Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Week 02

Lecture 10 : Variations of the PCR (Part 2)

Hello students. Welcome back. We are now at the last lecture of module 2 where we will be covering variations of the PCR in the second part. So, in the last part as we can see we covered all these types of PCR that are mentioned in green colour and we are still left with some other variety of PCR that we will be covering in brief in today's session. So, with that in mind it a lot of these will be again referred to in future classes where we will be detecting mutation analysis, we will be studying cancer detection, we will be studying pathogen detection, infectious disease so on and so forth. So, we will be directly going into each topic and we will be trying to cover them so that the concepts are laid out in this fundamental module of polymerase chain reaction.

We will start with hot start PCR. Hot start as the name suggest things are going to get hot to start with and by hot I means temperature high temperature. So, what does it do? This hot start PCR uses a mechanism by which it prevents non specific binding in start of the PCR. You know when PCR is being done in practical laboratory hands on we add all the reactions in the master mix and we add the tack polymerase the DNA polymerase in the last step immediately before adding the mixture to the thermal cycler.

Why so that the reaction does not start because DNA polymerase will start amplifying even at room temperature and by the time the temperature rises to 95 degree centigrade some primers or some nucleotides will be amplified anyway right. So, this is a high probability and this is also the temperature where primers may cross connect between themselves to form primer dimers. So, all of these things can be avoided if we start the reaction directly at a higher temperature. We start the reaction directly with denaturation that is what it is done over here by manually heating the reaction components to the melting temperature before adding the polymerase. But how can we do that? We cannot hold the tube which is 95 degree centigrade hot in our hands right it has to be done in a thermal cycler.

So the reaction is designed in such a way that the reaction will not start unless the

whole mixture is heated to 95 degree centigrade and it is achieved by it can be achieved by multiple means by chemical methods by using antibodies to the tack polymerase by modified DNA binding proteins which will only dissociate at 95 degree centigrade and allow the polymerase reaction. We can have physical barriers such as wax beads, we can have enzyme linked inactivation that will be I mean the inactivation will be inactivated only at higher temperature all right and for these special methods commercial hot start polymerase are available. So, in this diagram you can see is a physical wax barrier that has been added I mean that has been constructed in the PCR tube and that will only melt. We add the tack polymerase in the upper fragment upper segment and only at 95 degree centigrade the whole reaction the wax will melt and the polymerase will come in contact with the other component of the PCR and then the amplification will start. So, in comparison to traditional PCR which can have non specific products and primer dimers hot start PCR have got a very high I mean non specific I mean very low rate of having non specific products and very high specificity and very high filter products very high rate of achieving the desired product that we want.

So, this is the benefit of hot start PCR. So, increase sensitivity and specificity and which is ideal for when you are having more than one desired target amplicon. So, we have to prevent non specific products. So, preventing primer dimer formation non specific products specially for multiplexing. Now definitely it is good, but when something is very good it has to have some technological challenges and disadvantages.

In this case it is definitely costly than the traditional PCR we have to design the whole reaction mixture we need to procure antibodies or enzyme like inactivation mechanism or even commercially available hot start polymerases. So, that increases the cost of the experiment. Moreover since we are keeping the reaction mixture at 95 degree centigrade at higher temperature for a long time there is a chance of DNA damage right. Minded in traditional PCR we are just rising and then we are immediately bringing it down, but in hot start PCR we are starting with a higher temperature. So, to start with the template DNA might be damaged.

And one thing to note hot start PCR cannot amplify segment that is longer than 2 kilo base or 2000 base pair all right. So, these are the things little I mean bits and pieces that you need to remember for hot start PCR that will solve your most queries related to any short notes short answer type questions Viva or even multiple choice questions that will be getting in your assignments and proctored exam. The next type of PCR is touchdown PCR it uses almost similar concept we are starting with a higher temperature and then we are gradually coming down. Again the motto is same we want to prevent non specific primer formation we want to prevent non specific product amplification. So, what is done in initial phases the primer is amplified at a higher annealing temperature for first few cycles and then in phase 2 seen 10 to 15 cycle the primer is annealed at a higher

temperature and then the temperature is lowered for this the next few cycles the temperature is lowered.

