Comprehensive Molecular Diagnostics and Advanced Gene Expression Analysis

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Lecture 07 : Realtime PCR (Part 1)

Hello everyone, welcome back. We are continuing with our module of tools for molecular diagnostics and in today's lecture we will be covering real time PCR. Now real time PCR is a vast topic which comprises of many sections and subsections. So we will be dividing this whole lecture in two phases. In part one that is in today's class we will be discussing the general concepts regarding real time PCR we will try to establish how does it differ from traditional PCR and what are its advantages. We will be covering the basic concept of fluorescent dyes, FRET we will be explaining it all and also we will are going to see about what are the different probe designs and chemistry principles used in real time PCR and various dyes that are used and in the next part we will be going to the quantitation part.

So, in this part we will be starting with a recap. So, this is the general workflow of PCR you already know first we are extracting genomic DNA followed by we are running a PCR polymerase chain reaction which has got three steps of denaturation annealing and extension and thereafter we are visualizing the whole PCR process by running an agarose gel electrophoresis and viewing the whole thing in a UV transilluminator where we can compare the various sizes of the PCR product right. So, without these three the reaction is not completed. So, that brings a problem not to say that the traditional PCR that we discussed we termed it as one of the most beautiful tool most used tool the best tool it has got its shortcomings poor precision, low sensitivity, low resolution all compared to the newer variety.

The most problematic thing is it can discriminate only based on size only the size of the PCR product if we are synthesizing a PCR product that has got a similar size it will give same bands in agarose gel will have no idea whether those two genes are different right and moreover the results are not in numbers they are not quantitative. We can only conclude the PCR is successful or not even then we looked into various issues that a PCR can have troubleshooting for example, if primers are not designed properly we can have false positive bands of primer dimers. So, all of this can be solved by today's

discussion that is real time PCR. So, what is real time PCR? In traditional PCR what were we doing? We were running the PCR in a thermal cycler block there was no way to visualize what is going on after the reaction has ended we can take the PCR product and we run in agarose gel right. So, this is end analysis after it is done we can only look into it whereas, in real time PCR as the cycles are happening as the products are being amplified we can view it.

So, it is happening in real time the analysis can be done in real time. How do we do it? Mainly by using fluorescent dyes. So, almost all real time PCR system are based on some sort of a fluorescent dye detection system where we are using a fluorescent dye a fluorophore that is emitting a signal by binding to the PCR product to double stranded DNA and we can detect that with an inbuilt fluorometer or a CCD camera which is built into the machine. This is one an example of a real time PCR machine which has got an inbuilt everything is inbuilt here and we can visualize the whole thing in cycles. Whereas in traditional PCR if we say we are running the PCR 35, 36, 37, even 40 cycles we can only touch the machine after the whole total 40 cycles are over we cannot intervene in between.

Whereas, here it is possible right. So, and the most beautiful thing is the signal that is the fluorescent signal that is emitted by all the means or the fluorophores are directly proportional to the amount of PCR product in a reaction. Hence we can actually express the result in numbers we can quantify all right. Now one most commonly used acronym is RT-PCR right. We have heard it a lot it is 2023 and the word RT-PCR is known by every common man in the whole world thanks to the COVID-19 pandemic.

Often RT-PCR is falsely referred to real time polymerase chain reaction which is not true. RT-PCR means reverse transcription or reverse transcriptase polymerase chain reaction right is a different variety of PCR. We will be discussing that in our next class where we will be defining or studying various variations of PCR. Mind it RT-PCR is a variety of PCR whereas, real time PCR is a quantitative tool. So, RT-PCR this reverse transcriptase PCR can be done traditionally and can be done in a quantitative way.

