this case also DNA primer RNA primer is going to be synthesized and then this RNA primer going to be used by the DNA replication machinery and that is how DNA replication machinery is going to sit and it is going to synthesize the DNA as per the complementary T from this particular DNA right.

Similarly, the DNA strand will synthesis will stand here and it is going to synthesize this one right. So, in the second stage you are going to have the elongation which is going to be synthesis phase. So, the protein connects the correct sequence of the nucleotide into a continuous new strand of the DNA and then you are going to have the termination. So, it is going to stop the DNA synthesis. So, protein release the DNA application is over then the it is actually going to end up into the termination and that is how it is going to stop the DNA synthesis and protein are going to be released from the replication complex.

Now, for these three events you requires a battery of the enzymatic factors which are present in the cell and they are present in the correct ratio. So, that they one or other factor is going to come and this these events are actually going to be completely the controlled. So, let us see what are the different protein machinery what is required for initiation, elongation and terminations. So, you require the helicases, you require the primase and you require the single standard DNA binding proteins this is for the initiation ok. So, this is going to be the for first event that is the initiation then you require the DNA application and that is required for the elongation and you also require the tethering protein that is also required for elongation and termination is

always been done by the TIRP proteins.

So, helicases it is going to separate the two strands right that is how it is actually going to melt the DNA and melt DNA is going to have the two single standard DNAs and then the primase is actually going to have the RNA primer synthesis that so that it is actually going to provide the attachment point on to the DNA and that is how this that attachment point is going to be utilized by the DNA polymerase. Then SSB, SSB is going to prevent the re-endling of the single standard DNA or strands then you are going to have the DNA replications or DNA polymerase which is actually going to be participate into the synthesis of the new strands and then you also require the some of the tethering proteins that is actually going to stabilize the polymerase on to the DNA. Now, remember that all these enzymatic factors are present inside the cell and these factors are being present in a very adequate amount and there is a complete you know cellular machinery which is taking which is actually governing the presence of helicases, presence of primase, presence of SSB proteins. So, if I want to replicate these events into in vitro synthetic system then I need to add the helicase, primase, SSB at a regular time intervals right at a regular time interval and all these proteins will have their own half-life. So, it is important that it is very difficult to even the to manage these the regulation part right.

For example, when the helicase is separating the two strand the primase and SSB are actually going to be keep standing they are not doing anything once the DNA is been single-stranded and once it is going to open then only the primase will come and sit and it will actually going to synthesize the RNA primer right same is true here and this also right and while it is doing so SSB is actually going to go and bind the single-stranded DNA SSB will go will cover the nucleotides right that is how the it happens when you are doing the DNA applications and these SSB molecules are keep detaching from the DNA strands while the this been taken care by the DNA polymerase right. So, when the DNA polymerase when the primase will go and the primer is already been synthesized the DNA polymerase will come and sit and do and when the DNA polymerase is running it is actually removing all these SSBs right. This means SSBs are now been present into the solution right. Now helicase is also present in the solution right. So, helicase will actually going to interfere and then we do not know what will be the stoichiometry of these machinery which is required for DNA applications and so on.

So, that is why it is important that some of these events can be modulated by a physical parameters. So, that is what you are supposed to you know achieve when you want to do the polymerase chain reactions under the or the DNA application under the in vitro syndrome. So, if you want to achieve that you are actually going to do that by simply achieving all these three events in a in a by another method right. So, for example, for initiation you required a helicase in the in the natural system right, but the same can be achieved simply by heating the DNA because what helicase is doing it is actually breaking the hydrogen bonding right it breaking the hydrogen bonding between nucleotides. Same can be achieved by heating the DNA and you if you heat the DNA and if you heat the DNA at 95 degree Celsius or 97 degree Celsius then what will happen is that heating is also going to achieve the same thing it is actually going to break the hydrogen bonding between the nucleotides.

This means you can easily bypass the role of helicase simply by heating the DNA at 95 degree Celsius that does not mean that in the cellular system also that can be achieved. So, in the cellular system everything is being done at room temperature or 37 degree Celsius and that is why you require an enzyme to perform this task, but in the case of in vitro system because DNA is fairly stable right. So, you can actually heat the DNA and once you heat the DNA strands are going to be get separated and they will remain separated until the temperature is high and then you can enter into the another phase which is called as elongation phase and in the elongation phase you can actually be able to cool down this temperature right you can cool down to such extent the DNA will still remain as single standard, but it will not going to anneal it is not going to reanneal to each other and give you the double standard DNA and then what you are going to do is you are going to add the DNA polymerase you are going to add the DNA polymerase right and DNA polymerase is going to start the synthesis by taking the primer into the consideration and it is going to start the synthesis right. Once the synthesis is over and you can calculate how long this will take for doing the synthesis till this end you can actually be able to again go back to this and you can increase the temperature and this can continue for some cycle and that is how you can actually enter into the third stage where you can actually cool down the temperature and this can be achieved by keeping the reaction at room temperature or at 4 degree Celsius. So, once you bring the temperature at room temperature and 4 degree you are ideally going to do the termination.

