Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Week 02

Lecture 06 : PCR (Polymerase Chain Reaction) Fundamentals

Hello students. Welcome back to the second week and second module of the course on Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis. I am your course faculty Dr. Aarendam, I am a faculty of biochemistry in Dr. B. C. Roy Multi Speciality Medical Research Center at IIT Kharagpur and I will be your co instructor along with Professor Aritsri in this course.

Now in week 1, you have been taught the overview of various principles and steps of genetics you have been taught about the basic structural aspect as well as the replication and the how the process of basic genetics takes place right. We will use these concepts in this week and the week further where we will be learning various tools on which we can apply those concepts. Now in this very first class of the week 2, we will be discussing the most important tool that one should always know that is PCR or polymerase chain reaction alright. So, these are the concepts that we will be learning in this module.

We will be discussing about the basic concept of PCR. There are few ingredients that we will be needing for PCR. We will be discussing how the amplification occurs in PCR. We will definitely discuss what is amplification, what do you mean by the term amplification there are various steps of the polymerase chain reaction we will be discussing those, how we can optimize those steps in PCR we need something known as primer, how we can consider the various guidelines, how we can design the primers as well as we will be talking about where we can apply the PCR, what is the need of learning PCR. So, mind it the application of PCR will be naming a few, but all of them will be discussed in much details ahead in the course right.

So, right now those who have been interested in genetics I bet you must know the term polymerase chain reaction you some of you might have heard right. So, if you already know what is PCR then the lecture will be much more interesting for you. If you already know everything about PCR if you are post graduate student watching this I will also

suggest you to please take into the video because you will be learning definitely something new. And for those who are who have been listening to this term for the first time let me tell you polymerase chain reaction is a technique that is used to create copies of DNA fragment. Now see we can this is the photocopier machine right we put paper in the photocopier machine and by the press of a button the machine goes on and on and it will deliver multiple copies of the same source right.

We use the same analogy only in place of the paper we use DNA fragment and that DNA fragment will be amplified. Amplified means multiple copies of the DNA will be produced. Now instead of the source since we are using DNA we cannot use a paper photocopier for it right. So, the machine here is basically PCR machine you can say the correct terminology is thermal cycler the machine in which polymerase chain reaction happens which actually converts a source DNA into multiple copies of DNA is known as thermal cycler right. So, there are multiple many things which you can just I mean guess or try to decode from the slide itself first the name polymerase chain reaction as I told you it is abbreviated as PCR.

So, henceforth in any this whole course whenever the faculty members use the term PCR you should always know we are referring to polymerase chain reaction. Now from the name itself we can have some idea what do we mean. So, it is a reaction means something will be converted to something it is a chemical reaction that happens right. Next it is a it is the name polymerase in week 1 you have been taught about replication right you have heard about DNA replication, RNA replication here we are considering DNA for PCR right because the definition very definition is DNA segment replication. So, what do we need you might have guessed it right we need DNA polymerase.

So, the name polymerase. Now what about chain? Chain means you know small segments joined to form a long structure right chain reaction means when one product acts as a reactant for the next step. So, the reaction goes on and on and on. So, after step 1 the reactant 1 will be converted to product 1 this product 1 will act as a reactant for step 2 again this reactant will be converted to another product that will act as a reactant for step 3. So, it goes on and on and on for multiple cycles right.

Again the machine it says thermal cycler. So, cycler you already know there is some sort of cyclic reaction going on and the word thermal means the machine has got a capacity to change the temperature you this yes this machine is does only does that it changes temperature from a certain value to certain value again it can set that temperature to another value and it can do it in cycles again the whole thing will be repeated alright. So, this is the machine thermal cycler and we are using DNA to make multiple copies of DNA. These multiple copies are known as amplification we are amplifying DNA this is replication alright. So, before going into the details let us

understand what is the need for polymerase chain reaction what is the need for PCR right.

