Bio-Microeletromechanical Systems

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Module No. # 01

Lecture No. # 15

Hello and welcome back to the 15th lecture on BioMEMS – Bio microelectromechanical Systems. Let us do a quick preview of what had been done in the previous lecture.

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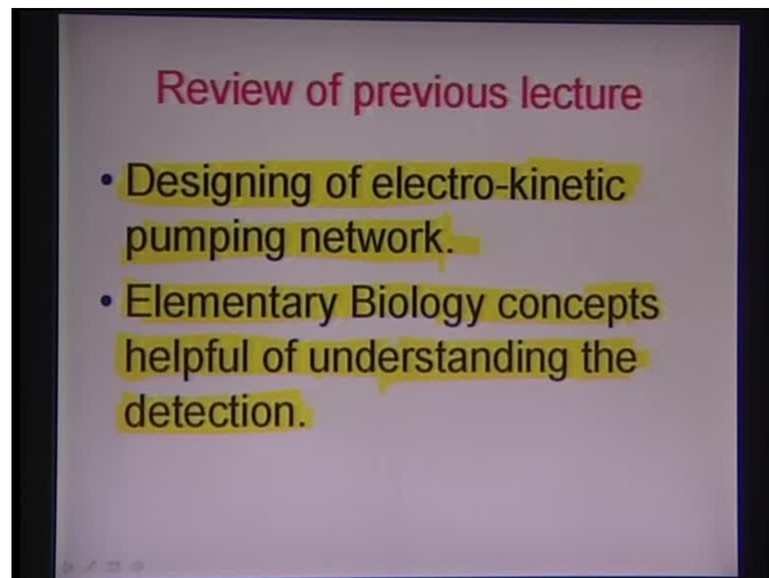

We talked about extensively with the designing of electro kinetic pumping network and then started developing or discussing some initial concepts of biology elementary level concepts, which are important for the understanding of the diagnostics especially clinical diagnostics technology. Essentially we started with a cell, we discussed what is the basic unit of life that the cell comprises of; essentially it is a gelatinous membrane, which actually contains a liquid called cytoplasm and there are several organelles like the Golgi bodies the energy have warehouses for the cell mitochondria, the plastic reticule of the protein warehouse so on so forth inside a floating around in this cytoplasm.

In center there is distinct organelle called the nucleus, which contains an area or region called chromatin, which essentially has a bunch of super coiled highly compressed DNA molecules in the form of double y shapes or chromosomes.

We also discuss that the human body - has about cells - has almost all cells which has 46 chromosomes except their productive once which have 23 and combined on fusion to develop 46 essentially. We discuss some facts regarding sizes and scales of the species for example, if you open up a chromosome and try to lay out in a flat surface stretch to DNA which comes out of a chromosome is approximately 6 centimeters long and contains about 510 to the power 6 base pairs of molecules in-sided. So, we then started discussing about the elementary DNA structure and talked at length about the Watson Crick model, which is like a double helical twisted ladder kind of structure.

We discuss some issues regarding the nomenclature, which is used for understanding the direction of placement of the sugar phosphate backbone. So there are 5 carbons in a sugar ring numbered 1 to 5 and the third carbon there is a ((phosphodised)) linkage which joins one sugar molecule to the next in the backbones and in fourth carbon there is a linkage the phosphodiester linkage, which goes all the way to the another sugar atoms.

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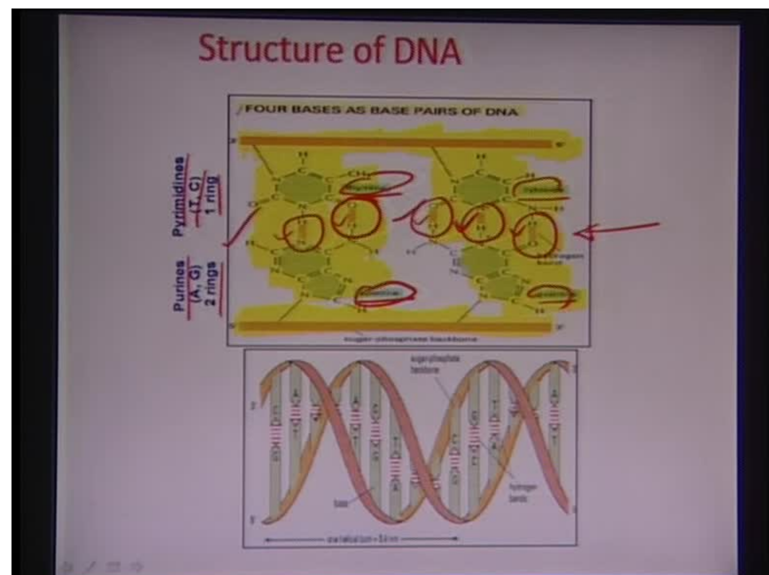