So, that what you see here is that the DNA synthesis under the in vitro condition can be achieved and you can be able to remove some of the crucial enzymatic factors what is been participating very extensively into the DNA applications, but you can actually be able to replace them by simply changing the physical parameter and that is actually was a groundbreaking discovery because that has discovered a technique which is called as the PCR or polymerase chain reaction. But as I said you know these any technique what you are actually going to devote or evolve does not come into a single day it is actually going to come into a very very long day of doing the different types of discoveries. So, let us see how the technique is been evolved over a course of you know couple of decades. So, there are different events which are been responsible for the development of PCR. So, in the case of 1950 there was discovery of mechanism of DNA replication by the Arthur Kornberg.

He discovered the first DNA polymerase and other factors like helicases and primases. So, it is started with the DNA replication it started with the basic understanding about the DNA replication and then the people have discovered the machinery right just now that what we have discussed right you require the helicases, primases, SSBs, DNA polymerase and so on. So, all that machinery is been discovered in the early stage of 1950 right. And then what you see here is there is a huge gap of so in the year of 1976 the people have discovered the first thermostable DNA polymerase and you will understand this when I am going to discuss about different steps of the polymerase chain reaction why it is important to have a thermostable DNA polymerase. Then in the year of 1983, Kanimoulas and synthesizes the DNA oligos probe for the sickle cell anemia mutations and that is how he has performed the polymerase chain reaction to amplify the particular gene what is responsible for sickle cell anemia and that is how he could be able to show or demonstrate that you can be able to perform the DNA applications or you can be able

to perform the amplification under the in vitro conditions.

And in the same year 1983 he also discovered how you can be able to do the repeated thermal cycling and so that you can be able to you know amplify a small segment of the cloned gene and you can be able to use that for reducing the large amount of DNA. Then in the year of 1984 the Kanimoulas and Tom White tried a different experiment to test the PCR on the genomic DNA and but the amplified product was not visible into the agarose gel. And in the year of 1985 the patent was filed for PCR and its application focusing on the sickle cell anemia mutations. And in the year of 1985 the use of thermostable DNA polymerase in PCR was started out of only two enzymes Taq and BST known at the time. The Taq was more found more suitable for the PCR.

And then in the year of 1985 the first announcement of PCR technique in Salt Lake City and then in the year of 1985 to 87 there were development of instrument for the PCR and its reagents. These are the some of the notable discoveries I am not saying that I am going to give you a comprehensive events of or comprehensive account of the events what is being discovered or what is being related to the PCR. Because there are many more probably things are being done apart from these things. But these are some of the important discoveries what has been done and that has been responsible for the development of the polymerase chain reactions.

Now let us come to the technique. What you are going to do is you are actually going to do a repeated cycling of the same replication. And that is how you are actually going to do the amplifier. How you are going to do that? You are going to do that by using a you know by using the simple technique like for example, this is the this is actually the template DNA ok or this is the starting DNA I will say template or starting DNA. Now this is a double standard DNA what you are going to do is you are going to do a denaturation. So, once you do the denaturation it is going to denature as a single standard DNA and it is going to form the two DNA strands.

This is going to be the strand 1 and this is going to be the strand 2. So, for example, if this is from 5 prime to 3 prime and this is the 3 prime to 5 prime then you are going to have this as 5 prime to 3 prime and this is the 3 prime to 5 prime. Now what you are going to do is you are going to add the primers right. So, you are going to add the primer. So, what will happen is the primer will go and sit on to the 3 prime end right.

So, it is going to sit on to 3 prime end. So, this is going to be on 3 prime end. So, PC has how this is going to be sit here right and then you are going to change the condition. So, that it will enter into the elongation phase you are going to add the DNA polymerase. So, it is going to utilize this primer and it is going to synthesize and it is going to go up to what because the template is going to be over. So, it is going to synthesize up to this same is true for this one also it is going to synthesize up to this one right.

Now what you are going to get is you are going to get the two copies. So, you started with one copy right and now you are after the first cycle. So, this is going to be after the one cycle you are going to get 2 DNA molecule right you are going to get 2 DNA molecule this is the one molecule and this is the second molecule. Now again if you continue the same thing right it is actually.

