Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Week 02

Lecture 09 : Variations of the PCR

Namaskar. Hi everyone. Welcome back to the course on comprehensive molecular diagnostics and advanced gene expression analysis. In today's class we will be starting with another important discussion that is the variations of the polymerase chain reaction. Now like last time this class will also be divided into two sections. We will be covering some variations in this section namely the topic of RT-PCR, multiplex PCR, nested PCR and asymmetric PCR as well as late PCR, late PCR and we will be covering some more variations in the later class.

Mind it polymerase chain reaction the variations consist of a wide variety of sub topics right. So, we have split the discussion into two. Mind it we already have discussed real time PCR right. Well I would say it can it is may be considered as a variation of the traditional PCR of course, but rather it is a different quantitative diagnostic method that we already made clear in details in previous two lecture classes.

So, right of the bat we will start with RT-PCR which stands for reverse transcriptase polymerase chain reaction. Mind it in last class we already discussed that this subtype of PCR can be done by traditional method or by real time method right. So, please do not confuse between real time PCR and reverse transcriptase PCR. When we refer to as qPCR real time when we refer to as RT-PCR it means reverse transcriptase PCR all right. So, what is reverse transcriptase PCR? It is definitely a modified PCR that amplifies the targeted RNA molecules.

Mind it in traditional PCR that we discussed in till now are always amplifying the DNAs all right. So, here we start with RNA molecules. So, we need to somehow convert the RNA to DNA. How do we do that? With the help of RT or reverse transcriptase enzyme that actually helps in amplification of the target DNA and it synthesizes a complementary stat it takes the RNA as a template and it synthesizes it is complementary DNA that is also known as cDNA right single strand cDNA. Then that cDNA is converted into double stranded DNA with the help of DNA polymerase and

that dsDNA molecule will now serve as template for the traditional PCR process.

The rest is absolutely same. So, there is an extra step of synthesis of complementary DNA from RNA molecule to start with ok. So, we need to know how the RNA molecules will be converted to DNA all right. So, again for RNA polymerase to act RNA dependent DNA polymerase that is reverse transcriptase to act. Mind it transcription means DNA dependent RNA polymerase, reverse transcription means RNA dependent DNA polymerase fine.

So, that also needs some primers to start with. So, there can be many type of primers. The first type of primers that we use in this RT reaction or cDNA synthesis reaction or reverse transcription are known as random hexamers. What are random hexamers? These are random combination of all six nucleotide sequences that are designed I mean that are provided in excess in the reaction mixture and that can arbitrarily bind to the RNA molecule here and there depending on their complementary sequence availability or complementary zone availability, then these will be extended with the help of DNA polymerase and finally, these segment will be tied by DNA ligase ok. So, this is how random hexamers act.

Simple RNA primers they will attach and they will be extended ok random hexamers. Next oligo DT primers what are oligo DT? They are DT deoxythymidine ok DT multiple sequences of T they will what we will do? They will attach to the poly A tail of the mRNA. We are amplifying a messenger RNA to start with right. So, messenger RNA has got a poly A tail oligo DT will latch on to the poly A tail and then they will be elongated. Thus we will form a complementary DNA strand which is I mean your DNA strand is complementary to the this gene or target gene of interest.

So, random hexamers and oligo DT primers mind it these are not sequence specific these all fall into the category of non sequence specific primers. It means what irrespective of what our target gene or target sequence is they can be used. So, any type of target mRNA target messenger RNA target gene we can use random hexamers because random hexamers can bind randomly anywhere. They will find some type of complementary zone invariably and every similarly every gene has also got its poly RNA I mean poly A tail over there also these primers can bind alright. So, mind it random hexamers combination of 6 nucleotides and oligo DT combination of multiple deoxythymidine nucleotides.

Next sequence specific primers means these are actually designed keeping the target sequence in mind. So, these will only attach to the specific mRNA that we want to amplify thus making this sequence specific primers thus making this RTPs are a very specific process. So, this is how region specific or sequence specific primer looks like

you might say well it looks like the same as random hexamers true, but given random hexamers can attach to any possible messenger RNA these sequence specific primers will not attach to our messenger RNA which is not our target of interest ok. It will only attach if and only if the target mRNA is present then it will amplify to form a cDNA and then the PCR amplification will be occurring. So, mind it 3 types oligo DT, random hexamers and sequence specific primers.