So, if we can look from a clinical perspective or in a question if you try to answer a question we will definitely find the need for PCR. Now all of us at some point of time must have witnessed some sort of episode of a detective serial or detective movie right where some sample is found at the crime scene and the what does detective do the detective collects the sample the forensics department collect those samples and most of the time all of the time they are matched with the DNA right. So, this DNA can be DNA of any suspect right DNA can be DNA of the unidentified victim to a DNA which is already in the police database. Needless to say often it is found that the sample is so minute that the amount of DNA collected from that sample may not be enough in order to match with the database right. So, what actually needs to be done you know a part of a DNA is matched with the part of a existing DNA database or a part of the DNA of a victim or the accused or the criminal right, but that sample you will be blurred the sample will be here sample sample will be skin sample semen sample anything you say the sample first is to be chemically processed right and then from that DNA is extracted.

Believe me if I collect 5 milliliter of blood right and we try to collect DNA sample from it the amount of final DNA sample will be in microliter it is very small the final volume and you see at the crime scene the samples are taken in swab. So, you can imagine the very small amount of DNA that is finally, received or collected or extracted, but scientists with the help of forensic scientists still do easily match those DNA samples how do they do it the answer lies in if we can make multiple copies of DNA that is known as amplification. This amplification or amplifying multiple copies of DNA can be done using two methods mainly right. Number one polymerase chain reaction or PCR that we are discussing in week this week itself will be deciding various bits and pieces of PCR will be in this class will be learning about the fundamentals in the next class will be learning about the variations what is RT-PCR what is real time PCR how we can detect mutation with PCR many things it will later the concepts will again come later right. And let me tell you at this point since this course is now being floated in 2023 we are in the post pandemic era.

So, not only scientists not only students not only science students know about PCR every common people have heard this term RT-PCR right for detection of COVID right anyways. So, then you got another answer I mean what is the application of PCR right. So, the next method by which we can amplify a segment of DNA is cloning this is a schematic diagram of cloning we will be discussing cloning in later modules we will now focus on polymerase chain reaction in this week right. So, as we all know we are going to again reinforce the concept PCR means to amplify a particular piece of DNA right segment of DNA. So, this is known as amplification which is numerous copies of a

DNA segment DNA sequence not only that how many copies numerous means it can make billion copies of a target DNA sequence in a short time right you can one can add simply conduct the replication reaction we can add the nucleotides one by one can take a long time just to complete one reaction and if we multiple reaction with billions of copies it will take ages we do not have time for that right.

In 20 to 30 minutes we can have billions of copies of this is the beauty of the thermal cycler right. So, basically it is nothing, but the DNA replication which you have been taught in week 1 this the thing is happening in laboratory the laboratory version of DNA replication whenever something happens in laboratory in test tube in a reaction vial it is in vitro and whenever something happens in body inside a living cell we call it in vivo. So, it is in vitro replication in vitro DNA replication right. So, this reaction happens in this type of locked vial plastic vials those are known as PCR tubes right anyway. So, when we are coming with I mean we need to study the amplification of a specific fragment of DNA it should be very clear since we are saying this repeated again and again you should always know that in vitro replication varies from in vivo replication.

In vivo replication what happens whenever cells are dividing for any reason whenever there is DNA replication in vivo replication divides the entire DNA it polymerize the entire DNA entire father DNA is converted into a daughter DNA right entire mitochondria maternal mitochondrial DNA right you get the point. So, the thing is in PCR we are not amplifying we are not replicating the entire DNA segment we are only replicating a fragment and what how do we get an idea what fragment we need to amplify that is denoted by primers ok. So, what are primers these are small sequences that are complementary to the flanking sequence, what is flanking sequence? Suppose this is our area of interest the red box we will only copy this region the this part of the DNA fragment will be copied this is the area of interest the segment of DNA lying adjacent to it right this part and this part these are known as flanking sequence all right. So, information from the flanks. So, of course, this flanks will also have a sequence we will design a short segment of DNA which is complementary to this flanking sequences ok.

You already know in DNA replication it starts with a primer all right same concept it is happening over here as well. Now in DNA replication we were starting with RNA primers right with the help of primase enzyme they were getting replicate synthesized first. Here since this thing is occurring in vivo we will start with synthetic oligonucleotide primer you are our acquainted with the term nucleotide. Nucleotide means nitrogenous base phosphate group and a pentose sugar. If you are not acquainted we can suggest you take up another course which have been floated by our team that is known as overview and integration of cellular metabolism which is also available in the NPTEL platform.