So, what you see here is that you are going. So, you started with the one DNA molecule you are going to have the 2 DNA molecule at after the end of the first cycle. Now if I do the same event again and again in the second event what will happen is this is actually going to give me the two templates right. So, it is going to give me the two templates imagine that this is same way I am going to show you right and again the same thing will repeat same is true for this one also this is also going to give me the two template this means after the end of the second cycle I am going to get the 4 DNA molecule and after the end of the third cycle I am going to get the 8 DNA molecule right and this will continue like after this you are going to get 16 molecules after 16 you are going to get 32 molecule and so on. So, after every cycle it is actually going to give you the double amount of DNA because the previous template previous synthesized DNA is also going to serve as the template right.

Now this is what is actually been achieved. So, PCR is a repeated cycle reaction that involves the mechanism of DNA replication. It results in the product of multiple copies of a DNA from a single one. The whole process involves three main events denaturation, annealing and elongation. A DNA fragment of interest is used as a template from which a pair of primer or a short oligonucleotide complementary to both the double standard of the DNA are made to prime the DNA synthesis where the direction of the synthesis or the extension is from 5 prime to 3 prime this means it is actually going to start from the 3 prime end as a template right as in as in as in in a DNA applications. The number of amplified DNA or the amplicon increases exponentially per figure such as the molecule of DNA give rise to 2, 4, 8, 16 and so forth after every cycle and if you want to calculate the amount of DNA right what you are going to amplify it is going to give you a with this equation which is called C is equal to C naught 1 plus e to power n where C is the final amount of DNA C naught is the initial amount of DNA and E is actually the efficiency and n is the number of cycle and S is the slope of the exponential phase.

So, E is always been calculated as 10 to power 1 by S and if E is 1 then the S is going to be 3.3 to 1. So, this is actually going to be used for calculating the amount of DNA what you are going to get because remember that after every cycle it is actually going to get amplified at double right. So, it is from 16 it is going to be 32 then 64 and so on. So, it is going to be an exponential application and remember that it is going to have the three events denaturation, annealing and elongations.

So, let us discuss about these events. So, that you will be able to understand more better about the DNA polymerase or polymerase chain reactions. So, polymerase chain reactions is going to be have the denaturation, annealing and elongations right. So, it is going to have the two round of denaturations you are going to have the initial denaturation which is actually going to denature the whole DNA. So, example you are talking if you are doing this from genome. So, then it is actually going to denature the whole genome right.

In the further denaturation it is actually going to only denature the small amount of fragment or a small fragment from where it is the primary is actually going to bind. So, in the initial denaturation it is going to be very long. So, you can heat the PCR mixture at 94 degree Celsius to 96 degree

Celsius for 10 minutes to ensure the complete denaturation of the template DNA. So, it is going to denature the complete genome and you are going to have the single standard two copies of the genome right. And then you are going to have the these events which are called denaturations, annealing and elongation on a repeated mode right.

So, these are going to be a part of the cycle right. So, you can see this is the initial denaturation right which is actually going to be called as stage 1. So, for example, in this case I have we have done it 95 degree Celsius for 5 minutes. So, it can be 5 minutes it could be 10 minutes. Then in the stage 2 you are going to do a repeated cycling. So, this is going to be a cycle this is a one cycle where you are going to and you are running this cycle for 30 times right.

In a single cycle you are going to have the denaturation, annealing and elongation. So, what happened in this denaturation? So, this is the first step in which double standard DNA template is denatured to form the two single standard DNA by heating at 95 degree Celsius for 15 to 30 seconds. So, for example, in this case it is 95 degree Celsius for 30 seconds. Then you are going to have the annealing. So, this is the annealing step where the temperature is going to be lower down not to that extent that the single standard DNA is going to be double standard DNA and going to form the double standard DNA, but good enough so that the primers and the should be able to anneal or should be able to bind the template according to the sequence complementary.

So, you are going to have usually 50 to 65 degree Celsius primers are allowed to bind the template DNA annealing time is 15 to 30 seconds. You remember that this time is being regulated in such a way so that it should actually allow the annealing of the primers to the template, but it should not allow the complementarity DNA should be you know should not form the double standard DNA. And then it depends on the length and the basis of the primers. And then you are going to enter into the elongation force. So, this is the synthesis step where the polymerase perform the synthesis of the new strand in the 5 prime to 3 prime direction using the primers and the dNTP.

So, dNTPs are the raw material what you require for the synthesis and average DNA polymerase adds about 1000 base pair per minute and step 1, 2, 3 make the 1 cycle and in general 30 to 35 to 40 cycles are performed in a typical PCR amplification. So, this is actually denaturation annealing elongation is going to be performed on a repeated mode. And for example, in this case, we are doing it for 30 times, but you can do somewhere around 35 to 40 cycles. After the cycles are complete, which are all the 30 cycles are complete, then you are going to have the final extensions or the extra elongations. And the reaction is held at 70 to 74 degrees Celsius for several minutes to allow the final extension of the remaining DNA to fully extended and then you are going to do the termination.