So, now you I mean might have got some idea how this RTPCR reaction can happen. So, there are 2 ways of doing it number 1 I will be discussing the 2 step RTPCR. In 2 step RTPCR first we carry out a cDNA synthesis using either random hexamers or oligo DT primers or gene specific primers alright. We can use any the choice is ours we will get a cDNA. So, till that step we need to do a separate reaction thereafter what will happen this cDNA will subsequently be used for another PCR experiment alright.

So, you see here this requires opening of the tube right we will first synthesize the cDNA and thereafter we are collecting that cDNA and we are adding the cDNA we are pipetting the cDNA to another reaction mixture another I mean polymerase chain reaction tube where we have added the specific primers to amplify the gene. Mind it the first set of primers are only to synthesize the cDNA and the second set of forward and reverse primers are for the PCR reaction proper alright. So, in 2 step we are first synthesizing the cDNA in one tube and then we are I mean utilizing that cDNA to make I mean for another subsequent PCR reaction right. So, that cDNA I mean in 2 step PCR we can plan this reaction in separate dates as well we can store the cDNA in minus 20 degree centigrade it will very stable and then we can subsequently use that right. And not only that we can I mean this is an option we are free to use some part of the cDNA alright the amount of cDNA that is generated we can keep that parts some part we can use for PCR and later we can use another part to run PCR at a later date the choice is ours ok.

So, you seen 2 step RT-PCR first cDNA stand is synthesized right and then in another setup that cDNA is added as a an ingredient of the second PCR reaction right. So, what is 1 step RT-PCR? 1 step RT-PCR the whole thing is done in one go cDNA the we use a thermo stable DNA polymerase which has got an inherent reverse transcriptase activity alright. So, we add everything to start with in a single master mix we add the RNA we add dNTP we add magnesium and we add reaction buffer everything is added and then we set up the cycle in such a way. So, that the first part of the reaction will be utilized in cDNA synthesis and then without even opening the thermal cycler without even opening the PCR tube we will start the cycle. So, that in c 2 over there the cDNA is produced and that cDNA is automatically utilized for a polymerase chain reaction amplification alright.

So, what is the advantage? Major advantage is in one step this is known as one step we do everything in one go there is no chance of contamination whereas, in case of 2 step there is a high chance of contamination because we are opening the reaction mixture. Of course, we take precautions to do it in a laminar flow hood and molecular value setup still one step is much more protected from any contamination. So, this is how one step PCR looks like I mean if we depict it in a flow reaction everything is added in one go there is no addition there is no plus we all complete the reaction in one flow. So, one step keeps on happening after another. So, reverse transcription first strand of cDNA then amplification it forms a complementary strand then so on and so forth the amplification keeps on happening right.

However, if there is a I mean scarcity in the quality of RNA sample that will affect the whole reaction whereas, in 2 step PCR we can actually control the amount of cDNA we are adding alright. So, mind it this can be a disadvantage of one step and moreover there is another disadvantage if we need to store the cDNA there is no such possibility we cannot store the product we have to complete the reaction in one step and ultimately we will get amplified product alright. So, if we need to repeat the reaction we need to start again from the RNA extraction procedure alright. So, this you should keep in mind both process have got their advantage and disadvantages that you can utilize on your I mean in your experiment depending on your situation. So, this is a pictorial diagram where we are seeing what I mean how both of them I mean one step and 2 step are executed mind it in one step we must use sequence specific primers.

Whereas, in case of 2 step we in order to since there is the cDNA we are free to use either oligo dt or random primers random XMRs or sequence specific primers sequence specific primers do apply for both. However, in case of one step there is no role of them ok. So, in one step we are doing everything in one reaction whereas, in 2 step first we have synthesism cDNA and thereafter we can use that cDNA to perform another PCR reaction ok. So, what are the applications of RT-PCR? Mind it RT-PCR anystudy related to RNA analysis alright. So, expression gene expression we are isolating the messenger RNA and then we are trying to see how many times it have increased what is the amplification what is the role of any drug alright.