Mind it real time PCR is actually abbreviated as QPCR the Q stands for quantitative PCR all right. So, do not make this mistake RT-PCR is not real time it is reverse transcription or reverse transcriptase PCR right. So, what are the advantages? Of course, it is quantitative we get the resulting numbers it is precise accurate we will see we can design sequence specific probes right we can do multiplexing in real time PCR very important multiplexing can be done multiplexing all right. It saves a lot of time suppose I told you the PCR reaction is not optimized in PCR troubleshooting we can when we are testing out for the first time it may so happen that our annealing temperature is not optimized our primer designing is not right and the PCR product is not actually

amplifying. In traditional PCR we have no way to comment unless we wait for entire 2 to 2 and half to 3 hours depending on what is the cycle settings and what are the number of cycles and then we need to run the whole thing in agarose gel and then only we can say whether the reaction is successful or not.

So, imagine in case of a failed reaction you still need to wait 3 to 3 and half hours because agarose gel also takes some time to run for example, 30 to 40 minutes depending on the percentage of the agarose gel concentration. So, real time PCR you can see right then on the screen itself every real time PCR device has got a computer screen computer it is built in so where you can visualize the whole thing. And right in the moment where we get a fluorescent signal we are sure that the amplification is happening or if we do not get the signal we are sure the reaction is about to fail then we can stop the reaction and we do not need to wait for the entire 40 cycles to happen. So, it saves time saves time so it is efficient right. So, all these are huge advantages of real time PCR over traditional PCR.

Now over here we should touch a bit on fluorescence and fluoro force. You know all real time PCR machines use a fluorescent dye. The fluorescent dyes have got a property basically photonic property we were discussing a bit of biophysics over here. So, a fluorescent particle can absorb a certain specific wavelength of light right. It then the electronic shift happens right it becomes excited and it goes from a lower energy state to higher energy state in that molecule right and then it can emit a different frequency of light.

Now, the wavelength at which a photonic energy is most effectively absorbed is known as absorption maxima or absorbance max and the wavelength at which light is mostly most efficiently emitted is known as emission max. So, there are two distinct range of frequencies and we can use optical filters to separate these two frequencies. This is general fluorescence fluorescence and this is used in real time PCR system. Why how it is so much favorable? It is because the brightness of emitted fluorescent light is actually directly proportional to the quantity of fluoro chrome present. So, the more amount of fluorescent particles are present the higher will be the intensity of emission alright.

So, this thing is used where we can actually send a fluorescent light signal for example, a xenon arc lamp which is built into the real time PCR by we can send it through a filter so, that a specific wavelength can be filtered that wavelength of light will strike a fluorescent particle in the liquid sample and then the fluorescent molecule will emit a specific wavelength of light which will again pass through a secondary filter and can be detected by a photo detector or a photo multiplier which is again inbuilt in the real time PCR system and it can be expressed in a number right. So, thus the amount of fluorescence can be converted to real number or electrical signal alright. Mind it this is

very important I if the signal is huge it can be detected using photo detector if the signal is very weak it can be amplified using a photo multiplier. So, everything is built into the real time PCR system. Now, next we will be discussing about the various probe designs and chemistry principles that are used in real time PCR.

Mind it the ground rule will always remain same they all rely on detection and quantitation of a fluorescent reporter dye it is a dye we will be discussing later what what various dyes we are using and there are other types of dyes as well that are used in real time PCR system. Coming to the chemistry these are the most interesting and most used type of probe designs we will again discuss what are probes and chemistry principles that are used in real time PCR cyber green, tachman probe, molecular beacons, hybridization probes, sunrise uniprimers, scorpions probe so on and so forth. Mind it these are the ones which examiner tends to focus during the exams there are there can be there are in fact, multiple varieties of probes, but we will be discussing the most important ones in this lecture. So, starting with cyber green it is actually a fluorescent dye which specifically binds to the minor groove of double stranded DNA right. Whenever it finds a double stranded DNA it will bind and it will emit fluorescence all right.