So this is the way the sugar atoms are all linked together and the first carbon on every sugar is also bonded to a base and there are two such complementary base pairs A and T adenine and thiamine, cytosine and guanine. Let us look at how these base pairs are

clubbed in structure. So this right here really is the DNA backbone made up of sugar and frustrated and the molecules as illustrated here are the base pairs; so as you see here (Refer Slide Time: 03:33) this is a thiamine T and this is an adenine A. Similarly, the cytosine and guanine these are all chemically classified into pyrimidine and this adenine and guanine are chemically classified as purines.

One more interesting factor is, if you see the linkage between these base pairs on the thiamine and adenine are connected using two hydrogen bonds here and here (Refer Slide Time: 04:00), whereas the cytosine and guanine connected using 3, so crisscrossing not permissible because it creates a thermodynamically unfavorable situation; three bonds if clubbed together essentially with two active sites would leave one bond, which is dangling vice versa. Therefore, a cytosine can never bond to an adenine or a thiamine can never bond to a guanine; it is only pair up in this unique manner, so it is a minimum energy configuration.

There is a huge negative charge on the backbone this phosphodiester linkage between the two sugar molecules as illustrated in the last lecture. Essentially having a PO 4 minus and the more the number of sugar molecules are more the number of the phosphodiester linkages and higher are the negativity of the DNA or negative charge of the DNA. If you put this in a solution there is a tendency of attraction between the different moieties including base pairs and therefore, the DNA really coils up and bunches bundles together and it remains in that form, until and unless it is forced through may be a set sieves using external electrical field etcetera.

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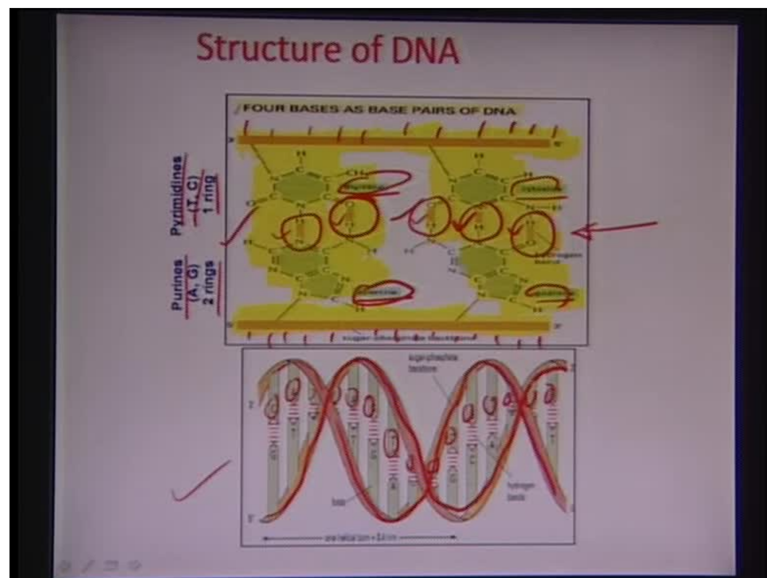

You can see here that in the figure below, we talk about actual structure the double helical coil; here is coil as you see, it goes in this twisted ladder kind of fashion. The connecting base pairs in between keep the strands integral touched to each other and bonded to each other. Therefore, the whole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