So, what will it do? In the first few cycles only the most specific primers that are designed for the target of interest that are designed towards the target of interest will bind. So, in the first few cycles only the maximum specificity the region which has the maximum specificity that is target area of interest will bind no other non specific product can bind why because non specific binding or annealing of primer generally happens at lower temperature. So, in any reaction whenever the annealing temperature is lower chance of getting a non specific product is much higher. So, we always start to design the annealing temperatures as high as possible to negate all the non specific products. So, what will happen in this case suppose there is a possibility that we cannot design the primer in such a way.

So, the I mean our region of interest has got similarity with other areas of interest as well in the whole target sequence right, but we know that our region of interest will be amplified at a higher temperature, but in any case since we need to bring down the reaction temperature anyway there is a chance of non specific amplification. So, with this we can prevent by using a touchdown model where initially when we are using a higher annealing temperature for the first few cycles only the specific primers will bind and by the time we lower the temperature they would have increased in such number for example, if I is the number of cycles for the initial higher temperature and J is the number of cycle for the remaining temperature the amount of product the amount of purified product the amount of product specific product that will be more than the amount of non specific products is 2 to the power I minus J. Simple mechanism the number of cycles see suppose this is the first cycle here only the specific primers can anneal an extent. So, by the time we lower the temperature this will already be doubled alright. However, in the lower temperature the non specific primer will start to bind.

So, if we already perform this step quite a few cycle of times. So, by the time this non specific primer starts to bind this will be hugely amplified a already and then subsequent cycles the specific products will simply out compete the non specific products alright. So, the shear number of the desired product of interest will out number the non specific product and thus in one single reaction we can minimize the amount of non specific product primer, primer, primer formation everything. This is the touchdown PCR model where which is widely used for genotyping, nutritional analysis because it has an enhanced specificity in target detection alright. So, two mechanisms of PCR which start with high temperature hot start and touchdown they are more or less similar in hot start we are using a physical barrier to prevent the reaction in touchdown we are designing the reaction in such a way.

So, that the more specific products the numbers take over during the reaction procedure. Next we are moving to a very important topic that is allele specific PCR also abbreviated as ASPCR. What does it do? It utilizes allele specific primers that selectively amplify the target sequence depending on presence or absence of a specific mutation alright. You already know what do you mean by alleles, what do you mean by genes from your fundamental class genes are the very basic segment of DNA which has got a function functional unit of a segment of DNA and alleles are the different copies of DNA in the homologous chromosome and there can be mutation in the alleles alright. So, this type of PCR is actually widely used in detection of mutation specific genetic mutation or single nucleotide polymorphism single point mutation any area of mutation will be easily detected.

It is also known by other names alright ARMS amplification refractory mutation system PAS is a PCR amplification of specific allele everywhere we are using the term allele because we are designing allele specific primers. So, any of them means the same thing the mechanism is the same. So, let us understand. So, this is an allele ok it is a sequence of a DNA in a gene. Now alleles can be two types number one which is known as the wild type allele or the normal mind it wild means normal allele that is present in an individual without any disease process.

However in this case you see A has been replaced by G mutation has happened and likewise the complementary strand T has been replaced by C this is known as mutant allele mutant allele is found in diseased person. We already know the sequence that this is the sequence of mutant allele and this is the sequence of normal person wild type allele. So, our goal is to find these sequence whether this sequence is present in the suspected case if it is present then we can pin point our diagnosis this is the case of mutant allele present and this is present alright. So, how do we do that? We need to select a segment of DNA where the mutation is present. So, this sequence should be known alright or the sequence where the mutation is there.

We are now discussing the wild type. So, definitely there will be one point where the mutation varies in the wild type and the mutant type. We need to select a segment of DNA in such a way so that the site of mutation is a centric means it is not exactly at the center if it is exactly at the center then this reaction will not be possible this process will not be easy you will understand why in a minute. So, selecting a segment of DNA where the mutation in the mutant allele will be a centric what do we do? We design primers multiple primers ok. One set of primer we design taking the whole upstream sequence in mind it we have selected a sequence we have selected a chunk of base in which the mutation area is present not at the center right.

So, this is a primer which is known as outer primer why outer primer? So, this is one

set of primer that we are using against the whole strand and we are also using another primer which specifically which is specifically complementary to the wild type sequence I mean normal sequence ok. So, now what will happen? Since you already have the concept of nested PCR this will now appear to be very easy to you because this will now amplify right this will amplify giving rise to a product and this will also amplify this will also amplify and in this case it will also consider this as a reverse primer alright. So, once these outer primers do amplify they will give a product of this much let us say this is 200 base pair. In case of the wild type allele since we are also using an inner primer we will also get another product which is short in length, short term because we are starting from here the amplification is starting from here and we will get another segment which is 150 base pair. So, this is all about the wild type normal person fine.