So, you can read up what do you mean by nucleotide oligonucleotide means multiple between 2 to 10 2 to 20 nucleotides are synthesized synthetically which are complementary to the 3 prime site of the target DNA. You can see it is we are attaching we are designing a primer in such a way that is complementary to the 3 prime sites of the flanking DNA sequences. Remember always primer is designed in such a way so that it is attaching to the 3 prime ends ok. It first attaches to the 3 prime end the process of joining of the primers with the flanking sequence or the target DNA is known as annealing ok also known as hybridization. So, these are the terminologies you should be acquainted with.

So, it means what 2 nucleotide strands are joining together to DNA segments are joining together by what bond you already know between A and T and G and C double bond between A and T triple bond between G C those are hydrogen bonds alright. So, when why do we need this primers? We need these primers in order to achieve the functionality of the DNA polymerase alright. You already know DNA polymerase cannot just start adding nucleotides from the 5 prime just by detecting the first complementary site. It needs a primer to start with that is what an RNA primer was doing. Here we are adding a DNA primer.

So, basically now it will continue to add 1 nucleotides to the 3 prime end of the primer from both sides alright. This will lead to the reaction compression will be again discussing them in details. So, this strand for schematic we have shown 3 with 3 nucleotides. So, TAA was the sequence in the 3 prime ends. So, we have designed a primer in such a way.

So, that is TTA complementary to the 3 prime end similarly here at the 3 prime end of the other flanking sequence alright. Now if we look at the direction of the primers this reaction it is going from left to right as our convention we answers in for English at least not for there are few other languages go from right to left anyway. From left to right we call it a forward primer same concept as it happens in DNA replication. And the primer in which the oligonucleotides are the nucleotide triphosphates are added in this way in this direction it is known as reverse primer. So, you see there are 2 primers that are needed why do we need 2 primers? In DNA replication the replication process could have happened with 1 primer as well right if you had 1 primer polymerase enzyme can add DNA sequences and we will have copies of this DNA right yes, but if there is only 1 primer only 1 strand of DNA will be amplified.

However, if there are 2 primers it will simultaneously produce 2 copies of the target DNA alright. So, remember this is the reason we need 2 primers 1 forward 1 reverse both are complementary to the flanking sequence and the product that will be form is

also complementary to the parent sequence alright. So, till now if it is not clear I suggest you pause this video go back come back here till now it should be very clear we are adding 2 primers to the 3 prime ends and polymerase enzyme is adding nucleotides 1 by 1. So, that the target DNA sequence is achieved or amplified alright. Next we come to the ingredients of a PCR reaction what do we need right basically the ingredients that we need are same as that we need in vivo.

So, if you know the DNA replication process in detail you already have some idea what we need as a cooking material in order to cook the whole reaction process right. So, we need all these things we need nucleotides adenine thymine cytosine and guanine we need primers we need the parental DNA sample from which a specific fragment will be analyzed we need a polymerase DNA polymerase enzyme and we need a buffer in which all the reaction takes place in cell it takes place in the you know inside the cells that provides the buffer and the whole thing occurs in a PCR tube alright. This is everything is put in a thermal cycler now here it is additional reagents may be included depending on what variations of PCR we are studying for basic PCR reaction these are all the things that are needed ok. Now I will say that not only you need to know what reaction you need to have some idea regarding a standard one PCR reaction these are the things that are needed ok. Approximately mind it these PCR reaction need to be standardized for every laboratory for every gene I mean for every segment of DNA for every target right and it will definitely vary.

So, more or less it lies within the range of what has been shown in the slide. So, regarding template DNA this is the amount of template DNA mind it nanogram per micro litre very very very small amount PCR buffer magnesium chloride 3 millimolar dNTPs means deoxynucleotide triphosphate alright 200 micro molar each primers forward reverse primer between these concentration DNA polymerase. So, basically nowadays everything is done using commercial kits and they will clearly say tell you instruct you what are the what is the amount of each ingredient you need to add in order to create a master mix. The every reaction thing that you is a master mix right mind it there is a sequence of adding DNA samples I mean adding samples before you put the reaction in the thermal cycler right you cannot add everything and wait for some time by then the reaction will start right. So, things need to be added one after another in a specific sequence and then the reaction will start ultimately the same thing you are mixing everything together, but there is a convention.