So, mainly used in diagnostics for example, detection of virus and I have already mentioned multiple times that the COVID-19 pandemic the main stay of diagnosis was RT-PCR which is reverse transcriptase PCR. So, COVID-19 made this term very much popular, but you should always know that RT-PCR is routinely used to detect many other viral retro viral diseases like HIV hepatitis C virus so on and so forth. Also many bacterial genes parasitic genes and fungal genes their mRNA are isolated and for any research scenario when we are trying to study any gene expression profile first we are isolating the messenger RNA and then we are amplifying them with the help of RT-PCR

it might be one step or 2 step and then it is extensively used. So, these are the applications of RT-PCR alright very very very important diagnostic tool rampantly used in all types of molecular diagnostics as well as research studies. Next we move on to multiplex PCR what is multiplex PCR? You see in traditional PCR the if we need we to amplify more areas of interest we need to start with more I mean multiple materials.

So, we can actually amplify 4 genes, but we need to set up 4 different experimental setups right. The phenomena or the type of radiation of PCR reaction where we can amplify multiple more than one target sequence using multiple set of primers in a single PCR reaction is known as multiplex PCR right. So, the pictorial illustration you can have a good idea. So, we are actually simultaneously amplifying several gene segments at the same time right. It was historically it was first used by Chamberlain for diagnosis of Duchenne muscular dystrophy dystrophy ok.

It is a muscular disorder where role of multiple genes were intended to be studied prior to that the only way it was possible was designing multiple primers and designing multiple set of experiments simultaneously, but the group attempted this and thereafter it became very popular it is known as multiplex PCR. So, if we look at the subtypes of multiplex PCR it is actually very easy to understand. The first type which can be designed is known as single template PCR reaction. Here the gene of interest is a big we are choosing a big section of interest which has got multiple subsections and we are designing the primer in such a way so that multiple areas of the single target will be amplified. See we have designed 3 primers to start with which are targeting the different areas of the same parental gene big gene.

So, one is targeting this area, one is targeting this area, another is targeting this area there are shifts alright. And ultimately we are getting our intention is to get 3 different amplicon which are generated from the same target gene from the same mRNA is used as a complementary source right. So, this is the first variety single template PCR reaction whereas another way can be multi template or multiple template where we are designing primers that are complementary to 3 different sources of gene to start with right. So, we are adding 3 different type of target DNA's or target genes in our PCR system alright. And then their amplification I mean their respective region are targeted using forward and reverse primer.

Mind it we are not one disclaimer please free your mind from any RNA ok. We are back to the traditional PCR system which deals with double stranded complementary DNA to start with alright. RT-PCR was a variation of PCR that started with single stranded RNA molecule we needed to synthesize the complementary DNA then those were used as a source. Whereas in multiplex PCR is a traditional PCR, but the number of products are multiple that can have a single source using multiple different primers that is a single

template or multiple source using multiple primers that is known as multiple template PCR reaction alright. So, these are two subtypes of multiplex PCR.

Now we need to be very careful since we are dealing with multiple primers all the primers will need to be designed in such a way because the reaction will happen in one step I mean in one cycle in one tube. So, the set of conditions will be similar. So, all primers need to be designed in such a way. So, that their melting temperature their GC content their cross complementarity everything is taken care of alright. So, that at the end of the reaction when the parameters I mean when multiple amplicons are generated we can I mean multiple amplicons whose sizes are different they can easily be distinguish using gel electrophoresis or by real time method using various type of different colored fluorescent dyes ok.

So, this is the concept of multiplex PCR where multiple amplicons multiple target gene can be visualized in the same reaction mixture ok. Next we should know about the internal control what is internal control? Internal controls are the ones I mean there are specific regions of a gene that may be present in all the target samples. Primers are designed for that target region that will amplify irrespective of our criteria of interest. It means suppose there is a bacteria alright we need to identify that alright. So, the bacteria we are trying to target a specific sequence of a bacteria to understand whether this bacteria is dangerous or it is not dangerous.

So, if that sequence is present the bacteria will be very virulent for example, MRSA or methicillin resistant staphylococcus aureus. If there is a gene which translates for an enzyme that destroys methicillin that will is known as super bug I am just giving an arbitrary example. So, we are targeting that bacteria. However, in the same PCR reaction we are so we need to design primers against that part of the gene which attributes this property to the bacteria. Along with that we are also designing a primer for a generalized section that is present in all bacteria.