So, when more and more PCR product are formed you you already know by the 40 cycles millions billions are produced by the end of 4 cycles to the power 16 copies are produced. So, as and when the number of double stranded DNA that is the PCR product increases cyber green dye which is already added in the system will bind and will emit fluorescence all right. So, what how does cyber green I mean how does this system how does this master mix I told you during PCR you need to add all of this agents in addition to for example, magnesium chloride which is already present in the reaction buffer this is known as master mix. So, how the master mix of cyber green based real time PCR chemistry differs we need to separately add this cyber green dye rest are almost similar. So, when more and more cyber green dye couples or intercalates with the double stranded DNA it will emit fluorescence and we can have an idea that the amplification has started to happen.

It is easier it is inexpensive mind it there is no probe required if this term is not clear it will be very clear after we are discuss after we have discussed few more mechanisms. So, mind it no probe is required hence it is actually inexpensive right it is less sequence dependent we do not need to be selective for sequences right it is less inhibitory for Taq polymerase it has got higher stability it has got higher resolution melting curves will be studying melt curve analysis wherever when we will see that cyber green dye plays an immense role right and it has got less mutagenicity less hazardous for healthcare professionals. However, the advantage of the specific qualities of cyber green becomes a disadvantage. So, it is a double edge sword less sequence dependent. So, what will it do?

It will actually bind to any double stranded DNA.

So, it can bind to primer dimers it can also bind to non specific reaction product. So, any unwanted double stranded DNA product irrespective of the size cyber green will bound and it will emit a fluorescence. So, it is a high chance that there will be over estimation of target concentration. And when the cycle is in very late stage when there are billions of copies there might be non specific products then there will be non specific background emission alright. So, early detection very good melt curve analysis the proper the reason.

So, considering all of them you will realize later that the very fact that it binds to primer dimers and non specific reaction products is utilized in melt curve analysis. So, these are the advantages as well as disadvantages of cyber green dye inexpensive non selective. Let us move on to the more specific and selective methods, but before which we need to know we need to be acquainted with the concept of fluorescence resonance energy transfer or FRET which is a very easy concept to understand basically there are two molecules or two dye alright. They are very close to each other need to be very close to each other specific within 1 to 10 nanometer. Then what will happen when one of them is in excited state mind it if a single fluorescent dye is present when it is excited it will emit its higher excited in wavelength.

When a however, in this phenomena of FRET it interacts with another dye in a close proximity and transfer its energy without emission of a photon. So, what will happen? Now the whole energy is with the acceptor molecule. Now this acceptor molecule can behave in two ways alright the acceptor can actually emit fluorescence alright. So, here instead of the donor emitting a fluorescence it is excited it transfer its energy to a something which is very close and it emits it is the acceptor which emits or it can absorb the whole thing that is known as quenching or often known as dark quencher. So, you see the in close physical proximity mind it 1 to 10 nanometer 10 to 100 angstrom no matter whatever unit you say or choose to represent the distance remains the same can lead to either fluorescence resonance energy transfer or FRET based quenching, quenching means absorption no reading.

So, you see in the first illustration we are talking about FRET based binary hybridization probes we will be reading it in detail. So, over here what is happening 2 molecules donor and acceptor run in close proximity whenever donor is excited the acceptor molecule will emit if they are not in close proximity there will be no emission from the acceptor alright. Our detector is designed in such a way it can detect only the emission wavelength of the acceptor alright it cannot detect the emission because since it is a fluorescent dye it will also have some emission of its own, but it will pass through optical filters and it will be absorbed. Our detector we can design detector in such a way

that only and only if there is an emission from the acceptor it can detect for that the acceptor needs to be very close to the donor if the distance increases there will be no fluorescence resonance energy transfer. We can use the similar principle in absorbing or quenching the emission.

For example, we see here R is known as reporter dye and Q is known as quencher whenever the reporter and quencher are in close proximity the reporter cannot emit because all its emission is being absorbed by the quencher. Whereas, when the quencher is separated from the reporter here FRET is occurring fluorescence resonance energy transfer is occurring, but it is leading to it is the dyes are the properties of dyes are such that whenever FRET is occurring one is absorbing the whole thing alright. But over here what is happening the reporter and quencher are separated and then it can it is free to emit there will be no FRET and no quenching alright. So, quenching will be released. So, in this case there will be the more distance will lead to more fluorescence mind it in quenching more the distance there will be more fluorescence the closer the distance there will be no fluorescence.