So, finally, you are going to hold the reactions at 4 degrees Celsius. So, that is actually going to terminate the synthesis and it is going to keep the reactions at 4 degree for a very very long extended period of time. Now, if I want to perform the PCR reactions, I first require to perform up you know, the setting up these cycles and the setting of these cycle has to be done into a PCR machine. So, what are the these cycles? So, you are going to have the initial denaturation which

is 95 degrees Celsius for 5 minutes, then you are going to have the you are going to set up how many number of cycle you want to perform. So, for example, if I want to perform the number of cycle at it for 30 cycles, then you in 30 cycles, you are going to have the denaturation, annealing and extension.

So, you are going to have the annealing for 95 degrees Celsius for 30 seconds, 55 degrees Celsius for 30 seconds and extension would be 72 degrees Celsius for 45 seconds. All these annealing and extension depends on the annealing extension especially if the extension depends on the length of the gene or length of the gene fragment which you are going to synthesize. So, as I said you know 1000 base pair per minute that is the criteria. So, if you are synthesizing for example, 1000 base pair right, then it is going to be 1 minute. Then you are going to have the final extension that is the 72 cycles for 5 minutes and then you are going to have determination or the holding that is usually been done at 4 degrees Celsius.

Now, what is the requirement or what are the different things you require for performing the PCR? So, if you want to perform the PCR, you actually require following information or following materials. You first require the DNA sequence of the target region must be known. So, that is very very important because and that is probably the one of the drawback of the PCR that you require the sequence of this particular DNA what you want to amplify. Then you require the primers. So, typically 20 to 30 base pair in size they can be readily produced by the commercial companies can also be prepared using a DNA synthesizer.

So, then you require the primers. These primers are been always been directed against this particular sequence and that is why you require this particular sequence. Then you require the thermostable DNA polymerase for example, the tag DNA polymerase which is not inactivated by heating at 95 degree Celsius. So, remember that when we were discussing about this right. So, if you do not have a thermostable enzyme or thermostable DNA polymerase then the moment you are going to enter into the denaturation state that is the 95 degree Celsius the DNA what the enzyme what you have added like the DNA polymerase is going to be inactivated. So, to avoid this only people have discovered the thermostable DNA polymerase.

So, that it when you are going to run these cycles for 30 cycle or 40 cycle when you reach to the denaturation stage right then you want to then your enzyme should remain intact and enzyme should not be get inactivated because then you are supposed to add the enzyme in a repeated cycle and then it is very very impossible or it is difficult to perform the PCR reactions. And then you also require the DNA thermal cyclers. So, machine which can be programmed to carry out the heating and cooling of the sample over a number of cycles. These thermal cyclers are actually very specialized machine and their job is nothing but to regulate the required temperature. So, that if they should incubate your reaction at a particular temperature until the people who have not discovered the thermal cycler what they were doing is they were you know manually changing the reactions they were manually changing the tubes from one temperature to another temperature.

For example, in the initial stages of PCR development they were having the three different

buckets right. So, they were having the three different types of incubators or water baths one is set as 95 degree Celsius the other one is as set as 55 degree Celsius and the third one is set at 72 degree Celsius. This one is actually doing the denaturation this one is doing the annealing and this one is doing the elongation. So, what they were doing is they were setting up the reaction into a you know a block right. And then this block is either first placed into the 95 degree Celsius then from here you are they are going to place it into here and then from here it is going to be place it here right.

And then from here again it will come back to this place again like that. So, it was very very cumbersome it was very difficult to perform and then during this you know shifting of the block from 95 degree Celsius to 55 degree Celsius or from 55 to 72 there will always been a change in temperature and other kinds of things happens and that also affects the overall productivity and overall quality of DNA what they get actually. But that time the electronics was not intact and we were not having the very good quality you know cooling machines and all that and that is why the people are not having the this kind of sophisticated thermal cyclers. So, this is a typical thermal cycler what you see is this is from applied bio system right. And the thermal cycler is a instrument that carry out the amplification via the polymerase chain reactions.

The device has a thermal block. So, what you see here is this is a thermal block and that has the holes right that has the wells. These wells you are actually going to place the your appendops or the reaction vessels. So, device has a thermal block which has holes where the tubes holding the reaction mixture can be inserted. The cyclers then raises and lower the temperature of the block in a discrete pre-programmed steps and you have seen the programming right that you are actually going to ask the system to you know increase the temperature to 95 you lower down the temperature to 55 and then hold the temperature at 55 for 45 seconds 30 seconds or whatever. And that can be done very efficiently into this kind of program in this kind of machines.