So, our final result will give us the amplicon of a general area as well as the targeted specific area. So, that is why we are using an internal control. So, mind it this may or may not be present in all the bacteria. However, the internal control the zone should be designed in such a that is present in all bacteria. Now since we are detecting multiple such product this is definitely a multiplex PCR, but why we are using the control to validate our result.

It means if there is an absence of the control gene or control amplicon. It would mean that our process has failed. So, if control is absent and the target is also absent we should not consider the bacteria to be not methicillin resistant for example. However, we should reject our whole run because the internal control has not amplified. Whereas, mind it in a

situation where both control and the target has amplified we are well and good we will definitely adopt those result.

In another scenario where the control has amplified, but the target has not amplified is it valid? It is definitely more valid and the absence is more significant because the our run is absolutely fine because control is has actually amplified. I am sure you must have understood, but if you have not understood I am giving one sort of specific example we will again deal with this in much detail when you are studying detection of infectious disease using polymerase chain reaction all right. So, this is about the internal control. Now the advantage multiplex PCR is basically safe and time consuming cost effective method because we do not need to repeat multiple experiments over and over again everything can be done singly in one tube fine. So, what are its application? I have already told you you have some ID can be used in diagnosis of multiple infectious disease.

For example, bacteria causing meningitis, bacteria causing polio, different type of vibrio I mean diarrhoea causing bacteria. For example, this is a picture where we are diagnosing various types of gene in a vibrio species. Vibrio is a bacteria that can cause cholera right this entry. So, vibrio para hemolyticus species we are actually detecting over here that can cause dysentery and we are detecting simultaneously detecting multiple genes. So, thermolabile thymolysin, thermostable direct thymolysin.

So, based on that the virulence can be determined do not go into the specific example, but how we are doing it we are simultaneously multiplying genes and those amplicons are then added to a lateral flow cassette. So, that we can easily diagnose them ok. So, one example is detection of multiple such species or multiple such species simultaneously. Not only that it is also very very important multiplex PCR apart from diagnostic laboratory for genotyping, mutation analysis, polymorphism analysis, micro satellite detection as well as gene deletion analysis. There are many many many process where multiplex PCR saves a lot of time and brings a lot of convenience in our experimental situation alright.

However, definitely there are some limitation every process has got some limitation the main limitation is working with so many primers alright. The use of multiple primers is the main inhibition you need to design the primers in such a way so that they all work together. Cross complementary will absolutely ruin our chances of getting any desirable product and due to this the efficiency of the amplification efficiency is actually very low. What is amplification efficiency? The zone, the lock phase where every product is multiplying itself twice that is often not the case in case of multiplex PCR. So, we need to I mean there are some pros and cons at the cost of something.

So, we are sacrificing the efficiency for convenience alright. Multiple pair multiple primer will lead to a potential problem. Next we want to nested PCR. What is nested PCR? A nested PCR in order to increase the sensitivity as well as specificity of the reaction means we do not want any unwanted product. We first design the PCR in such a way that there are two sets of primers.

We are targeting a large gene to start with and the next set when that first target region is amplified we are targeting designing a primer which will only amplify a region which is which lies in between this targeted region. This so, first suppose this is our target sequence. It may be so, that the target sequence may have got some similarity with any other sequence. It may be present alright. However, if we design the primer in such a way so, that first we target only this sequence.

So, only and only after this sequence have been amplified we are now using the second set of primer. So, this will only amplify the region which is present in this. So, this double check double filter actually cuts the need of I mean the possibility of unwanted primer. So, the first primers that are used to target the first gene that is the upstream region that are known as outer primers alright. And the second more specific primers that targets the smaller region are known as inner primers or nested primers.

So, we are nesting one reaction within another ok. So, the traditional method was earlier how it was done it was I mean one set of PCR was done and then that PCR product was used I mean specific nested primers was then again added to that specific PCR reaction to make this process much more specific. However, as we have already noted opening of the tube gives a chance for contamination. So, nowadays to address this a single tube nested PCR right STN PCR reactions have been developed where both set of primers are added in the reaction vessel and an extended PCR is performed alright. And how do we know the ultimate the product can be diagnosed traditional method by running the whole product in a 2 percent agarose gel lithium bromide or it can be designed in such way so that it is visualizing real time using a fluorescent dye.