Whereas, in case of FRET based binary probe closer the distance more will be the fluorescence more the distance there will be no fluorescence you get my point. Here the distance is directly proportional to the fluorescence here distance is inversely proportional to the fluorescence in case of quencher. Anyway you need to remember quenching means absorption no emission. So, based on this quenching principle the first type of probes that are designed are known as Taqman probe these are hydrolysis type of probes. So, you see a Taqman probe is actually a probe which has a sequence and which has got a reporter and quencher die that are actually in close proximity means whenever the sequence is like this when it is intact the quencher can actually prevent the reporter from emitting.

So, there will be no fluorescence right. This part is designed so that it can bind to a specific PCR product or the complementary DNA all right. Now what happens so before the reaction before the amplification the Taqman probe already binds to our product of interest or the target region of interest. Now what happens when the that DNA where Taqman probe is already bound is being read by the polymerase during the extension phase all right. So, primers annealed probe will also anneal with the DNA.

Next when it is amplifying what will happen the 5 prime to 3 prime exonuclease activity of the Taq polymerase will cleave the Taqman probe. Mind it again be very careful this is the typical property by which Taqman probe varies from all other probes. The Taqman probe is actually destroyed how it is destroyed during extension phase the Taq polymerase has got a 5 prime to 3 prime exonuclease activity. So, it will cut it will cut it will destroy the in between connecting strands. So, when amplification is completed the

reporter and quencher will be separated and then the reporter can emit its fluorescence.

So, more and more amplification means there will be destruction of more and more probes and that will lead to more and more fluorescence all right. And as always the signal is proportional to the amount of amplified product in the sample all right. I hope if it was problematic you can pause the video go back watch it again then it will be very clear. Taqman probe it binds first then it is it anneals first then during extension it is hydrolyzed right and it increases fluorescence. So, what are its advantages it is highly fluorogenic it is very easy you just add probe along with your PCR master mix.

Not only that the beauty of it is you can design a probe in such a way since it is sequence specific we already know what is our target area of interest we are trying to amplify the target area of interest. So, without the target area if the target area is not amplified the Taqman probe will not bind moreover. So, this is very high sequence specific right moreover suppose we want to amplify two such genes together we will be discussing multiplex PCR, but the gist of it is we are amplifying two products simultaneously in a single reaction setup there also we can use the Taqman probe because it is signal sequence specific. So, we can do easy multiplexing with Taqman probe. However, the disadvantage is being since we need to synthesize the probe we need to do there is a genetic engineering involved.

So, it is expensive and it needs designing and challenging where you need to position the probes where the reaction mixture is reaction is not hampered all those are challenges, but it is widely used. So, nowadays we are in a such in advanced medical engineering era that biotechnological engineering that these probes are easily synthesized by commercial companies and we can utilize them in multiple molecular diagnostics. So, another advantage of this is similar condition for primers and probes. So, we need to make sure the reaction is occurring first right and also we need to make sure that the annealing temperature is optimal for both the primers and probes. Next elevated background so, the quenching capacity of the so, in the later cycles when there is an elevated background I mean background noise the quenching capacity of the remaining probes will not be highlighted properly.

So, it is better to study or analyze the reaction in early phases alright we will be studying this in much more detail in the next lecture we will be studying the quantitation in real time PCR system. Moreover, this probes cannot be utilized in subsequent reactions. So, we need to add a lot of probes keeping in mind the reaction will use it all up whereas, the all the other types of some other types of probes will see that they are they can be reused alright. So, there is no end point analysis because the probe has already degraded. So, these are the few advantages and disadvantages of TaqMan probe and the basic principle of how a TaqMan probe works.