Now let us consider the mutant type, in mutant type there is a mutation right. So, what do we do? For the mutant type we also at the same primer considering the outer sequence because the parent sequence will be the same this part and this part is the same alright. However, in case of mutant type we design a primer which is specific to the mutant allele it will only align with the mutant allele and we design it for this strand mind it in case of wild type we were we did design a primer for this strand you will understand why very soon. Now these will amplify alright considering the outer primer they will amplify like this can you tell me considering the inner primer how they will multiply this will now consider this one as its reverse primer. So, amplification will only happen in this area and you guessed it quite right the outer primer will give rise to a 200 base pair similar product like this whereas, the inner primer for the mutant type will give rise to a smaller 50 base pair.

In this case because we have designed the primer in such a way now consider the fact if now what happens actually in when this is amplifying we are getting this type of base pair right. However we do not have a I mean we can run controls and patients sample together in a PCR tube alright. So, for both the alleles we if both mutant and wild type are present mind it when both the alleles are mutated right we get homozygous mutation when one is normal one is mutated we get heterozygous alright. So, we can differentiate homozygous heterozygous type of mutation and disease presentation with this. Now consider the fact where we have added all the three primers in the reaction mixture we will get these three variety of length of base pairs.

Now if the if we chose the DNA sequence where the mutation was lying absolutely in the middle we would have no idea no way to differentiate whether this mutant product is varying from the wild type product. How do we get the length we can simply run a gel electrophoresis and by pairing the length of the PCR product with the molecular weight marker we can easily get. And this is the basic concept again it will be discussed in detail when we are discussing any type of inherited disorders where mutation is present alright,

but this fundamental concept should be absolutely clear we will make you understand how the pattern is when it is homozygous how the pattern is when it is heterozygous, but I am sure you can now brainstorm your way you can have a read from all the sources that I will provide you in the reference very soon to understand even before that module how the homozygous and heterozygous pattern will be there and how we can differentiate them using allele specific or ARMS PCR alright that is done. Next we move on to colony PCR what is colony PCR again this is a PCR colony what have colony bacterial colony we mainly refer to bacterial colony we can have some fungal colony as well in culture media alright. So what happens bacteria is grown on a culture media with some treatment with some experimental setup exposed to it and it undergoes some transformation by transformation means there is a change in some genetic information and we want to detect that genetic information.

What could have been run traditionally we could have isolated the bacteria we could have isolated the whole DNA I mean first of all not only that we need to extract the bacteria with the help of plasmid with the help of cloning we need to amplify the segment and then we can detect whether the and then we can also multiply using PCR if the product of the cloning is not adequate. So there are multiple steps involved in order to identify the target change of DNA due to a transformation in the bacterial colony. However in colony PCR we directly we bypass all these steps alright bypass all these steps by simply taking up the bacterial colony and directly re-suspending them in a PCR reaction. How the colonies are picked up as you can see in the picture these are picked up using a sterile toothpick or a pipette tip alright. We are picking it up we are re-suspending them alright in a mixture in the PCR tube and then PCR amplification is performed using the primers.

So what does it do? It actually bypasses all the steps we do not need to isolate DNA we do not need to restriction digest ok this is very much cost effective and very quick compared to the traditional method by which we would have needed to isolate the bacteria cut the gene of interest using restriction endonuclease and then went for traditional PCR alright. So how what does it do? It actually helps us to rapidly screen the transformed cell and confirm the presence of desired DNA insert alright and amplify the portion of construct and specially it is very useful in quick and efficient screening of bacterial colonies. Mind it I told you in the last lecture a simple change in bacterial gene may give rise to huge resistance for example, methicillin resistance, staphylococcus aureus right superbugs. So all those can be easily identified. Again it will be discussed in much details when we are discussing detection of infectious diseases but mind it this name colony PCR is extensively used for bacterial colony.