The detection depends on you. However, if cost is an issue since we are desiring I mean we have designed the experiment in such a way that we are only getting the final targeted sequence with the help of 2 primers the cost effective agarose gel electrophoresis can give a very specific result alright. So, this is just another pictorial representation where the first circle first cycle is done with a more wider primer may it may have been so that if we are using the absolute the first small targeted sequence it could have amplified this region as well. However, the possibility of the target having same complementarity in a bigger in a smaller target zone is actually very low. What I mean to say suppose our gene of interest our target of interest is this much very small.

This small possibility can be present here, here or here. However, if we are cutting off this section to start with the possibility of our target primers or specific primers to bind elsewhere and amplifying them is almost nullified. Since we are chopping of the target sequence to start with and then we are adding a nested primer it means whatever amplification we will get will definitely belong to our target sequence of interest alright. So, these are the advantage of nested PCR the non specific amplification are absolutely eliminated it is because the nested primers will not find anything primer, dimer etcetera everything non specific artifact is absolutely ruled out there will be no such amplification of non specific products alright because we will only prime any specific product from the outer target sequence alright. So, PCR specificity is very high in case of nested PCR. So, what are the application? Since it is designed in such a way that only very specific area will be amplified therefore, it along with PCR specificity it has got very high sensitivity in cases where there are low abundance of target.

For example, DNA in complex tissues DNA from sample which are formalin fixed or paraffin embedded where by traditional extraction method a very small amount of DNA might be found and we cannot absolutely risk a non specific amplification. So, for valuable samples nested PCR gives a very specific diagnosis and simultaneously both sensitivity and specificity are increased. Again pathogen detection in clinical diagnosis very crucial for early diagnosis because it is sensitive enough to detect low concentration alright very important. So, we can easily rely on this for early diagnosis forensic DNA analysis wherever the sample trace amount of sample is available for DNA profiling this is a method of choice. Molecular cloning and sequencing again for fragmented DNA amplification it is very much preferable and viral quantification all with low level anything which deals with sub optimal sample we can actually use nested PCR to our advantage.

Limitation definitely susceptible to contamination if we are using reaction which has got extreme sensitivity ok mind it it is not a false positive ok. We are not getting something that are not used I mean that is amplified outside or target area of interest right. However, if we are using an open tube method where the first set of primers are amplified and then we are transferring that primer or that amplified zone to the second specific primer opening means general contamination ok and that is a problem for that single tube nested PCR is the method of choice. And mind it in all cases single tube may not be designed because both the primers need to work in such a way both the primers may have their varied temperature of annealing etcetera. But ideally if a certain PCR is followed the step of contamination is almost nullified and again it is costly because we are using two separate primers to achieve a result all right.

So, anything which involves multiple primer multiple reaction multiple cycles will definitely increase the cost, but that is the expense we need to bear in order to avail the

high sensitivity as well as specificity of this type of PCR. Next asymmetric PCR what is asymmetric PCR? This actually varies from previous types of PCR in a very strange way mind it till now whenever we are studying any PCR reaction whenever we are designing master mix or the amount of products that need to be attained or added to the reaction mixture we should always add equal amount of forward and reverse primers right because both are amplified simultaneously in a similar way. However, in asymmetric PCR which is actually used in special cases where we need to amplify only one strand from the targeted double stranded DNA. What do we do? We add one primer in much excess compared to the another primer. You see both of the primers were amplifying the target DNA parent DNA in such a so that in every cycle two amplifiers are applied to two amplicons were produced and they were amplifying both the strands in this way.

However, if we add one primer in excess what will happen after few cycles the limiting primer which is the primer in small concentration will be depleted and after few cycles only the primer the abundant primer will amplify the first targeted reaction or the first target or the first strand this is the targeted strand alright. So where they will be and at the end of the experiment what do we do? We can compare the amount of double stranded DNA and single stranded DNA but there are many methods we can do it in a fluorescent way to achieve our target of interest. So where where do we need this sort of peculiar requirement they are used for DNA sequencing, hybridization, probe design, site directed mutagenesis, aptamer generation, cloning, library construction also in in vitro transcription reaction where we need to generate single stranded RNA right from a DNA. So first we need a single stranded DNA we isolate a single strand and then we treat them with the RNA polymerase to do in vitro transcription reaction. So mind it these are the situation where we prefer to start with a single strand of DNA.

