BioMEMS and Microfluidics
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Lecture – 15

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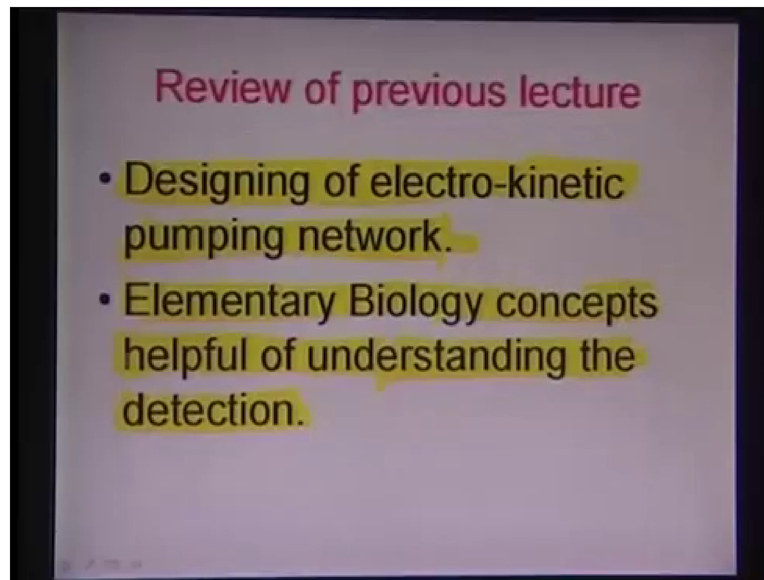

Hello and welcome back to the 15th lecture on BioMEMS. to a quick review of what have been done in the previous lecture. We talked about extensively in the designing of an electro-kinetic pumping network and then, started developing or discussing some initial concept of biology elementary level concepts, which are important for the understanding of the diagnostics especially clinical diagnostics technology.

So, essentially we started with the cell we discussed, what the basic unit of life that is the cell comprises of essentially it is a gelatin membrane, which actually contains a liquid cytoplasm and there are several organelles like the Golgi bodies and energy warehouses for the cell mitochondria the endoplasmic reticulum the protein warehouse so on, so forth. Inside floating around in this cytoplasm and center there is a distinct organelle called the nucleus, which contains an area called a region called chromatin, which essentially has a bunch of super coiled highly compressed DNA molecule, so the form of double y shapes are chromosomes.

We also discuses that you know the human body has about cells has almost all cells, which as 46 chromosomes except the productive one, which is 23 and the combine and fusion to develop 46 essentially. And we discuss some facts regarding sizes and scales of this species

for example, if you pull chromosome and try to lay out on a flats surface stretched DNA, which comes out to be chromosome is approximately 6 centimeters long and contains as I has about 510 to the power 6 base pairs a molecules inside it.

So, within started discussing about the elementary DNA structure and talked at length about the vaccine creak model, which is like a double helical twisted ladder kind of a structure. We discussed some issues regarding the nomenclature, which is used for understanding the direction of placement of the sugar phosphate backbone. So, there are five carbons in the sugar ring numbered 1 to 5 and the third carbon there is the phosphate as the linkage we joins one sugar molecule to the next in the backbone.

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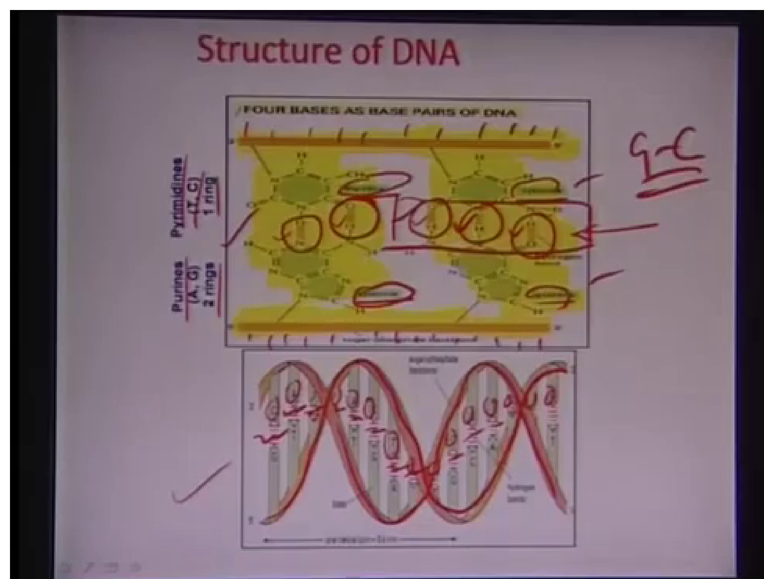

The fifth carbon there is a or on the fourth carbon there is a linkage the phosphate ions the linkage, which goes all the way to the you know to the sugar atoms. So, this is the way the sugar atoms are all linked together at the first carbon on every sugar is also bonded to a base and there are two such complimentary base pairs A and T adenine and thymine, cytosine and guanine. So, let us look at how these base pairs are clubbed in a structure.

So, this right here really is the DNA backbone sugar and phosphate and the molecules as illustrated at here are the base pairs. So, as you see here this is a thymine T and this is an adenine A, similarly the cytosine and guanine these are all chemically classified in to pyrimidines and this adenine and guanine are chemical classified as purines. And one more interesting fact there is a if you see the linkage between these base pairs on the thymine and

adenine are connected using two hydrogen bonds here and here, where as the, the cytosine and guanine are connected using three.

So, crisscrossing is not permissible, because it creates a thermodynamically unfavorable situation three bonds if clubbed together essentially with two active sides only one bond, which is dangling are vice versa. So, therefore, a cytosine can never bond to an adenine or thymine can never to bond to guanine it is only pair of this unique manner. So, that is a minimum energy configuration. So, there is huge negative charge on the backbone this phosphate linkage between the two sugar molecules as we illustrated in the last lecture.

Essentially, having a PO4 minus and the more the number of sugar molecules and more the number of the phosphate linkages and higher is the negativity of the DNA or the negative charges of the DNA. So, if you put this in a solution there is a tendency of attraction between the different moieties including the base pairs therefore, the DNA really coils up and bundles together and it remains in that form and until and unless it is force through may be a set of sieves using electronic fields external electric field etcetera.