Anyway before going forward we need to know that this DNA polymerase is a special variety of DNA polymerase which has been shown here Taq polymerase what is so special about it we will understand very soon. So, see if we will understand it very soon when we do actually know what are the steps of a PCR reaction. So, PCR reaction is distributed in 3 distinct steps denaturation, annealing and extension alright annealing.

The process of denaturation means you already know DNA are double standard we need to in order for the polymerase enzyme to act we need a single standard of DNA where the primers will latch into where the dNTPs will be added one by one. So, we need to separate the DNA fragments.

So, the step of denaturation is nothing, but heating the sample to 95 degree so that the both the DNA strands will be separated the hydrogen bonds will be broken alright we will have double stranded 2 separate single strands of DNA for the ingredients of PCR to work with and amplify them alright. Now what happens the next step is known as annealing. Annealing means what we need to reform the hydrogen bonds right the hydrogen bonds have been broken. Reform the hydrogen bonds not between the 2 parental original strands, but with the primers. So, it is the temperature which is actually set based on the primer characteristics right.

So, that the primers can optimally bind or hybridize or anneal with the 3 prime flanking sequences of the target DNA alright. This is known as annealing and as you are seeing this varies the range varies from 50 to 65 degree centigrade of course, there are optimal temperatures and this again varies from primer to primer target DNA segment to target DNA segment what gene we are amplifying there are multiple factors alright. We will be discussing how we need to have some idea about the annealing temperature ok. Next step is extension, extension after the primers have annealed we are again rising temperature of the thermal cycler the PCR tube the reaction mixture. This step activates the DNA polymer a special type of DNA polymerase or Taq polymerase which now adds the nucleotides one by one.

This is the actual polymerization step where the primers the length of the primers will be extended by adding one by one nucleotides by the Taq polymerase and this phase is known as extension till we reach the entire flanking sequence alright. So, Taq polymerase is a special variety of heat resistant polymerase DNA polymerase that is isolated from the that have been isolated from the bacteria thermos aquaticus thermos aquaticus they have been found in hot springs alright. So, you see what was the problem what would have been the problem if we did not get if we do not have Taq polymerase can we do that with a normal DNA polymerase we can do that we can denature the DNA, we can anneal the primers and the normal DNA polymerase will not act on at 70 degree centigrade right. There will be a lower optimal temperature as happens in our body I would say 37 degree centigrade then what will happen as soon as. So, one reaction will be completed no harm, but as soon as we denature the DNA for the next cycle the polymerase will be destroyed because they are not heat resistant they are normal DNA polymerase for example, DNA polymerase 3 in our system is heat labile L A B I L E L A B I L E heat labile whenever we say the term labile it means susceptible it will be destroyed and this Taq polymerase is actually heat resistant it is a variety of heat

resistant DNA polymerase.

So, the beauty of it is with this small amount of DNA polymerase Taq polymerase that is in the reaction mixture we can continue the reaction ok. So, basically now we are looking at the graphical illustration that will help you to understand how this whole cyclic thing is occurring. So, as you always have understood or we have summarized in the previous slide ideally the temperature is 95 degree centigrade unless stated otherwise ok. So, in the first step DNA is polymerized sorry DNA is denatured the two parental strands are separated into daughter strand ok. This is also known as melting of DNA ok the term we use was melting.

So, when the DNA is completely denatured it is already separated we will discuss later melting temperature something known as Tm we will be discussing later, but let me tell you right now. So, that you can recall later melting temperature is the temperature at which the entire DNA strand is half denatured. So, at some point of time it will be fully denatured right. So, we are giving temperature in such an excess that the whole thing is getting separated, but of course, for every segment of DNA inherently it can be calculated based on the amount of hydrogen bonds AT and GC bonds what will be the temperature if we provide that it will be fully denatured fully separated. The temperature at which only half of the hydrogen bonds have been broken are known as melting temperature ok.