Now, how this machine actually do? So, these machines are always been using the Peltier system and the Peltier system is cooling and heating the system or very efficiently. It is much better than the compressors actually. So, each module is composed of the N and P type of thermal semiconductor material and the Peltier phenomena occur at the junction of these two similar conductors. So, when the current is passed to the junction a transfer of heat occurs from one side of the junction to the other and QL is the absorb heat on the cold side and the QH is the release heat on the other side. So, because of this the you know the absorption of heat or the release of heat you can be able to maintain the temperature very precisely and that too at a very very rapid rate.

Now, let us move on to the what are the material required for the performing the polymerase chain reactions. So, as I said you know you require a template DNA. So, you can have the 1 picogram to 1 nanograms of template DNA for a viral or the short templates, but you require a 1 nanogram to 1 microgram for genomic DNA. So, depending upon the source of the DNA you can be able to use the different types of products, different amount of DNA actually. So, you can use 1 picogram to 1 nanogram if it is a viral or the short DNA short templates, but if it is a genomic DNA then you are supposed to use 1 nanogram to 1 microgram.

Then you require the primers. So, you require two different types of primers you require a forward primer and a reverse primer which means you require a primer for doing the synthesis of the leading strand and you require a primer for doing the synthesis of the lagging strands. Although there is no leading and lagging strand in the case of polymerase chain reaction because the synthesis of both the strands actually completed at the same time, but just for making you more familiar with the DNA replication I am using this kind of terms. So, you require two different types of primers forward primer and a reverse primer and you are going to add the primer in the range of 0.

1 to 0.5 micro moles. Then you also require the magnesium chloride because magnesium chloride is going to work as a cofactor for the DNA polymerase. So, you require the 1.5 to 2 millimolar DNA polymerase for the Taq DNA polymerase and then you require the dNTPs. So, you require the four different types of dNTPs you require the dATP, you require the dTTP, you require the dGTP and then you also require the dCTP. So, you require the ATGC that is what is required for the synthesis of the DNA and the typical concentration is 200 micro mole of each dNTPs.

Then you also require the Taq DNA polymerase, so Taq DNA polymerase you require the 0.5 to 2 units per 50 micro reactions. Remember that the PCR is going to be performed into a large volume and I will explain you this when we were going to talk about the restriction digesters and other things. So, remember that when we were talking about restriction digestion I said you know that restriction digestion has to be performed into a larger volume such as 50 microliters whereas the PCR you are supposed to perform also into the larger reactions, but ligation when you are going to do it has to be done in a smaller volumes. Now, let us talk about the template DNA. So, template DNA could be of multiple types it could be a genomic DNA, it could be a DNA fragments, it could be plasmids, it could be a viral DNA or it could be a tissue sample.

Whatever would be the source you are supposed to use the amount of DNA accordingly it should be a blood actually, it can be a blood sample, it could be a blood sample from the crime side or some place. So, that also can be used. So, from the blood sample also you are going to use the DNA for detecting a particular fragment. So, these are the different types of sources of the DNA templates. You can have many more things from the tissue you are actually going to get the cells and from the cells you are supposed to isolate the DNA and that is going to be served as a template for performing the preliminary chain reactions.

Then you require the primers. A primer is a short DNA stretch that serve as a starting point for the DNA synthesis. In PCR you require the two primers which bind each of the single standard DNA flanking the target sequence. These are called forward primers and the reverse primers. The primer have a sequence complementary to the sequence in the template strand where they are supposed to start the synthesis.

Then you require the enzyme. So, you require the Taq DNA polymerase. So, Taq stands for the thermos ecutus which is a microbe found in the 170 degree Fahrenheit hot springs in the

Yellowstone National Forest and the Taq DNA polymerase is stable in a high temperature and act in the presence of magnesium. The optimal temperature for the Taq DNA polymerase is 70 degree Celsius. Now, what is the December? These are the advantages that it will not get denatured when you are doing the different cycles and all that, but it also has the disadvantage. So, when you are actually going to use the Taq DNA polymerase, it is going to have the different types of determinates. Taq polymerase lacks 3 prime to 5 prime exonuclease proofreading activity commonly present in the other polymerases.

So, it means it is actually not going to check whether I have added the correct nucleotide or not. So, it is actually going to make you, it is going to synthesize the DNA, but may have the errors. Then Taq DNA polymerase misincorporate one base pair into the 10 to power 4 base pairs, right, which means out of 10,000 bases, it is going to incorporate one base pair. Although this number looks small, but when you are going to do a synthesis of the DNA synthesis of a large DNA fragments, it could actually accumulate the mutations, right. In the first cycle, it may mutate the number 5, it may in the second cycle, it may mutate the number 8, it may third cycle, because you know, this is, this we are talking about in the each cycle.