So, you can see here in the figure below we talk about the actual structure the double helical coil here is coil as you see it goes in this twisted ladder kind of fashion and the connecting base pairs in between keep the strands integral and touch to each other and bonded to each other. And, so therefore, on the whole DNA molecule is nothing but, a bunch of sequences of these different base pairs and essentially the complimentary is defined by what sequence this base pair here as and, so therefore, DNA has really a very important advantage of a kind of self-assembling if you know one strand or one sequence in this particular molecule.

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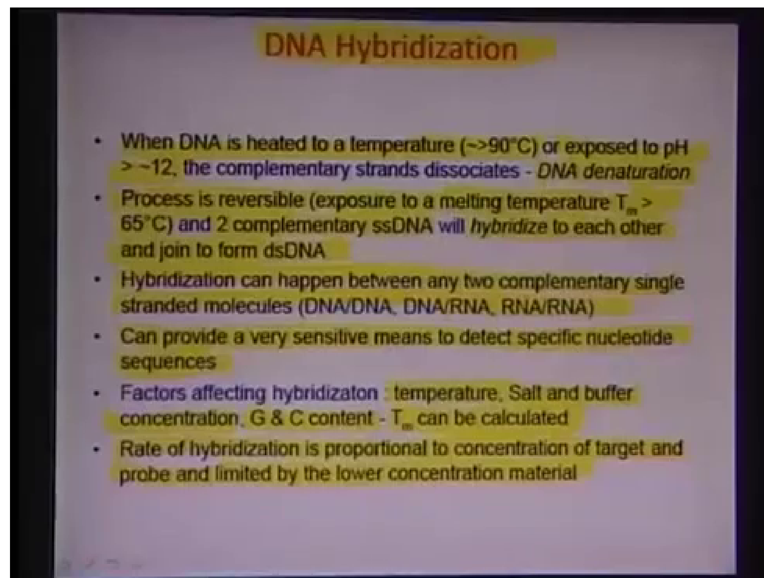

Let us talk about some other aspects of DNA, which is important for detection and diagnostics, what is DNA hybridization. So, essentially when a DNA is heated to about more than 90 degrees Celsius are even expose to pH of less than 12 or greater than 12 there is a tendency of this complementary strands to kind of split apart along hydrogen bond. So, there is a cleavage between the different hydrogen bonds in between the base pairs along this directions is if we look at these are the hydrogen bonds really and they would be tendency of cleaving the molecule away from each other, but cleaving these hydrogen bonds.

So, such cleaving can occur where you have such subjecting in DNA with that kinetic energy, which means that essentially you are providing free vibration to the different strands, which are bonded together thorough the base pairs the hydrogen bonds. So, the go above the certain frequency of vibration on the strand separate and another ways to put it in the pH solution extremely basic pH solution for it to kind of split apart. So, therefore, this is also known as DNA denaturation this closes of DNA splitting apart also known as DNA, DNA denaturation this process; however, is reversible.

So, therefore, once it happens other exposes to melting temperature of T_m greater than less than 65 degrees Celsius, but again less than 90 degrees would result in the two complimentary single standard DNA structure, so formulated joining back together. So, this we know as hybridization, hybridization is essentially the joining of two single standard DNA

molecules on a certain meting temperature, which is about 60 degrees are little more for the molecule to begin integral back to normal.

So, there is a development of hydrogen bounds between the base pairs and this strands typically join together to form a double standard DNA molecule. So, this is what hybridization essentially is further hybridization is happen between any two complimentary moieties like DNA and a DNA or DNA and RNA. The difference between RNA and DNA essentially is that RNA is a single standard structure and thymine group in a RNA is replaced with another base called the eurocell essentially that is what the primary differences between a RNA and DNA.

So, the hybridization can take place between two DNA's DNA and RNA or RNA and RNA and essentially all you thing, which is needed for the hybridization to happen, realize it is the complementarity, complementarity of the two joining strands. So, this really can provide a very sensitive means of detection this hybridization protocol and you can detect specific nucleotide sequences. So, essentially if you know what you are trying to detect or if you at least have a hunch of what you are trying to detect you could really design and exact complimentary sequence and try to hybridize your target, with the complimentary sequence.

If, so there is a in this process there is a hybridization, which happens and there is a effectively some kind of immobilization, which is taking place, because of this hybridize hybridization you can say that the target had the same sequence of the complimentary sequence of the sequence that you decided for. So, the flank which is there to capture is also known as the capture probe and the other portion, which kind of comes and binds of to the capture probe is the target DNA.

So, it is a very sensitive very specific means of detecting sequences on nucleotide enough, so extremely rapid. So, there are several factors, which effect this hybridization process variation in temperature salt and buffer concentrations and essentially the guanine cytosine content are solve the things, which would really vary this hybridization property logically that make sense, because you know more is a number of cytosine and guanine more is the triple hydrogen bonds.

So, the two base pairs guanine and cytosine as you saw before join with three hydrogen bonds and this particular illustration here it joins with three hydrogen bonds. So, therefore, more is the G C content or more is the guanine cytosine content in DNA more is a bonded

essentially between both this strands. And, so hybridization can more difficult and that melting temperature or the melting characteristic may change, because of the presence of the extra hydrogen bond in more abundant manner in such a molecular sequence.

Therefore, you know temperature, salt and buffer concentration and also the G C content can really be critical for finding out the hybridization rate and also of the melting characteristics of the particular target molecule. One more issue here is that the rate of hybridization has been found we propositional to the concentration of the target and probe. And essentially limited by the concentration of the lower material or a lower abundant the one which has a low concentration is limiting concentration.

So, therefore, hybridization can be characterized are effectively proportional to the concentrations of a both the species, which are binding the target molecular as well as the capture probe. And the limitation is a impose by the precious with the lower concentration with there is more number of targets and the capture probe hybridization will be limited by the capture probes all is there is more number of captures probes in the target hybridization is essentially be limited by you know the target. So, they won't be any binding after while, because target is unavailable, so that is what this process of hybridization as.