The only concept that you need to know is we are bypassing all the steps of DNA isolation, restriction, digestion we are directly re-suspending bacterial colonies in a PCR

tube they are centrifuged and then PCR primer amplification is being done using the traditional method to identify the target area of interest. This is colony PCR it is basically a modification it is a process which we need to it is not much different from the mechanism of PCR is not much different but how you do it and why you do it are different compared to the traditional ways of treating a bacteria in a culture colony culture media alright. So next is digital PCR again this will be discussed in a great lot of detail when we are when we will be learning cancer detection, mutation, liquid biopsy all those things ok, but we will just discuss the fundamental concept. The digital PCR or DPCR is basically molecular biology technique where in a PCR mixture using oil emulsor technology the target DNA is distributed into multiple fragments or multiple partitions in each droplet there is a target DNA there may be or there may not be target DNA there may be any background DNA control DNA alright. So this is a technology that has been developed by very small for example, one tube may contain 120,000 droplets alright.

So in each droplet all the components of the reaction mixture are there it is engineered in such it is a innovation in biomedical engineering and then the whole product is amplified. So in each droplet there will be a PCR reaction that will be happening and ultimately by tagging with fluorescent probes each droplet can be quantified actually helps in absolute quantification of DNA target DNA. So you can see each droplet may or may not have any target DNA it may have nothing. So ultimately when the droplets are being analyzed it will give us a signal whether fluorescence is present or fluorescence is absent in a binary manner and each drop can also be quantified and this absolutely negates the need of a standard curve. New concept, but very important this is also often referred to as digital droplet PCR alright and commercial kits are available for this type of reaction, but now a days it is very much used as I mentioned in liquid biopsy, NGS negotiation sequencing, library analysis, gene expression, micro analysis everything.

Again it will be discussed in detail in the future modules, but just we are laying the fundamental concept over here so that when this term is uttered you are familiar with how it happens alright. Next we move on to long range PCR. What is long range PCR? You know traditional PCR there is a limit to which the amount of targeted sequence can be amplified for example, 200 to 1000 base pair and believe me the amount is much lower in case of real time PCR it is even lower 75 to 150 base pair in case of real time PCR. It may be more depending on the amount of specialized product that are coming up now a days, but in general it is small smaller. What if we need to amplify products that are longer? For that we need long range PCR longer means over 5 kB up to 25 to 30 even 40 kilo base can be amplified, but we need special cases special situation special tag polymerases with high processivity and high fidelity.

So that the extension rate is enhanced the proof reading capabilities are more we need specialized reaction buffer we need optimal reaction condition. So all of this will help us to achieve a scenario where we can amplify longer target based product. For example, those are needed in whole genome sequencing mutation detection over a wide a range of interest. So extensively used in cloning, genotyping, sequencing everything where we need to amplify long range targets commercially available long range PCR enzymes are available for example, PFU Q5 etcetera from various companies that helps us to achieve this scenario, but definitely they come at a price. In situ PCR what is in situ PCR? The name in situ means in the natural environment in the cell we are not doing this in a PCR tube.

So, how it is done in situ PCR is a technique that combines PCR amplification with spatial localization means we can now see where in the tissue the DNA is there. Because we are actually amplifying the DNA in the location where it is originally present. So we are not isolating the DNA we are not extracting the DNA this in situ PCR eliminates the need of DNA extraction. What is done the processes? So of course, first the tissues are fixed in the slides alright they are dehydrated they are fixed they are treated with proteinase K there are multiple steps how it is. So basically three steps number one tissue pretreatment where it is fixed in a slide that assembly can be I mean for that is immuno labeled with digoxygenin DUTP labeled antibody right there are primers and enzymes that are specifically designed to work in this type of scenario using this plate in the small wells in the tissue.

And after the whole thermal cycle is done in each pocket of the tissue it is immuno detected using anti-digoxygenin gold linked antibody alright. Also when the products are amplified it can be enhanced using silver dyes and finally, visualized in the microscopy. So what do we see ultimately? Maybe in while doing microscopy we cannot see the DNA at level of I mean they are very small right, but however, if the DNAs are amplified in the tissue and the present it has got a typical signature if we can label that target DNA of interest or gene of interest in the tissue we can easily save microscopy that this is an area where it is muted and that has lead to all the problems for example, say cancer. So finally, these are the types of visualization we do have in in situ PCR. What are its application? Localization, cellular localization of gene expression and detection of specific DNA sequences in tissue sections we are doing it directly in the tissue benefit done in natural environment and it eliminates the need of prior DNA extraction alright.