Next are molecular beacons. So, molecular beacons are also probes all they are hair pin in structure. So, they are also referred to as hair pin probes. So, as you can see the probes are designed in such ways that there is a self complementary part and it forms a stem alright. And there is a loop this circular part is the loop which actually contains PCR product specific complementary nucleotides. So, the specific nucleotides they can bind to the PCR product.

Now the stem is designed in such a way that the 5 prime end is attached to the reporter and the 3 prime end is attached to the quencher, but due to the self complementary stem like design both are in close proximity and there will be no fluorescence that will be emitted. So, the fluorescence is quenched when the hair pin loop is closed. What is the beauty of it? Now let us see what happens as the PCR reaction proceeds. So, when the reaction continues the newly sized PCR products are denatured by high temperature simultaneously the self complementary part where there was a bit of double standing in the hair pin loops they will also open up. When the extension has happened in the next cycle mind it in the next cycle when it has opened up now the specific PCR specific target sequence can bind to the PCR product in the next round of primer and in the next round of PCR.

Thus what happens the hair pin loop is opened and the quencher and fluorescence reporter are separated and it can emit fluorescence right. So, the more and more product will be there the more and more fluorescence signal will be generated. So, one thing we need to know that in case of molecular beacons the Taq DNA polymer is the reaction mixture is designed in such a way so that the DNA polymerase has got I mean it is designed in such a way that there is no 5 prime exonuclease activity. So, this molecular beacons are actually displaced they are not hydrolyzed mind it very important molecular beacons can be reused they are not hydrolyzed these are not hydrolysis based probes. You just need to remember that they are displaced not degraded ok.

Why because we use a polymerase which is devoid of 5 prime exonuclease activity there will be no 5 prime exonuclease activity in the polymerase that we use for molecular beacon based reaction right. The entire reaction mixture is actually provided by the commercial company where we are ordering our molecular beacon based on our target gene of interest. Advantages high specificity low background noise post PCR analysis is possible because we have got multiple probes it is possible it is not needed mind it when you are analyzing we can do it in real time, but still there are situation in which post PCR analysis might be required in order to nullify the background noise and the many factors. Again since it has got specific target sequence it can be used in multiplexing and very important it can be used in allelic discrimination greater specificity than linear probes. Linear probes can also be used in allele specific you can design the probes in such a way.

So, that they are complementary to the specific allele in case of detecting genetic mutations, but hairpin probes are much more specific ok. It is this post PCR analysis where we can also have an idea about the SNP genotyping analysis that we will be discussing in later part of the course in much details where we are discussing PCR based mutation analysis. Next disadvantages of course, when something is so good, but the mechanism is so complex again there will be design challenges there will be expense challenges there can be inter molecular competitive binding all right. And there can be low signal levels why because the proximity of reporter and quencher are very high. So, we need good photo multiplier system, but once the amount of PCR product increases the amount of signals can be amplified all right.

Next sunrise uniprimer probe it is almost similar to molecular beacon the only difference see it looks almost same it has got a stem it has got a loop, but one the 15 polymer poly a tail is present in the one primer end all right. So, what will happen it is the probes are designed in such a way so that it binds to the poly t segment of a primer. So, in this case when you are using sunrise uniprimer probe one of the primers are designed in such a way that it has got a poly t tail where which binds to the poly a of the hair pin loop. Thereafter the whole thing opens up rest of the mechanism is same it will bind to the target specific complementary sequence in the PCR product and the quenching will be removed. So, in this case it has been mentioned that quencher dye is dapsil and the reporter dye is FITC which will fluoresce once the hair pin loop opens up all right.

So, this is a sunrise uniprimer probe. Next we discuss about hybridization probe they are actually this specific hybridization probe that we are discussing are actually FRET based binary probe FRET based binary probe. So, binary means there are two probes all right we will show the picture and you will be able to understand. So, there are two probes this is the 5 prime end and this is the 3 prime end this is the 5 prime end and this is the 3 prime end. So, there are two probes which are designed keeping in mind the sequence. So, suppose a short segment of sequence is selected and one short segment is complementary to the upstream and one short segment is complementary to the downstream.