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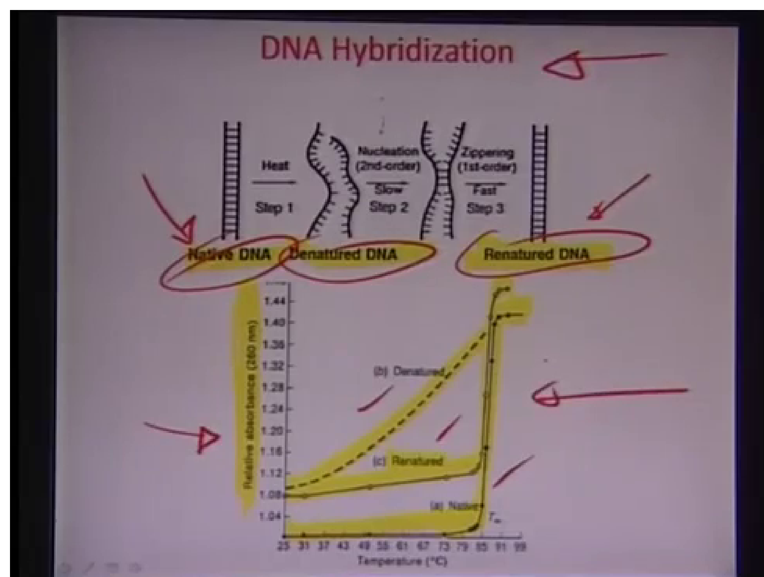

So, if you really look at some of these kind of relative absorbance strands at 260 nano meters you can see that the way that DNA would kind of go from it is native state in to the denatured state and then, again get renatured does change the relative observance of light at 260 nano

meters. So, this is the denatured case where you are just heating it and as you see here it with the arise in temperature there is a sudden change in the rate of rise of the relative absorbance around 90 degrees, which is also effective by the denatured temperature.

In the case of renatured DNA there is of almost all there is no absorption are almost very limited absorption are tell the temperature reaches, let say about 80 degrees of, so beyond, which there is a sudden rise in the relative absorbance. So, the way that absorptions to light behaves for this different processes is also to an extent different. For a native DNA this would happen somewhere around again I would 90 degrees of, so the absorption criteria would change might would happen quite towards the end of the spectrum almost around 80 degrees also that would be a sudden rise on the absorption.

So, you can actually have differential characteristics as you can see here of optical absorbance between this three states denatured, renatured and native state of hybridized DNA these characterize are important to for essentially a determining the hybridization process you have to understand here that the DNA molecule being extremely small about 20 to 30 Armstrong's in radius about a few microns in a length. You have to rely on the optical data or a like data is given here in this illustration for finding out the signatures and that can give some information what the state of the DNA is in essentially.

So, for DNA hybridization arrays it is critical that is characterizations done in advance and the calibration. So, obtain can be compare to the binding characteristics later on with any unknown target is our capture probes to determine what the target can possibly be in terms of sequences.

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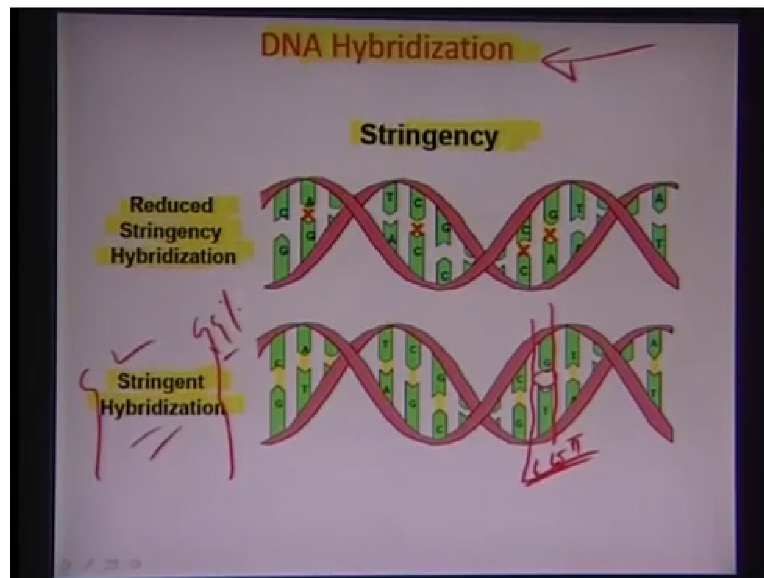

There are certain other issues that I would like to discuss here about hybridization, so hybridization essentially is not a full proof process there may be illustration, where there is a stringent binding like for example, here if you look at this particular figure here you have almost symmetry based on pairs supposed to each other like for example, C and G are binding and T R binding E and A R binding this is C G, this is GC, this is C G this is AG and a T, so they do not bind.

So, essentially this is something that is they are in the molecule by virtue of expression, where does not bind. So, here there is essentially will not be any bond, which is developed similarly the A and T here would again be something, which is remaining. So, here they would be a bond let me just erase this for convenience. So, there is no bond in the G T case you have another A and T here at they would be a bond.

So, essentially as you are seeing here most of the base pairs facing each other complimentary and they would do the binding, where there is only one base pair here, which is a non complimentary and there won't be a any characteristics binding in this particular region of the guanine to thymine binding does not exist. So, therefore, still it is very, very stringent very, very perfect stitching process or hybridization process as suppose to this other case here on the top, which shows about reduce tendency case, where as you see there are lot of base mismatches.

So, there is A and G in this region, which is a mismatch there is C and C region, which is again a mismatch again C and C are again A and G.. So, there are, so many mismatch in such a particular situation the DNA still binds, because the number of bonds, which are holding it together are more probably the number of bonds, which are not getting formulated, because of and non-complimentary base pairs facing each other. But, again the forces given by the binding pairs of problem much, much more, so that the DNA can still be help together.

But, the problem here is that it is not that stringently bond bonded to you know both the flanks are not very stringently you wanted to each other as in the case of the highly stringent case hybridization. So, it is desirable that you know specially for the read out aspect as well when you are trying to decide for, what is there on target by looking at the capture probe that we go for a stringent hybridization case, where almost 99 percent of molecules to bind to each other because there is exactly complementary of each other.

So, therefore, this is something which is very important to designing hybridization generates the focus of the target of the capture for really should be able to have highly stringent hybridization process for the sake of clarity and fast readout.