So, for example, in the first cycle, it may mutate the fifth residue, right. In the second cycle, it may mutate the 8th, right, it may mutate 11th. So, that is how it is actually going to keep accumulating the mutation when you are doing the 36 or 37 cycles. So, that is why it is important that we should not have these kind of mis-corporations. For a 400 base pair target with contain an error is 33 percent of the molecule with the 30 cycles. So, if you are actually amplifying a 400 base pair DNA, right target DNA, then it is actually going to incorporate 33 percent of the new molecule after.

So, whatever the molecule it is going to produce out of those the 33 percent molecules are going to have the, the you know, mis-incorporated nucleotides. So, error and the error distribution is going to be random. This means what we are going to be emphasize is that if you use the tag DNA polymerase, it is actually going to incorporate the mutations into the amplified DNA and that is not going to good if those mutations are going to change the downstream applicable, the applicable utility of that DNA for the downstream applications. So, what is the alternative? Alternative is that you are going to use the other kinds of DNA polymerases. So, you can use the PFU DNA polymerase which is from the pyrococcus furioces and it process the 3 prime to 5 prime exonuclease proof reading activity and because of this it actually can cross verify whether it has added the correct nucleotide or not.

The error rate is only 3.5 percent after 20 cycle more amount of primer is added to avoid the primer dimerring and for an as explored gene the primer used in the closely related species are being used. Remember that what we have said, we have said that the prime requisite of a polymerase chain reaction is that you should have this information about the sequence of the DNA. But what if you do not have the information about the gene information or you do not have the sequence information, then in those cases you can be able to synthesize the primer based on the closely related species. So, if you use the closely related species for example, you do not know the sequence in human, but you may know the sequence in monkey or you may. So, that

sequence information you can use and it may actually give you the amplified product and that you can actually be able to sequence and verify whether it is actually that sequence or not and that is how you can be able to overcome this kind of limitations.

Now, let us see how you are going to set up the PCR reactions. So, the first setting up the PCR reactions you are actually required the template DNA, primers, magnesium chloride, ENTPs, tag DNA polymerase, buffers and water. So, what you are going to do is in a 50 microliter reactions what you are going to do is you are going to first going to add the water, whatever water as per the calculations, then you are going to add the buffer, tag DNA polymerase buffer, then you are going to add the DNTPs, you are going to add the primers, you are going to add the magnesium and then you are going to use the tag DNA polymerase and that is actually going to make you the PCR mix. Now, into this PCR mix you can be able to add the template. So, you can actually be able to use the different types of templates if you want and that is why you are going to add the template at the end and as soon as you add the templates then you are supposed to put it into the thermal cycler and you are going to close this lead.

This lead the top lead also has a heating block. So, this what you see here is this is actually a heating block and it is actually going to stop the evaporation of the material from the your append-ops or your tube actually. So, because the top heating block is also going to be maintained as 100 degree Celsius. What is the role of this? The role of this is that suppose this is a tube and you have kept the PCR reaction at the bottom. So, this is going to be placed in this block which is actually going to maintain the temperature of 95 degree Celsius, 55 degree Celsius, 70 degree Celsius.

So, in that case what will happen is it is actually going to evaporate although it is closed by the lead. It is going to be closed by the lead, but it is actually going to evaporate. So, all these are going to evaporate and they will actually going to accumulate on the top. It is going to accumulate on the top surface. Now, if it is accumulating on the top surface then it is all the enzymes and other things will not get a chance to interact with the reaction mixtures. So, to avoid this what you are going to do is you are going to keep a heating block on the top and what is the temperature of the heating block? The temperature of the heating block is 100 degree.

So, even if you have a temperature of 95 degree Celsius at here the top temperature is 100 degree. So, because of that it will all the liquid will come down because it will evaporate, but the top chamber it the temperature is more. So, that is why it will not go up it will actually be present at the bottom only. So, it will all the liquid will remain as the liquid in the tube and that is why it is actually going to perform the reactions. Now, let us take I will take you to my lab for a demo how you are going to set up the PCR reactions, how you are going to perform the PCR reactions and how you can be able to set up the reactions and analyze the results. In this video, we will be demonstrating how to set up a PCR reaction and analyze the results using other room gel electrophoresis.

PCR or polymerase chain reaction is a widely used molecular biology technique to amplify a particular segment of DNA. It is also employed in biomedical research and forensic medicine.

The main application of this is polymerase chain reaction is ronin. To set up a PCR reaction, we need template DNA, site specific primers, DATP mix, nucleus free water and Taq polymerase. For a 50 microlitre reaction in a typical concentrations of 10 to 100 nanograms of template DNA used and 5 picomoles of each primer will be used.