So, we are clear with the denaturation step mind it deals with breaking of hydrogen bonds. The next step annealing from 95 degree centigrade is rapidly cooled the cooling happens in seconds alright. If we just leave it to an incubator the reaction could have been done incubator it will take hours to cool down it might take hours in the closed system to reduce the temperature, but not the thermal cycler has got a heating mechanism it has also got a cooling mechanism right. That is the beauty of a thermal cycler that is why it costs so much it is simply not a heater it is a cooler refrigerator and heater built into one and it does in a rapid very quickly a heater or a refrigerator takes times to rise and I mean let down the temperature thermal cycler does it in a flash. So, the reaction is then rapidly cooled to a desired range with which leads to annealing hybridization of the primers to the single stranded templates.

We have got single stranded templates we already know by now the primers DNA primers will attach to the 3 prime ends ok. Next mind it primer will anneal to sequence that are complementary to them target sequence because the primer is a DNA primer mind it in in vivo replication we are using RNA primer. So, it was directly binding to the strand for which it is meant for DNA primer of course, it will always bind to the complementary strand. We already know whenever a strand is binding it is binding to a complementary strand is a DNA sequence. So, both in case of RNA and DNA the

complementary bonds AT, G and C will be the only ones where primer binds.

Primer cannot bind anywhere we are designing primer in such a way so that it is binding to the complementary sequence and we are designing in such a way that it is complementary to the 3 prime flanking sequences of both the ends of the single stranded. Next so, after we allow to cooling of around 50 to 60 degree centigrade the primers will attach through hybridization this is known as annealing. And in the next step what happens the extension the actual so, in here again in annealing reformation of the hydrogen bonds. So, in extension phase what happens the immerse the end of the primers are now extended with the help of adding the nucleotides one by one with the help of Taq polymerase so that the entire segment is amplified the entire target sequence is amplified. So, now, we from one parental strand original DNA we have 2 copies of the 2 copies of that right.

So, after first reaction which has got 3 steps we have got 2 copies of the previous DNA right. So, next what happens this reaction as I told you the product of this reaction will act as a reactant of the next reaction. So, after the first 3 steps this was the first step region to be amplified we targeted with the flanking primers and this is the region that have been amplified. The new strand will behave as this so, the we have got 2 copies now right they will act in the as a reactant of the next cycle. So, those will be melted again so, we will have 4 copies of the DNA right 4 single strands each of them will be hybridizing with the primers in the annealing step and then each of them will also be extended.

So, one very important concept you should understand that the original DNA sequence might be very long right that as we are making multiple copies the daughter copies are only produced in the desired length right. So, after first reaction of course, the parental strand when the ultimately reaction reunites after the entire amplification in the first cycle one strand will be long that is the parental strand whereas, the replicated strand will be short. But as we continue to grow the amplify the DNA the copies that long copy will be remaining one only parental copy, but the further copies will be of the desired size and the math is very simple after cycle one there are 4 strand of DNA because there are 2 copies after cycle you can see cycle 2 there are 8 strands cycle 3 there are 16 strands. So, so on and so forth so, you can just imagine and since this reaction is happening within 1 and 1 and half minutes in a very short time we can have for example, 2 to the power 20 which is basically 1 million copy we can have 2 to the power 25, 26, 27 it is a billion copies of DNA right. So, this are this product the PCR product the copies that are made from the parent DNA are known as amplicons alright.

So, we have multiple amplicons within a short amount of time. So, if we just look into the reaction proper basically what happens this is the DNA template we are adding we

are now seeing the ramp this is called ramp view R A M P it is looking like steps alright ramp. So, first the temperature rises denaturation temperature it is cooled for a primer anneals and then again extension 72 degree centigrade and again it goes for denaturation annealing extension denaturation annealing extension alright. So, it happens and we can set the number of cycles if we set it 25 cycles this whole thing will happen 25 times and we will end up with 2 to the power 25 copies of the single target DNA 1 billion copies alright. You should note that after this 25 reaction happens the thermal cycler adjusts itself in such a way so that in the 26th reaction it will not go up to the denaturation temperature rather when the cycle has been completed it will sharply decrease to 4 degree centigrade alright it will come way down it will come way down the temperature say here if it is 0 right it will come to 4 degree centigrade why because suppose we are all busy scientists are all busy right we cannot exactly it may not be possible for everyone to sit in front of the thermal cycler machine right to stop and collect the sample whenever the reaction is complete still we are very meticulous we do sit in front of the thermal cycler machines all the doctors that are doing this reaction in the diagnostics may do that.