Now, with these two probes they are attached with single different type of fluorophores in the 3 prime end of the first probe donor is attached and 5 prime end the acceptor dye is attached to the second probe all right. So, this is the phenomena. Now, what happens during real time PCR the excitation is performed with the specific wavelength and during the annealing step both of them will hybridize hybridize means annealing they are forming hydrogen bonds and this is the FRET based means it is the that using that mechanism where the proximity of the two dyes are leading to emission because we are

sending an excitation signal to the donor and we are programmed or detector in such a way that it can detect the emission by the acceptor. So, PCR probes actually both of them are aligning in head to tail arrangement and when they are close together the FRET is occurring and more and more emission which is actually proportional to the amount of increase in PCR products. So, this is the principle of hybridization probe which has got their advantage and disadvantages mainly advantages which are similar to other such fluorophore based probes the main disadvantages being we need to design specific probes because there are a this is a compatibility between donor and acceptor fluorophores and the donor and acceptor fluorophores need to be separately arranged to the two sequences.

So, there is a design challenge that remains the disadvantage. And the last variety that will be discussing are scorpions probe or scorpion primer. So, many text books say scorpion and many articles can also say scorpions ok. So, they are both the same. So, here this is the construct in which the probe and primer are again designed together right at the 3 prime end there is a primer and there is a PCR blocker as well. So, what do they do? Again the reaction mechanism remains the same this part whenever there is a loop there is a complementary sequence that can bind to the target area of interest upon annealing.

So, in the initial cycle the primer which is actually attached to the probe hybridization and the extension occurs due to action of polymerase alright. Remember this primer cannot be amplified in the other direction due to presence of a PCR blocker this hexagonal structure is PCR blocker. Now, extension will happen after the extension has happened and the next denaturing cycle the probe will open up and can bind to the complementary sequence that is the PCR product and again the fluorescent reporter and the quencher will be separated and there will be extension of fluorescence I mean emission of fluorescence alright. So, quenching will not happen once the fluorescent dye and quencher are separated. This is also hairpin loop this is a scorpions probe is named in such way because of the design which is proprietary.

So, these are the commonly used fluorescent probes among them we have discussed stack man molecular beacons, FRET probes, scorpions probes, hybridization probes there are few other probes which are expensive, but they are also used. And last part of our discussion will be discussing dyes we have I have extensively used the term dye, reporter and quencher. So, reporter dye is the acceptor dye which actually emits fluorescence and quencher dye is the dye that absorbs the quenching and prevents its emission. What does the reference dye do? This is very important it may not be mentioned because the reference dye do not actively participate in the PCR reaction, but instead it provides an internal reference there is a background emission which can be normalized during the data analysis. The very purpose of reference dye is to provide a background data for example, a blank reaction that we do in spectrophotometry and it

can be necessary used for correcting any fluorescence fluctuation because the fluctuations that are happening in reporter and quencher reporter dye will also happen in the reference dye and we can subtract and get the idea.

So, these are the few examples of reporter dye, quencher dye and reference dye that are commonly used in real time PCR system. So, that brings us to the end of our discussion and these are the summary that we have discussed today mind it real time PCR which will hence referred to as qPCR we have discussed the concept how does it differ what are the advantages from traditional PCR we have discussed the phenomena of FRET and quenching what are the various probes and chemistry principles and what are the dyes that are used in real time PCR. In the next class we will continue with the quantitation how it is done these are the few references these are the few important text books which are which I should always recommend to everyone of you to at least have the e book version mind it the additional not important because these are important basic facts which have not changed this is the earlier chapters in this book and you can browse these articles to have some idea. So, that is it for this class I thank you for your patient hearing I will continue with the next class. Thank you for your kind attention.