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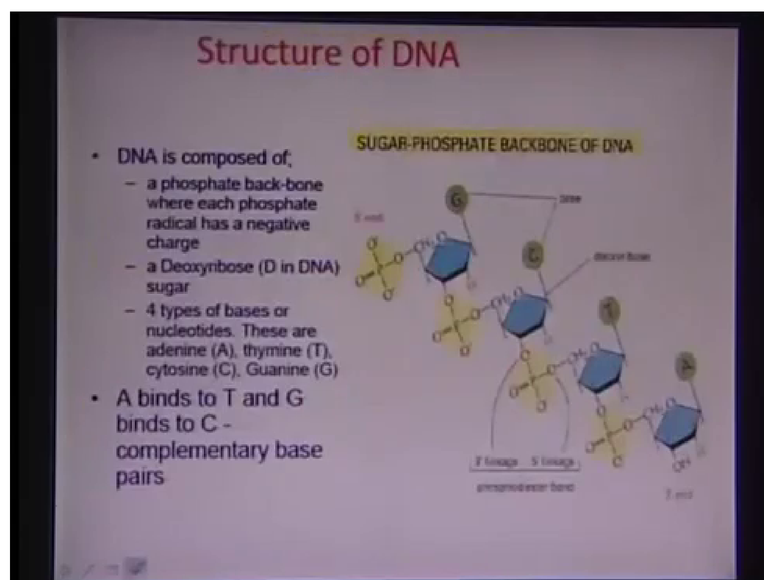

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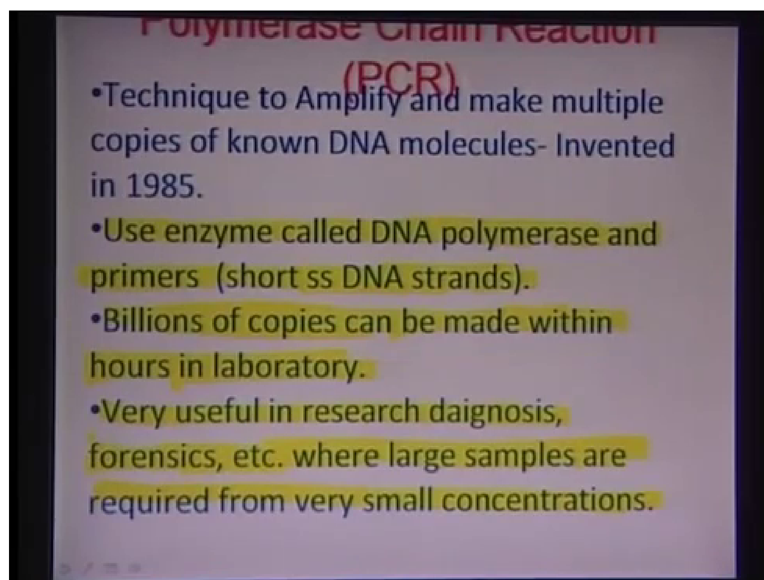

So, there is another you know kind of very interesting phenomena with DNA, which was invented way back in about 1985 this is known as the polymerase chain reaction. So, essentially this also process, which was invented by scientist by the name of Kary Banks Mullis and he was applied he is essentially awarded the noble price for this discovery this reaction is really something, which is able to give a breakthrough I would say in field of molecular biology and identification especially diagnostics.

We look at what this reaction is, but before starting it let me just retreat some concepts of sensing mechanism and sensors. So, one of the interesting aspects of a senses is how to amplify of a week signal. So, when we a talking about certain sequence of bases in DNA it is essentially bunch of may be few molecules and if attaching normal probes are you know we are actually by probes what I mean is a fluorescence probes or we are attaching detection mechanism, which is very, very normalized without really very high sensitivity.

Then, there is really a problem of identification of the signal. So, therefore, it is important to enhance the signal, so that we really get a very detailed very good data for confirming whether DNA with the certain base pair sequence are present on sample of interest. And, so it is almost in the interest of a proper sensing to develop amplification protocols for the signal. One way of amplifying would be just to take the protons generated by something like florescence using a probe and then, trying to kind of collect this, amplify this and go ahead you know with the amplified signal.

Another way could be that if I can somehow chemically copy the base pair sequence in-vitro; that means, outside in pretty much similar manner as it happens within the cells then thus that can be a great advantage, because essentially we are coping certain size certain region the DNA sequence, which is unique to a particular organism or a particular cell of interest and therefore, the idea would be that you take particular sequence and try to chemically use certain steps.

So, that we can exactly copy identical sequence is make multiple copies that where the signal can be increase. So, that is what the PCR reaction is if you look in to the way that cells replicate and divide essentially this is what happen that DNA zips down or zips open. And then, there is replication of the individual daughter strands, which actually get double the number and they are exactly split it to two daughter cells during the cell division process.

So, the same chemistry or the same mechanism can be used in in-vitro as well and, so Robert Mullis, the first proposed this PCR are polymerase chain reaction. So, the issues which are important here are following one is that the amplification process is driven by an enzyme called DNA polymerase and there are also identification sequences called primers, which are short single standard DNA strands and you put that in the solution at the very beginning before starting the chemical reaction I will just about a minute, so schematic would give you better understanding what this primal are, what they do really.

Some characteristics in PCR proposes the billions of copies of DNA molecules can be made within about hours in laboratory and essentially it is very useful in the reaches diagnosis forensics, where large samples are required from small concentration. And other interesting when I got mention is the area of forensics during investigation protocols from just blood stain of a patient are the person how was actually conducted the crime it is important from that little stain to decide for information about the finger printing aspect of the person it gives an idea who the person is essentially.

So if you look at that aspect PCR is essentially immensely vital, because how do you take stain, which is probably there for the next to the last few hours after any such criminal event has happen how do you detect from small amount of sample, which is the finger prints of essentially the people who are there on the sight of the crime are about the entity of the person who are the suffered who is the victim. So, forensic science really relies a lot and PCR

as well apart from diagnostic science for this techniques. So, let us look at what this interesting technique is really.

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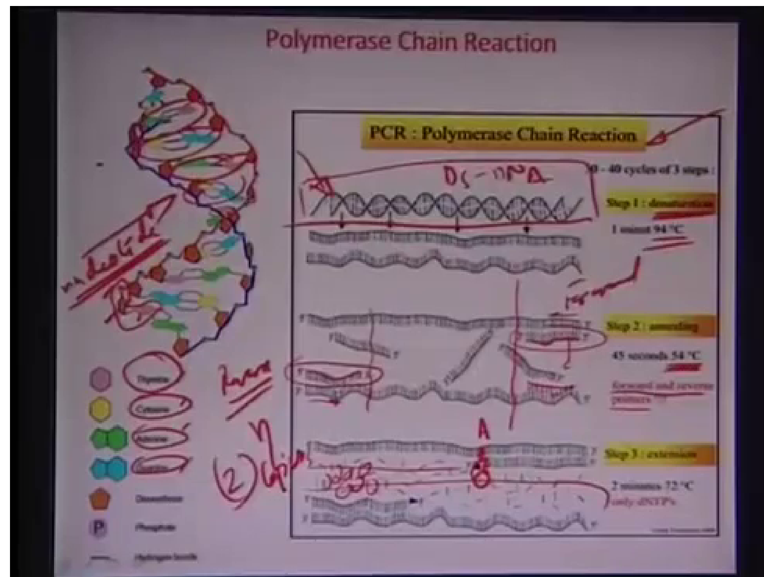

Essentially, it is a three step thermal cycling process which does the following. So, the first step if you look at, so again I would just like to retreat this is what a DNA structure is really looks like it is like a double helical structure with base pairs which are connected in a complimentary manner to each of the right. So, let us think that is that double helical structure here, which is kept in the solution with the bunch of different other chemicals.