However, if there are some delay say 10, 15 minutes, 20 minutes even an hour the machine is capable enough to store the samples in 4 degree centigrade and extracted the PCR amplification product the PCR products will be stable at 4 degree centigrade. However, for long term storage it should be stored at minus 20 degree centigrade right from 4 degree will directly store the DNA sample to minus 20 degree centigrade. Now let us understand the entire workflow of PCR. So, this was the basically one step a thermal cycler reaction is the main step main process. So, we are making the master we are extracting the DNA first we are making the master mix we are distributing the reaction in the PCR tubes and then we are starting the reaction.

So, first of all we need to identify what we need to amplify right next we will design the primer in such a. So, it is specific to 3 prime finding sequences we need to optimize the PCR reaction by selecting the proper annealing temperature what ramp what cycle we need to do alright. Next the whole thing that the reaction is complete will be a simple colorless liquid alright, but whether the product has amplified or not what product have been amplified if any unknown product is amplified what is the characteristics all those analysis has to be done post PCR that is actually done by agarose gel electrophoresis which is abbreviated as AGE. So, it may go according to plan or there might be some error whenever we are starting it for the first time there might be various reason for reaction failure. So, we need to be aware and to be ready with the troubleshooting and once everything is done when the we are satisfied with the result we can rerun the thing again and we can visualize with the help of agarose gel electrophoresis.

So, these two steps are often for the very first few standardization processes once it is

done one become master of your technique we can simply optimize and then analyze without much troubleshooting alright. So, let us discuss something in brief about agarose gel electrophoresis. So, why? So, as I told you it is a colorless liquid. So, we cannot see I mean we do not have any idea. So, the master makes the amount the even if we have 2 to the power 25 billion copies of DNA that will not increase in size the liquid suppose 20 microlitre of master mix was there in one PCR tube after the reaction it will remain appear as such right, but in it whether it has amplified or not that is our area of interest right.

So, suppose our target is a PCR product is 340 base pair what is that the number of bases base pairs are 340 ok this is the standard denotation of a length of a product. So, what do we do? We run it using agarose gel right we load we make 1 percent 2 percent depending on again your lab standardization agarose gel we will make lanes we will add the agarose gel we will do the process of electrophoresis along with that we will also load a known thing that is known marker what is a marker it is a mix of multiple base pairs or multiple lengths of DNA segments whose length are already known. Next we will visualize this how do you visualize definitely with the help of UV transilluminator by adding a dye known as ethidium bromide or ETBR we will be discussing again. So, we will add ethidium bromide to the agarose gel electrophoresis buffer it is ok ethidium bromide we will come back to this slide ETBR we will add some ethidium bromide to the agarose gel electrophoresis buffer and then we are not discussing the process of electrophoresis here in detail. So, PCR is done I mean electrophoresis done with the PCR products and after it the electrophoresis is the bands if PCR has happened properly the bands will be visualized and it can be compared with the ladder or the known molecular weight marker to know about the target of PCR product.

So, in this case if the target base pair is 340 it has beautifully showing that is between the 300 and 400 base pairs right. So, this is the entire process of PCR. So, we need to know a bit about this dye see DNA is actually colorless right. So, in order to visualize that we need a UV transilluminator, but we also need a fluorescent dye so that we can visualize that. So, that in conventional PCR it is done by ethidium bromide it is added to electrophoresis buffer I told you it is a abbreviated as ETBR it is done by ethidium bromide how does it act it acts by this term is very important if asked in Viva you must always utter this word intercalates ok.

It intercalates between the base pairs alright between the so you can see these are the regions where ethidium bromide latches on to the spiral helix right. So, deoxyribonucleotide bands actually or gels DNA when they are intercalate when they are bonded strong with ethidium bromide when UV light is exposed it emits the fluorescence alright. So, basically the color image it looks like a pink glowing band right we have mono it is a monochrome version. So, it is looking like that. So, we can either put it in a

gel doc these are equipments that have inbuilt fluorescent cameras which can shed UV light and then capture and give you this beautiful image right or you can place the gel on a manual UV transilluminator where you just put the gel and you switch on the UV light then also you can see the bands very beautifully.