So, this is an earlier version of thermal cycler which contains display unit where we can observe the parameters and change the parameters. This is a hard shield, this is sample holder and inside there is a Peltier system which can maintain the temperature fluctuations. For setting up a PCR reaction, initial denaturation at 95 degrees Celsius, 3 minutes and these steps we will use 30 repeats where initial denaturation will be 30 seconds and annealing it, extension time should be given 1 minute per kB. And here final extension should be given 10 minutes and hold at 4 degrees Celsius, 10 minutes.

Once the PCR reaction is completed, we have to analyse the results for amplification. For that we need agarose and TAE buffer. First we have to weigh agarose and mix with the TAE buffer. It will not dissolve easily, so we have to heat it in microwave oven until it get dissolved.

Now agarose got dissolved in TAE buffer. We have to let it cool down up to 53 degrees Celsius. Now before pouring we have to add lithium bromide for detection purpose. Now the gel got solidified, we have to take out the gel and keep it in the electroporotic apparatus. We have to gently remove the comb, lose the knobs and keep the gel in the apparatus.

Make sure that the buffer is submerged in the gel. We have to fill the remaining part with 1X TAE buffer. Generally for analysing the DNA samples we will use agarose gel electrophoresis. This is the power pack and this is the electrophoretic apparatus. This is negative electrode and this is a positive electrode.

We can change the voltage from here. For loading of sample, we have to mix PCR reaction mixture with 5X loading time. So after loading is over we have to cover the electroporotic apparatus with the lid and we have to adjust the voltage. Then start rough. After the agarose gel electrophoresis we have to visualise the amplified product. This is the chemiduct MP where we are going to visualise the amplified product.

Now we have to keep the gel and close the thing. We have to select here application nucleic acids, ethidium bromide, exposure, optimal exposure or we can select manual also. Then we will acquire the images. Now we can find here this is the DNA ladder, this is the PCR amplified product. We can transform it into transform or save this image into JPG.

In this video we have discussed how to set up a PCR reaction and how to analyse the PCR result. We have also shown how to use a thermal cycler and what are the components of thermal cycler. So during all these process we have to take some precautions to get better results. Like all the time you have to keep palmarise engines and your primers on ice. And other thing we have to remember is while running the agarose gel electrophoresis always wear the gloves to prevent any contamination with the ethidium bromide.

So ethidium bromide is a carcinogen. So it is not very likely to cause any cancer but we have to make sure that we are providing this kind of touching ethidium bromide. Now once you set up the PCR you are actually going to face the different types of problems. You are going to face the multiple types of issues like you are not getting the amplifications, you are getting the amplification of the non-specific bands and so on. So how you are actually going to encounter or how you are going to provide the solution to that.

So you are going to do the optimisation. So optimisation would be for the reaction cycles you are going to do a hot star reactions. So hot star means you are actually going to keep the reaction into the machine. For example like and this what we do is we actually keep the tubes into the machine and then we turn on the machine. So what happen is it is actually going to first enter into the stage 1 where it is going to do a 95 degree Celsius for 5 minutes. Then it will enter into the cycles and it is going to do the stage 2 and then it will enter into the stage 3.

On the hot star what you are going to do is you are going to turn on the machine and let the machine reaches into the stage 1. So once the temperature is at 95 degree Celsius then you are going to open the lid and then you are going to insert your reactions. What is the advantage? Advantage is that it will not going to allow the non-specific amplification. So what happen is when you are even if you are keeping the reactions on 37 degree Celsius or in ice some primers actually go and bind some amount of DNA is get you know get access to these primers. So primers actually goes and bind to multiple places non-specifically and that is going to you know initiate the synthesis of the non-specific DNA.

So to avoid that what you can do is you can just go with the hot star. Then sometime you are actually going to optimize this primer sequences you are going to optimize with the different types of enzymes. For example, you may not get the better amplification with the VFU DNA polymerase you may go with the tag DNA polymerase and so on. And then sometime you also going to have the additives. So there are different types of additives what you can use for overcoming the false positives or your actually going to use it for taking care of the non-specific amplifications. For example, if no product is produced or there is no correct size product produced then you can actually be able to use the check the DNA quality.

First thing what you do is you check the template whether the template is correct. Then you reduce the annealing temperatures because you allow suppose you are annealing at 65 suppose you are annealing at 62 degree Celsius. Then you lower down because you are not getting any products. This means you should allow the annealing temperature. So you maybe do it like 59 or you may do it at 58. See whether you are primary annealing sufficiently or not you can increase the time also you can increase the time like from 30 to 50 seconds or so on.