What are the disadvantages? It is not widely used again because of the low reaction efficiency. The only reason why the PCR has gained so much operality because it was amplifying twice exponential. However, that will not be the case when the limiting primer is depleted the reaction will definitely slow down alright. And the concentration of one primer lowers its melted temperature below the reaction of annealing temperature. So we need to design the reaction in such a way we need to design the cycle in such a way so that there are room for an annealing of both the primers alright.

So just by adding amount of primer in excess we can achieve the process I mean we can amplify single target, but that is not much efficient alright ultimately one primer will be limited and the reaction efficiency will be lowered. So how to overcome this? So this has been overcome by tweaking the reaction in such a way so that the target primer suppose the forward primer and the reverse primer does not vary in amount, but they are characteristic alright. So the lower concentration primer has got a higher melting temperature than the more concentrated primer to maintain the reaction efficiency. The

forward and primers are designed in such a way so that their melting temperatures are different mind it when we are studying PCR we discuss that the forward and reverse primers should be designed in such a way so that their melting temperature does not vary by 5 degree over here we are intentionally altering that. So what will happen we are first allowing the both the primers to anneal alright and subsequently we are designing the cycle in such a way so that we are providing a lower temperature so that only the limiting only the abundant primer will anneal and thereafter again we are increasing the temperature the ramps are designed in such a way so that there is a room for attachment of both the forward as well as the limiting as well as the abundant primers alright.

And we are reading when the we are detecting with the help of two different type of probes for example, the single stranded DNA are detected at low temperature with the help of fluorescent probes and the double stranded DNA at high temperature can be detected with the help of cyber green dye which actually specifically binds to double target DNA. Now you see again the reaction is definitely will slow down if both the parental strands are not amplified to start with alright. So in first situation it was the amount of primer that was limiting here again it is the temperature that is also playing a factor ok. So here along with the amount of primer that along with the concentration the temperature is also altered so that after first few phase of reaction which are exponential the later phase of the reaction were only single stranded DNA the single targeted strand is being amplified over and over again it is linear alright that is why the reaction is named linear after the exponential. So this is the exponential phase after which the reaction becomes linear so LATE PCR.

So what is the advantage where we can utilize this type of reaction we can again utilize this type of reaction in precise genotyping as well as mutation analysis if it is combined with high resolution melting HRM mind it will be discussing HRM in detail when we are discussing PCR dependent mutation analysis. Apart from that single nucleated polymorphism detection as well as in case of varied template initial template concentration it can be done accurately if we are adapting this LATE model in case of qPCR right and again when detection of low abundance variance this is very important particularly useful in case of heterogeneous population if the target gene we need to study we can actually do that with the help of just by amplifying a single target alright and this type of PCR also reduces a chance of forming primer-dimer by improving the specificity and we also need to know that is suitable for amplifying complex structures for example, the template if they have got a high probability of forming secondary structure due to high GC content often we may not get an ideal template for a PCR. So in all those case of complicated scenario we this variety of this modification of PCR has led to the solution of achieving the amplification of the target sequence which can be later utilized for further study. So these are the references for today's topics mind it what topics what all topics did we cover we covered to start with RT PCR that is real time not

real time reverse transcriptase PCR thereafter we studied multiplex PCR mind it RT PCR was in one step or two step in the next we studied multiplex PCR then we studied nested PCR asymmetric PCR and then LAT rate PCR. The next part of our discussion which is will be which will be the last discussion of this weeks module will be covering the other remaining variants of PCR so your concepts will be very clear when we will be discussing the use of PCR to detect cancer infectious disease inherited disorders so on and so forth these are the references which you can go through at your own will your own pace it will be very easier for you to follow along if you are interested in reading more about all this topics so I thank you for your patient hearing and I will see you soon in the next class till that stay tuned and thank you for your attention.