So, this bit you need to know about ethidium bromide it intercalates the DNA it emits fluorescence upon UV light radiation. So, now we are slowly coming to the end of our lecture. So, next we go on to PCR optimization. So, what are the things conditions that is actually very ideal for you to keep in mind so that this reaction is completed mind it all seem very easy right, but when you are trying to design a PCR reaction whatever it is for diagnostic purpose whether it is for research purpose whatever it is chances are for first time you have very high chance of failing. Failing means you complete you do everything in your capacity, but still you see when you finally, run the gel there are no bands right.

So, things we need to make sure ideal it should not happen if you have done everything right it should not happen right. So, the things that we need to keep in mind in order so that the reaction does not fail. Number one optimal concentration and proper cleanliness and no contamination. So, proper purification technique using absolutely aseptic precautions and you need to do it in a hood with a laminar flow you need to make sure there are multiple steps in DNA extraction process we are not going into that, but make sure you are using proper gloves you are using profuse amount of ethyl alcohol because it sterilizes everything you cannot touch your hairs and everything because DNA's are present everywhere anyway. So, basically you need to maintain a sterile environment you need to maintain proper temperature while DNA extraction right not only that.

So, this will ensure you have a high quality DNA you should also use proper purification techniques after extraction you can purify the DNA with proper commercial kits right if you are making the buffer in your own lab make sure it is not contaminated right. Next designing the primers very important if the primers are not designed properly the reaction will invariably fail. So, there are multiple factors when deciding the primer if for example, GC content appropriate length that is very important if it is not happening properly or you know there is a condition that is known as primer dimer formation means if the primers are complementary to each other you have designed the primers in such a way. So, that they are complementary to the 3 prime ends of the both segments, but if they are complementary to each other they may choose to bind to each other and amplify them that is known as primer dimer formation. So, self complementary primer should be avoided this is one of the trouble shooting right these are the most common that is why we are highlighting.

Next primer concentration very important you need to standardize if you are following

any standard literature you should always go by that of course, you can modify and mix and match and standardize in your own lab, but always you should follow a standard protocol not only primer concentration, concentration of the magnesium chloride, concentration of PCR buffer, concentration of everything you need to determine right. And also the proper annealing temperature of the primer you need to set properly in the PCR machine often there are PCR machine. So, for example, you will often see that the reaction has failed you can increase by 0.

5 then the reaction takes place if not you can decrease by 0.5. So, the change happens in such a way minute variations well the beauty is there are machine. So, imagine you need to redo the whole thing. So, suppose you set the annealing temperature 53 your supervisor or guide tells you that you need to do it in 53.5 or 54 you have to again do the whole thing right, but we can avoid that by using something as gradient PCR. Again PCR machine is what suppose these are the slots of a thermal cycler thermal cycler looks like this when you open the equipment there are holes you need to put the PCR tip.

So, you can design the machine in such a way. So, that a gradient is created in the annealing temperature everything else will be same every tube will be going through denitration annealing and extension when it comes to annealing suppose this row will be set to 53 this row will be set to 53.5 this row will be set to 54. So, you can have a situation where you can suppose of 10 rows from 53 to 55 and you can actually put suppose 6 tubes in each. So, you if you do not have standardized you can have some flexibility and you in one reaction itself you can have some idea what is the ideal annealing temperature and next when you repeat you set your machine into that specific annealing temperature ok. Next and mind it whenever you are doing a PCR it is always advised to prepare a master mix tube in triplicate means 3 copies of the same sample same tube should always be run because any error in one tube you will have 2 more samples to read right triplicate very important.

So, next we will study about the primer designing consideration or the primer designing guidelines. So, what are the important things that we need to keep in mind? Number 1 of course, the primer sequence the primer length the percentage of GC content GC bonding what is GC clamp what is the melting temperature what is annealing temperature everything should be considered when designing a primer we will go through them in brief one by one. So, as you already know primer is complementary to the flanking sequence a 3 prime flanking sequence of the target region as already told you should avoid complementary sequences between primers that will lead to primer dimer self complementary primer should be avoided right. Again you can see this sequence if there are so much repeat ok it may be so that the primer forms a hairpin loop between itself it is complementary to itself right it is not complementary to the sister or forward or reverse primers are not complementary complementary itself may be these type of

sequences are present in multiple fragments you know tata box and all that. So, it may be so that it actually binds to another target sequence you know I mean instead of a target area of interest.