So, the first step PCR reaction would execute is that basically denatures DNA by reading it the offer about one minutes of, so to temperate of about more than 90 degrees Celsius. So, essentially you are breaking open the hydrogen bonds and you are not doing that the cost of braking the sugar phosphate backbone you just simply speeding the two hydrogen bonds are the two base pairs the parts and the process separating this strands with the strands individually still remaining intact, but getting separated from each other that is denaturation.

So, the first step is denaturation and the second step you design these this intelligent identification sequences called primers. And there are two sequences here one with starts and binds at the forward direction and other in the reverse flank of the other gene or the other plane for interest of the other strand of DNA and essentially this gives you an identity and idea about the base pair the sequence DNA.

So, PCR is an effective process, because without looking any way into this particular flank and just by developing some identification complimentary strands 20 base pairs, 30 base pairs may be about 50 base pair long, which kind of binds to the sequence, which are there in the both planes of this DNA one in the let say one strand and other in the complimentary strands the reverse direction we have done. So, this identification is good now, for giving as an idea of what the sequence are on particular DNA molecule.

So, the primers is again kind of you go and kneel to the daughter strands, which have been formulated by denaturation and that happens in the certain temperature this temperature about 54 degree Celsius. And other very interesting factor here is that why is a needed to have two set of primal forward and reverse and what would happen really if we start doing this synthesis from same flank on both ends of the DNA molecule, so this is very interesting aspect.

If there are two complementary strands just opposite to each other the primers, which exact complementary of each other would bind the each other rather than getting bound to strand therefore, you have forward and reverse sequence and why both sequence are needed is that you have to really copy both the molecule. So, the idea is to be able to exactly duplicate the copy into two folds the number of molecules the originally represent will solution, so this is the kneeling step.

So, once the primer binds you have other moieties are other chemicals species within the, the chemical reaction are the parent solution, which does the remaining job. And this species which are there include the enzyme which is also called take polymerase there are these individual sequences called DNTP's are essentially nucleotides and what nucleotide is really is one of these base pairs A T C G bonded to the first carbon of this sugar molecule and part of this phosphate.

So, essentially the base pair the sugar in the phosphate all together this is essentially what a nucleotide means. So, there are four nucleotides with this base pair varying between adenine thymine cytosine and guanine. So, there is a sugar phosphate thymine there is a sugar phosphate cytosine there is a sugar phosphate adenine and then, there is a sugar phosphate guanine these four base pairs are present in the solution. And then, there are these enzymes which would essentially be intelligent molecules bio molecules which would look at what is present on the DNA flank to be sutured.

So, if you find out that here we have the enzyme would try to pick up the T molecule and affects this here and the not only that it also tries to suture the phosphate linkage to the earlier sugar, which is already been sutured here. So, not only the enzyme picks up the base pair particular base pair the phosphate sugar in the base pair and suture it to the complementary base pair on the DNA. But, also sutures the phosphate connecting both the sugars the other sugar is fixed and the sugar is which is mobile is on the particular DNTP or the nucleotide.

So, that way you can actually get a whole coverage very rapid manner and the enzyme can suture quickly by adding the base pair you know, which joining at to the daughter strand and kind of bridging whatever has been left open and zipping up the DNA. Again, but mind you this new DNA molecule has been made from molecules individually present in the solution by help of the enzyme the Taq polymerase is also goes up to an extend of doing proof reading.

So, if there is some by mistake if there is something or which has been a wrongly sutured or there is an not a good enough match between the base pair which is there on the daughter strand and the particular DNTP that has been picked up. So, the Taq polymerase is always has this tendency of kind of proof reading and cleaving back the in completed or the wrongly sutured base pair back it to the solution. So, it is also a self-proof reading mechanism it is a very stringent process, because errors happen while copying DNA inside cells the rate of above 1 in 10 to the power 9 such events.

So, it is such as highly stringent such a highly accurate error free process and if it were to be a little more in the intensive we will have a lot more mutations and they would not be a stability in the way we have evolved over past. So, just because of the sturdiness of the DNA molecule we have been able to evolve in a manner without with minimum possible mutations, because of this error rate while copy is extremely loads about one in 10 to the power nine such suturing events.

So; however, once the enzyme kind of copies the DNA it zips up and makes two strands out of the one strand that had been or one DNA double DNA double stranded DNA that had been opened up here or denatured here. So, now, if you repeat this cycle for n times you should ideally get about two to the power n copies of the parent number of molecules, which were there in the solution and this is exactly the replica fun other there exactly coping the sequence that was there on the parent molecule. Thus essentially you have amplified the chemical

signal in terms of creating identical molecules throughout you know the particular chemical reaction process. So, now, let us look at some of the other aspects.

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So, essentially you have here you see double standard structure you are split it open and bonding it to primers. And then, you are completing it through an enzyme and these are on different temperature scales 90 degrees, 55 degrees, 70 degrees Celsius and then, from again you do repeat the cycle by heating it an denaturing bonding in to the primers again and then, you know trying to extended.

So, you have essentially again another denature and heal an extendure and this continues and as you see here there is a slow growth in the number of sequences and these are all the chemicals chemical signals which are amplified products made from suture DNA out of the template which was given by the original DNA. So, it is essentially contains information about the sequence and exactly copy the sequence in the in the following manner.

So, some take homes here are that primer essentially as sorts strands of nucleotides which are complementary to specific to target DNA which is to be amplifier hence the n sequence of sort regions of target DNA to be copied this and therefore, so now, how it is important in the diagnostics again very critical question. So, if I am able to somehow sequence a particular area define area in a certain region or the gene of a cell of interest and if that particular region is able to address particular entity or a cell of interest.

So, I know now that there is a database where we have a sequence of the DNA and we also have the name of the moiety which contains the DNA, so it addressing the different entities. So, you have let say certain materials cell which has a cretin region of the DNA which is unique to that particular cell if you have an another cell why you have another region of the DNA or a you know another sequence in the DNA which is very, very unique to why. So, it addresses the x y. So, on etc now the whole idea is that if I want to explore a particular sample for a target of interest I want to find out rather let say an fruit sample i have a certain bacteria x.