Inverse PCR, what is inverse PCR? This is a technique where we are identifying flanking sequences adjacent to known DNA sequence. For example, we have got a sequence that is known and we want to find out the sequences that are adjacent to it. For example, consider a transposon ok this is a jumping gene. Now this transposon can latch

on to any gene of interest or it can travel across any gene suppose it has latched here. Now we know the sequence of transposon right, but we do not know what are its adjacent area.

So that situation is very handy and we can detect these flanking adjacent sequence using inverse PCR. So what is done? We treat the whole sequence using a restriction enzyme you know restriction enzyme has got a I mean property so that it will cut in specific areas where the specific base pairs are present right. After cutting these is self ligated to form a circular type of DNA alright and the primers are designed in such a way so that it is complementary to this known sequence, but they are oriented in the inverse order they are oriented in the reverse direction. You see primers oriented they are facing this normally when you are amplifying any target sequence the primers do face this side alright, but we are not trying to amplify target sequence we are trying to amplify the adjacent sequence. So when it is circular what happens the whole now this primer can now find this as a template and then it can amplify the whole circular DNA.

So template is the restriction fragment that has been self ligated. Now what happens this after the whole thing has been amplified this area can be restriction digested again and we will get some idea about the known DNA unknown DNA sequences we can use bioinformatic tools we can use BLAST all those things can be used and thus it is very easy we can put this in a sequencer machine to get some idea about the non unknown sequences, but this is a way to get hold of the unknown sequence we now know for sure that this is the unknown sequence where the which lies between the known sequence by just converting the linear into circular and then opening up alright. So this is the concept of inverse PCR where can be it applied detection of flanking sequences of transposable element and identification of genomic inserts. And the last part of our discussion is ligase chain reaction. So we are done with almost all major types of PCR that are important for gene expression analysis and mutation analysis one specific type of PCR have not been discussed deliberately that is methylation specific PCR that will be discussed in great details when we are studying epigenetic diagnostics of epigenetic diseases using polymerase chain reaction alright.

So what is LCR ligase chain reaction it is not a variation of PCR alright it is we can say it is a complementary alternative means of amplifying a DNA. So we discussed amplification of DNA can be done by cloning molecular cloning polymerase chain reaction this is another method where we are using four probes mind it in PCR we have studied also they are using two primers. So we are using four probes that is how LCR is different from PCR and we are using two enzymes in PCR we used Taq polymerase here we are using thermostable polymerase as well as thermostable DNA ligase. So what happens suppose this is our template we design four probes in such a way so that two probes will bind absolutely to this sequence and another two probes will bind to this

sequence and the five prime end and the three prime end of the probes are closely in proximity after the binding has happened. So what happened after the binding has happened using hybridization technology this will be sealed by DNA ligase mind it if there is a gap even after hybridization suppose the probe has been designed in such a way that there is a gap this will be amplified by the polymerase ok and then finally, the gap will be sealed by ligase, but if you are designing the probe in such a way that is of exact match then there is no need of polymerase.

However, you know target sequences are big and probes are generally small so we need both polymerase and ligase, but you see using even using four probes at the end of the reaction the target DNA has duplicated and so with each cycle just like PCR we get an exponential amplification of DNA. So in the next cycle another four probes will bind to another parent DNA so this can be utilized in detecting the target sequences. However it is very important to know that it was originally developed to detect single point mutation alright a single nucleotide again single base mismatch why because you see if there is a single base mismatch the probes will not hybridize properly you see there is an overhang over here in case of single mismatch there is a the three prime end and the five prime end of one adjacent probes will not match. So even if there is amplification there will be no ligation. So this is enough to prevent one mismatch is enough to prevent the ligation and amplification and in case of mutation there will be no ligation later this was also used in amplifying other normal DNA, but nowadays LCR is not much used compared to PCR.

However, theoretically it is much more accurate compared to PCR alright. So in case of mutation the TM is designed in such a way that only and only at the right area at the specific if the only specific target of interest is present they will anneal in case of any mismatch one primer template duplexes mismatch will not be tolerated and there will be no ligation. So this is in short about ligase chain PCR. So this is the total amount of number of PCR reaction that we have covered. So we did cover real time PCR in previous class reverse transcriptase PCR one step two step multiplex nested asymmetric and linear after the exponential PCR in the previous class and in this class we have covered allele specific arms, hot start, colony, touchdown, digital PCR, long range in situ inverse as well as ligase chain reaction.

So there we have covered a lot each can have short note question depending on what type of examination you are facing. So these are my references for today's class and I thank you all for your patient listening. We will see you in the next module very soon.