Then you can increase or play with the magnesium concentration because some of the enzyme requires the higher concentration of the magnesium and some enzyme requires the lower concentration of magnesium. So you do not know the what is the optimal magnesium concentration required for tag DNA polymerase to give you the optimal product. Then you can

add the DMSO to add the assay so that it can also give you the some advantage and it going to give you the better amplified product. You can use the different thermostable enzymes as I said in an example like PFU versus if you use the tag DNA polymerase. The tag DNA polymerase will give you the product but PFU may not give you the product because the PFU's Km values are on a higher side compared to the Km values of the tag DNA polymerase which means tag PFU requires the more amount of templates compared to the tag DNA polymerase.

Tag DNA polymerase is more efficient in terms of giving you the product but it is having a problem of the it does not have the proofreading activity whereas the proof PFU has a proofreading activity. So you would suppose to change the enzyme you may actually that may also help in getting the right product. At the end if the thing happens then you should throw out all the primers and you should start the new primers and because sometime the primer also get degraded and 50 get crypted and all that and that is how they may not binding to the correct DNA sequence or they may not binding to the DNA sequence at all. Now if you are getting the non-specific product so you are going to have two condition only no product or you are getting more product then you can actually be able to do many of these things which are you know reverse like for example you can increase the annealing temperature, you can reduce the magnesium concentration, you can reduce the number of cycles and you can also try the different types of enzyme.

We gave you example of PFU versus tag DNA polymerase. Now once you are done with these PCR you are going to analyze the PCR onto agarose horizontal gel electrophoresis right and what you are going to see here is that you are going to get the product. So for example you are going to run the three different reactions you are going to run it along with the marker so that you will be able to know what is the size of the amplified product. So this is actually the negative control so you are not getting any DNA amplified DNA except you are getting the template DNA and then this is the amplified product what you are going to do and these are the primers right what you have used. So remember that in a negative amplification primers are not being consumed and that is why you see a primer whereas in the case of the positive amplification there is no primer right because all the primers are being used up in giving you the amplifications. So once you are done with the analysis of the PCR, the PCR is complete the amplified product is loaded onto the agarose gel and observed under the UV a weak water blank reaction is included to monitor the cross contaminated DNA source as a template.

The percentage of agarose gel depend on the size of the DNA to be visualized generally 0.8 to 1% agarose is used for analyzing the 0.525 kB DNA while a DNA larger size or genomic DNA is visualized in gel with the lower percentage of the agarose. Now why we are doing the PCR we have the you know speed easy of use and sensitivity and robustness and what are the limitations of the PCR as I have discussed right we need a target DNA sequence information you require the fidelity of the DNA applications and so on. So you require the target DNA sequence information so that you can be able to do a primer designing for unexplored ones and you can actually be able to have the boundary region of the DNA to be amplified must be known. Tag DNA polymerase no proofreading mechanism so it is going to have an error of 40% after 20 cycles so short size and limited amount of PCR product is going to be formed then you are going to have up to 5 kB it can be easily been amplified but up to 40 kB can be amplified with some modification

but cannot amplify with the gene which are larger than 100 kB.

So for these kind of large DNA gene fragments you will not be able to amplify with the help of the PCR. The PCR cannot be used in a genome sequencing project because you cannot be amplify the whole genome and that is why you can be able to produce or synthesize that. So these are the some of the limitations so these are some of the theoretical aspect of the PCR what we have discussed in this particular lecture what we have discussed we have discussed how the PCR is been evolved and how the concept of the PCR came by taking the inspiration from the biological phenomena of DNA applications. So people have discovered how the DNA is been synthesized into the cell by the discovery of DNA polymerase by the author Kornberg and his colleagues. But how to modify those events how to modify those things are been always been achieved once the people have discovered the tag DNA polymerase and other kinds of enzymes and then they actually started doing the experiment with the different types of physical parameters and so on so that they can be able to break the double standard DNA into single standard DNA so that they can be able to avoid the role of the helicases, primases and all that. And that is been achieved by mostly by the Carey Mullers and his colleagues and that is how he has developed the technique of polymerase chain reactions.

And in a polymerase chain reactions you require the three events you require the denaturations you require the annealing and you require the elongations. And all these are been performed by the varying the temperature from the 95 degree Celsius to 55 degree Celsius to 72 degree Celsius. And by doing so you can be able to amplify a DNA fragments and then you can be able to get the desirable product. At the end we have also discussed about the different types of the limitations of the PCR and we have also discussed about the troubleshooting part. So with this kind of background information we would like to discuss more about the technical part how you are going to design the primers how you are going to set up the reaction and so on.

So that we are going to discuss into the subsequent lecture. So till then we would like to conclude our lecture here and in our subsequent lecture we are going to discuss some more aspects related to polymerase chain reaction. Thank you.