So, how do I do that? So, I essentially try to read if there is a sequence which was addressing at which has been found out to address x in the particular sample and for doing that you develop primers which can go and attach to the n flanks and forward one in the reversed direction and copy you start copying the DNA. So, if I put such primers there with exact complementariness on the first twenty sequences maybe on the either side of the known DNA sequence or the target DNA start sequence.

Then, we essentially put the primer and run PCR process and there is a signal growth because of the growth in the chemical signal growth in the number of copies I would be able to selectively say that the bacteria x or pathogen x that I was looking at or looking for is present in the, because the primer was design for that particular and it went there and its to that particular sequence of DNA and it successfully amplified how that can be possible without that sequence being present here.

So, this kind of yes no answer about the presence or absence of the particular pathogens the other protocols, which are develop substantially there has been lots of research in this area, where there are real time fluorescence based deduction techniques, which also talks about not only yes no type of answer about the presence or absence of species, but also about you know the intensity the concentration or the availability of that species of interest.

So, therefore, if a signal if signal is stronger then definitely it is an appropriate to assume that that the parent concentration of the DNA strand that we are looking that is huge number. So, there is a proportionality in the concentration of what you are detecting to the signal that you are getting from system. So, that in the nutshell is what primers do also DNA polymerases is an enzyme which takes nucleotides from the amphibian solution and starts to construct the complementary sequences and also you do need an adequate supply of this nucleotide.

So, this called DNTP's deoxy ribonucleic triphosphates it can be a dATP; that means, with an adenine group dCTP with this cytosine group similarly with a guanine group and with a thymine group. So, this is all four dATP dCTP dGTP and dTTP are mixed in a certain ratio and actually a certain equivalent ratio and this is fixed based on how many molecules is suppose to copy based on how many molecules is suppose to copy and also on an idea to they should never fall short.

So, the reaction should not be limited by the unavailability of these nucleotides are given in access almost always in the particular reaction. So, therefore, we are more or less now kind of to this PCR process now there are certain concentrations that we need to investigate for designing any PCR process and we will be actually doing the design here subsequent slide it is. So, essentially again the denaturation is the process of a cleaving the molecule there is a DNA loses the secondary structure hybridization.

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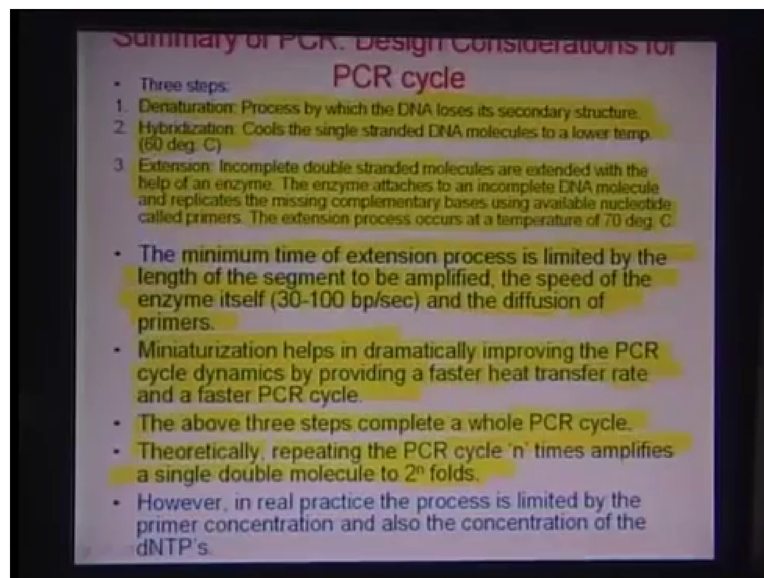

On the other hand, cools and the single standard DNA molecule lower temperature let us say 60 degrees of, so and the hybridize the primer and extension is a essentially you know when they incomplete double standard molecules are extended with the help of an enzyme that polymerase the enzyme attaches to the incomplete molecules and replicates the missing complementary bases using available nucleotide called primers the extension process occurs at a temperature of above seventy degrees also.

So, these three temperatures states are very critical and this essentially can be achieved in a thermal cycler, which is conventionally design in the manner where there is a huge aluminum block with the various through holes and essentially this is used as a thermal cycling modality for holding together with vials, the PCR fluid on it. And then, what happens the block is circulated at a different temperature points by using a Peltier cooler and resistance heater and the one of the earlier forms the PCR, where really PCR is such a process done using just dipping into different solution at different temperatures.

So, let say you have water at ninety degrees this water at again seventy or sixty degree. So, you keep on dipping this vial in different solutions PCR would be successfully executed, but the rapidity of diagnostics forced the technology to change towards an automatic temperature control system. Now, with the all the simple view one important aspect that micro scale devices have to all for as you have talk earlier is also the low thermal mass and also the greater amount of surface area to volume ratio heat transfer essentially is again surface area dependent property.

Therefore, it is a most amenable to assume that in such a case when there is a thin form. Let say fluid in certain chamber and we are trying to oscillate the temperature are vary that temperature of that particular film of fluid you basically use you know are basically get a better efficiency then rather thermally cycling volume here is the surface area is a very, very high over the volume.

So, you are taking let say which was earlier in a vial on the top of the chamber and spreading it into a thin film and in the in the process the molecules have that film would be subjected to more amount of a heat then you know all clumping together in a droplet form in a certain while. So, therefore, micro chips have been designed off and on for doing this activity of polymerase chain reaction and there has been successful models which can generate amplification at a great speed and with great rapidity and accuracy by exploring the micro scale a micro systems design and architecture.

So, some design considerations for the PCR reaction itself is that if you look at time scales of the various cycles the denaturation the hybridization or kneeling or the hybridization or kneeling the cycle. Then, the processes really rate limited by the way that the enzyme would be able to stitch the different base pairs make a completed DNA strand and process is really

the slowest type of the whole PCR process denaturation almost always occurs the primer binding also does not take much time in the extension does take the lot of time.

Because, it is actually the enzyme which goes over a certain base pair tries to read, tries to see there is complementary arounded and then tries to make a hydrogen bond of formulate a bonds. So, it is a catalyst it does some catalyst such a catalyst and sutures the phosphate group on the sugar on the last sugar. So, therefore, it kind of zips first of all makes the zips and slowly starts the zipping the DNA. So, that' why it is a slowest step of the process.