So, these are very important. Next primer so these things should be avoided right primer length optimal primer length should be sequent between 18 to 25 it should not be too long it should not be too short this much you should remember GC content very important the GC content the number of Gs and the Cs in a primer this is a primer sequence should always be in such a way that it should be around 40 to 60 percent if it is too high or too low the there will be problem too high will increase the melting temperature too low the binding strength of the primer will not be ideal. So, one thing regarding GC we should note that in the last 5 bases right it should not be more than 2 Gs or 2 Cs right very important it should be there right in the last 5 bases you need to have C and G because it leads to what is clamping? Clamping means attaching with a spatula or a clamp you know GC clamp means since it is a it has a strong bond 3 hydrogen bonds the primer will anneal very strongly, but much I mean too much G and C will again ruin it all right. So, it needs to have 2 G and C in the last 5 bases, but not more than 2 right very important. Next we should also consider melting temperature annealing temperature while designing primers what is melting temperature I told you the temperature during the DNA is half denatured right. So, maximum difference between primer temperature right if we are designing 2 primers we need to design both the forward and reverse primer using bioinformatic software for example, primer 3 primer 3 and blast all the softwares.

So, we need to calculate the melting temperature of each primer segment by considering the amount of A T G and C we should check whether the forward and reverse primer melting temperature the difference is less than 5 degree if it is more than 5 degree the reaction will definitely fail ok. Not only that we also need to have some idea about the annealing temperature right the primer whenever we are hybridizing the primer whenever one extension reaction has occurred we need to again denature the whole DNA right the temperature to the primer actually melts. So, directly depends on the length and G C composition of the primer. So, too high annealing temperature will produce insufficient primer template hybridization if it is too high ok. We need an optimal temperature so that the primer anneals right because we are cooling if it is too high it would not happen and if it is too low there will be so much non specific products it will bind anywhere.

So, we need to make sure in every case it is it should not be too high it should not be too low it should be optimal right. So, remember too high annealing temperature will lead to insufficient primer template hybridization and too low will lead to non specific products there will be products your target might be amplified, but some other thing will

also be amplified and that will not help that will confuse our diagnosis. On what it depends? It depends on the length and G C composition of the primer and also annealing temperature will also depend on how the primer melting temperature is which is calculated by this. So, one thing leads to another.

Lastly we should know what are the advantage and application of PCR. So, why why do we like PCER what why this is the very basic tool on which everything has been designed? It is because of it is simplicity the method is actually easy it is very sensitive it very short time we can get so much desired product and it has been validated extensively over the years using multiple standard laboratory operating procedures right and the reagents and the equipment are extensively available. You saw when we were going to the PCER pand COVID 19 pandemic right every primary care center every mid to low hospitals railway hospital tertiary care hospital district hospital were provided were converted to COVID center where we are provided with RT-PCER machine and this whole thing was done by trained every who everyone technicians are trained to do this. So, it is doable right we are not talking about any fancy thing which needs a lots and lots of infrastructure is not doable. So, it is not only easy validated it is feasible and reagents are available and it has got multiple application. So, genotyping viral load detection RT-PCER COVID 19 we all know have been discussing it can be used in again in application of that can be done in cloning for the vectors mutation detection sequencing microarray forensic pattern re-testing infectious disease detection and so many things are there right everything believe me will be taught in much detail in their own separate modules right will be understanding every comprehensive thing about genetic as well as proteomic analysis in this course.

So, this are the summary these are the take home messages that we discussed in this now you should be able to go back and know that what we discussed what are the ingredients of PCR, what are the method of PCR amplification, what are the steps of PCR, what are the optimization steps in PCR, what consideration we should do while designing the primer and what are the application. So, these are my references for the slides ok these are the text book and these are the links where you can find the figures and so I thank you for your patient hearing and that will be it for today. So, we will in the next class will be continuing with this concept and will be learning about all the variations of the PCR RT-PCER real time PCR so on and so forth and we also discuss about the genotyping how mutation detection using PCR. So, that is it for today I thank you for your kind attention.