So, normally the minimum time of extension process is limited by the length of the segment to be amplified the speed of the enzyme itself is about thirty to hundred base pair per second and also the diffusion of primers are important aspect in a PCR reaction. So, therefore, the enzyme can suture only at a rate of about thirty to maximum about hundred base pairs per second.

So, the maximum speed the enzyme can actually go a kind of do the suturing action is about hundred base pairs in one second and the way that the primers would diffuse and essentially all chemical reactions are to a majority to a major extend limited by the diffusion step on diffusion rate. So, here we have to ensure that the primer really goes and hits the right molecule which has just been denatured and it is just ready for kneeling the primer on to it.

So, the primer has to really diffuse to the whole mass and just because that is thermally cycling process there are conductive currents, which are there in the medium which could help or aid this position diffusion, but still it does have some rate limitations. So, that is another aspect. So, there are two aspects one is the enzyme the rate at which deduced and then other aspect is a diffusion of primers in the other like human the DNTP’s or ionic transport across the solution which maintains certain pH, so and so for miniaturization.

So, the miniaturization definitely helps in grammatically in moving the PCR cycle dynamics by providing a fast and heat transfer rate as we have already talked about and. So, therefore, there will be almost always a faster PCR cycle time, because of this miniaturization and above three steps complete a whole PCR cycle. So, repeating these steps for n number of times would essentially theoretically mean repetition of a single molecule to the 2 to the power n fold.

So, essentially repeating the PCR cycle ten times amplifies the single molecule two to the power n holds of the same molecule in the same sequence identical copies the question is happen this way for if you looking into the way that diffusion kinetics works that is a major problem. So, therefore, reaction exploit the set at the initial phrase because the primers are not really diffuse to the point where they suppose to the DNTP's also are not very well diffuse.

So therefore, that is a question of delivery rates of these different moieties to the exact sight of their suturement and because that the limitation in real factor the PCR process is flow at the very outside it picks up the man after while, because of the exhaustiveness of the, because of the exhausting of the concentration of one of the most species in the solution it falls down back again. So, therefore, in real practice somehow process is limited by the primer concentration also the concentration of the DNTP's and the diffusion rate, which it on the other hand cools and the single standard DNA molecule x.

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Amplification Factor (Γ)

- In practice there exists an amplification factor which is dependent on cycle no.

  $\Gamma = [1+E_{PCR}(n)]^n$ where n is the cycle no. and E_{PCR} is the PCR efficiency which is a function of cycle no.

So, for n < 30 cycles, $E_{PCR}(n) = 1$

for n > 30cycles, $E_{PCR}(n)$ drops

So, there is a important factor here call the amplification factor gamma which is of some importance in practice their existence simplification factor, which is also dependent on the cycle member. So, normal is 2 to the power n. So, amplification efficiency should be one. So, $E_{pcr}(n)$ here should be one that is not what happens and. So, therefore, this varies also is a function of the number of cycles. So, gamma is equal to $\Gamma = [1+E_{pcr}(n)]^n$ where n is cycle number C is the PCR efficiency, which is also a function of a cycle numbers.

Therefore, we see that for the initial few cycles let say for n less than thirty cycles, now Epcr is one it is was assumption that diffusion can taken place. In the molecules are distributed all though out the throughout the matrix of the chemical reaction and for number of cycles more than 30 cycles the Epcr drastically drops down. And this kind of platters growth rate for the DNA of a particular sequence. So, therefore, in the gamma here one plus Epcr efficiency PCR function of n it or n would 10 to 2 to the power n for n less than 30 and would be much smaller than this 2 the power n for a for a men value. So, it will be much smaller 2 the power n for n value more than 30 cycles.

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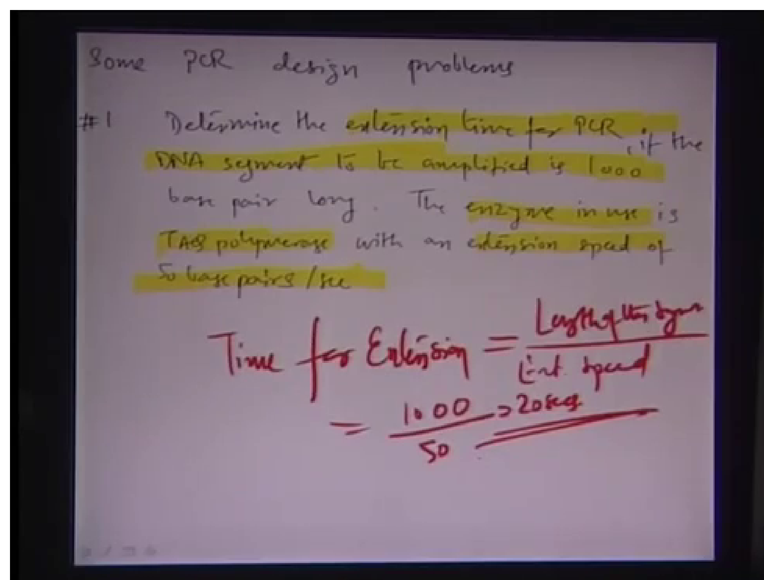

So, that is what in the dynamics of PCR processes, let us physically looks at for examples. Let say we want to determine the extension time for a PCR process and what is essentially given is that the statement to be amplified about 1000 base pair long and the enzyme and uses Taq polymerase, which is at the suturement rate and the extension speed of above 50 base pairs per second. So, the time that it would take really for on the extension is around 20 seconds. So, time for extension equals the length of the segment divided by the extensions speed that is about 1000 by 50 that is about 20 seconds.

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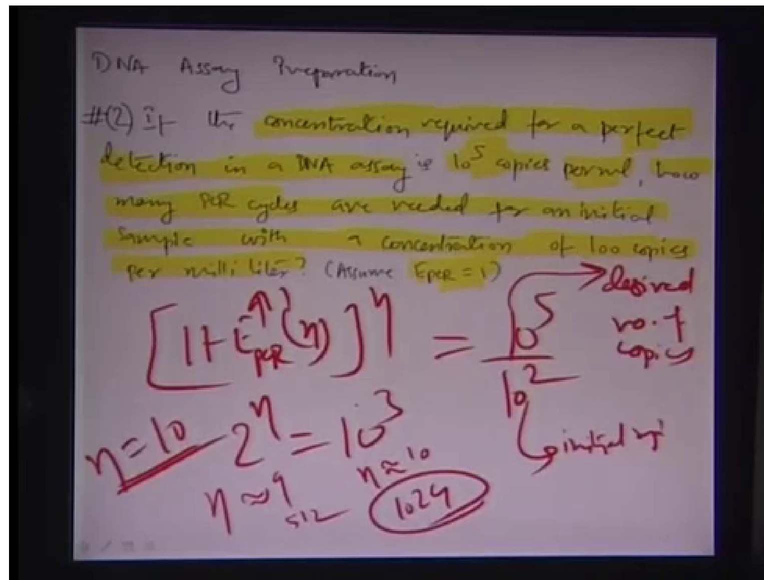

Let us do another example, where we are suggested that concentration that is required give me a minute here. So, the concentration required for a perfect deduction in a DNA is about 10 to the power 5 copies this was the final concentration of the DNA there should be PCR reaction should be and that is per mL, of the sample we need to find how many PCR cycles are needed. For an initial example with the concentration let say 100 copies per mL we have to assume any $E_{pcr}=1$.

So, we really write down this equation $\left[1+E_{pcr}(n)\right]^n=\frac{10^5}{10^2}$ where 10^5 is design number of copies at the end of PCR process and this is initial example Epcr being 1. So, this happens to be about 1000 therefore, the n can be accordingly determined. So, here therefore, if n equal to 9, then 2 to the power would be 9 about 512 a convert 10 about it is 1024.

So, essentially it is some between 9 and 10, so we can say that you know the number of cycles here is about 10. So, this concentration would reach and about 10 cycles or 10 thermal cycles, so this particular reaction.

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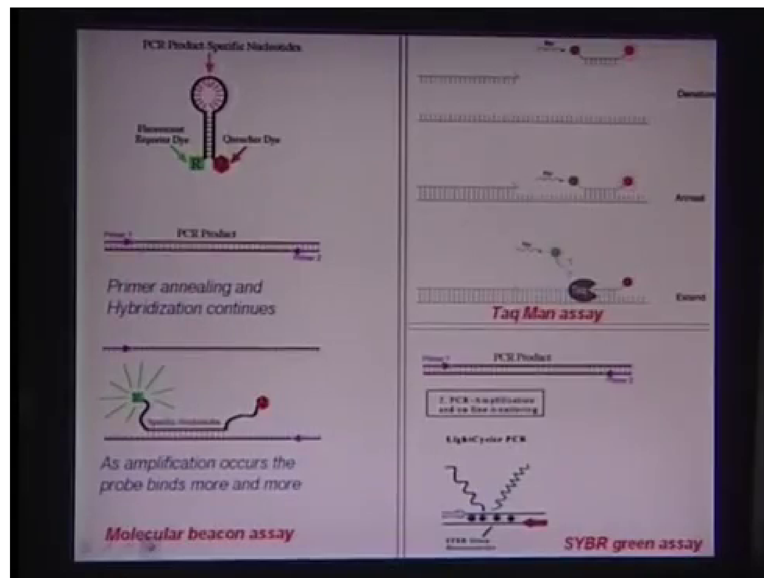

So, I would like to also introduce another very interesting area that once the DNA has been copied into several you know several different copies how do you read this signal really on the DNA how do you certain that the DNA file is the molecular state. So, either you have to get a characteristic spectra or you have to get some kind of signature which can show you terms of intensity growth or in terms of other factor that the DNA is increasing more than number of copies DNA is increasing.

So, there any deduction methods to establish these increasing signal one of them being fluorescence and essentially be florescence is again a physical phenomena where there is a tendency of getting a certain orbital configuration in a manner where in there is a transitions of a electron which takes place from a ground to exited state, but then the electrons comes back in a step.

So, it actually comes back to lower exited state first and then a bunch of different electrons all together come back on to the ground state and the very fact that it goes from higher to lower excited states it is nothing but, a energy loss and it is called loss it is essentially vibrational loss , because of the bond vibrations in a particular molecule. But, the same time what is interesting to know is that such orbital state is developed there is a continued high quantum yield emission from particular agent, which would develop essentially those states in this case or in the case of the DNA as you know the DNA structure is like a twisted ladder.

So, it is like a cage and there are certain fluorophore molecules, which kind of rime with the size of the you know the on the DNA also about two to three nanometers and they easily go and fit inside this cage. Now, they do not bond chemically the way they go is that there would go they would sit inside the cage and then there is the because of this presence of.

So, many sp 2 sp 3 orbital's in the especially in the base pair regions there is a tendency of formulation of this ground exited states and suddenly there is an increase in the quantum heal. In terms of emission such a configuration is exited using the excitation the excitation frequency of the particular die of interest it would emit at a very large rate at a high quantum healed and that would also determined you know the number of copies DNA molecule, which are their within this solution.

So, fluorescence essentially is the mean of determining this signal from which we can also interpret how many DNA molecules have a have been amplified of copy what is also important here to mention for me to tell you is a that fluorescence is definitely method of a certaining the real time kinetics of the reaction. So, as PCR the process happens it goes in goes on that the growth in amplification of the molecules on a real time basis or the fluorescence spectra does report, what is going on if there is a slow growth of the molecules it reports the slow growth of the fluorescence if there is a rapid growth in the mole molecules.

It reports an exponential growth of the if there is some kind of a plateauing action happening, because one or more of the react depleted in the overall reaction and other copies are not getting formulated anymore the florescence signal would also report the same. So, it kind of gives at real time snap shot from time to time of the whole PCR reaction in this is also known as real time PCR or q PCR, quantitative PCR process. So, let us look at some these different dies or how innovatively we can make a combination of the dies in the primers.

So, that the artificial process can be successfully executed, so there are several techniques, which have been cited of and on literature and lot of research existing or is going on this area, where in the developed different dies and also different configuration of this size or conjugation of this dies to the different constituents of the PCR reaction. So, what I can recapitulate or what we can actually tell at this time there are this point of time.

There are about close to four different techniques through, which fluorescence can be reported at the first technique is a just intercalation it is an essentially case where there is a molecule which would just go into DNA structure on start florescence the second case is you

know combination of essentially quenching of die molecule and these are bound two flanks of a primer. this technology also known as the TAQ man PCR or the TAQ man assay the third cases molecular beacon, where we talk about hairpin loop DNA, which has a fluorophore quenchers with exactly do the same thing by separating the two and getting a signal. And on the fourth case there is again some kind of a you know FRET technique of florescence reason is energy transfer technique where in two fluorophores are different wave lengths are excitation and emission and with some connection between the or what close by. So, bring them a part would change their emission characteristics. So, in the next lecture we would like to discuss some of these aspects and then do some device design for PCR micro chips.

Thank you.