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DNA Hybridization

- When DNA is heated to a temperature (~>90°C) or exposed to pH > ~12, the complementary strands dissociates - *DNA denaturation*
- Process is reversible (exposure to a melting temperature T_m > 65°C) and 2 complementary ssDNA will *hybridize* to each other and join to form dsDNA
- Hybridization can happen between any two complementary single stranded molecules (DNA/DNA, DNA/RNA, RNA/RNA)
- Can provide a very sensitive means to detect specific nucleotide sequences
- Factors affecting hybridizaton : temperature, Salt and buffer concentration, G & C content - T_m can be calculated
- Rate of hybridization is proportional to concentration of target and probe and limited by the lower concentration material

Let us talk about some other aspects of DNA, which is important for detection and diagnostics. What is DNA hybridization? Essentially, when DNA is heated to about more than 90 degree celsius or even expose to a pH of less than 12 or greater than 12. There is a tendency of these complimentary strands to kind of split apart along the hydrogen bond, so is cleavage between the different hydrogen bonds in between the base pairs along these directions. If you look at these are the hydrogen bonds really and they would be a tendency of cleaving the molecule away from each other by cleaning these hydrogen bonds.

Such cleaving can occur were you have such subject in the DNA either to a kinetic energy, which means that essentially you are providing free vibrations to the different strands which are bonded together through the base pairs by the hydrogen bonds. They go above a certain frequency vibration and the strand separate and another ways to put it in a pH solution an extremely basically pH solution for it to kind of split apart.

Therefore, this is also known as DNA denaturation; this process of DNA splitting apart is also known as DNA denaturation. This process however is reversible. Therefore, once it happens as an the exposure to a melting temperature of a T m greater than let us say 65 degrees celsius but again less than 90 degrees would result in the 2 complimentary single stranded DNA structure, so formulated joining back together. This is known as hybridization. Hybridization is essentially the joining of two single stranded DNA molecules on a certain melting temperature, which about 60 degrees or little more for the molecule to be again integral back to normal; so there is a development of hydrogen bonds between the different base pairs and the strands typically join together to form a double stranded DNA molecule.

So this is what hybridization essentially is, further hybridization can happen between any two complimentary moieties like a DNA and DNA, DNA and RNA. The difference between RNA and DNA essentially is that RNA is single stranded structure and thymine group in RNA is replaced with another base the called euro cells. Essentially, that is what the primary differences between RNA and DNA.

So the hybridization can take place between two DNAs; DNA and RNA or RNA and RNA. Essentially all things which are needed for the hybridization to happen or realize is the complementarity of the two joining strands. This really can provide a very sensitive

means of detection, this hybridization protocol and you can detect specific nucleotide sequences.

Essentially, if you know what you are trying to detect or if you at least a hunch of what you are trying to detect, you could really design an exact complimentary sequence and try to hybridize your target with the complimentary sequence. In this process there is a hybridization which happens and there is effectively some kind of immobilization, which is taking place because of this hybridization. You can say that the target had the same sequence or the complementary sequence of the sequence that you designed it for.

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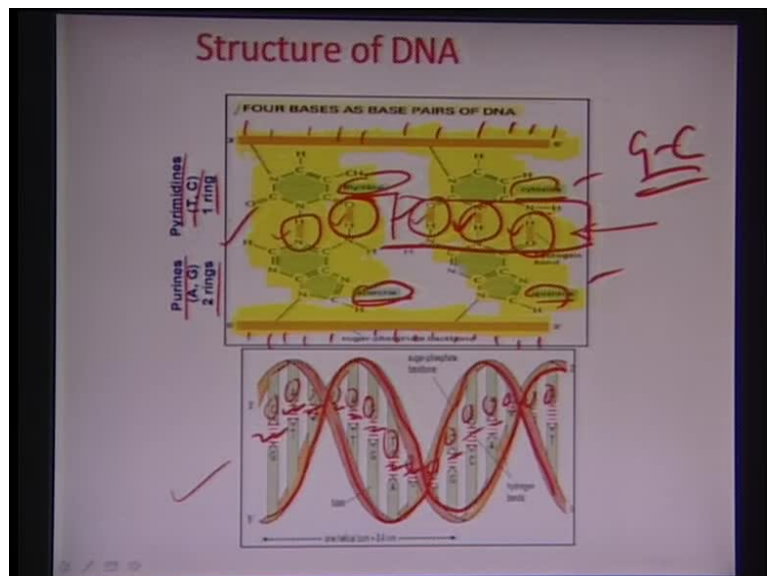

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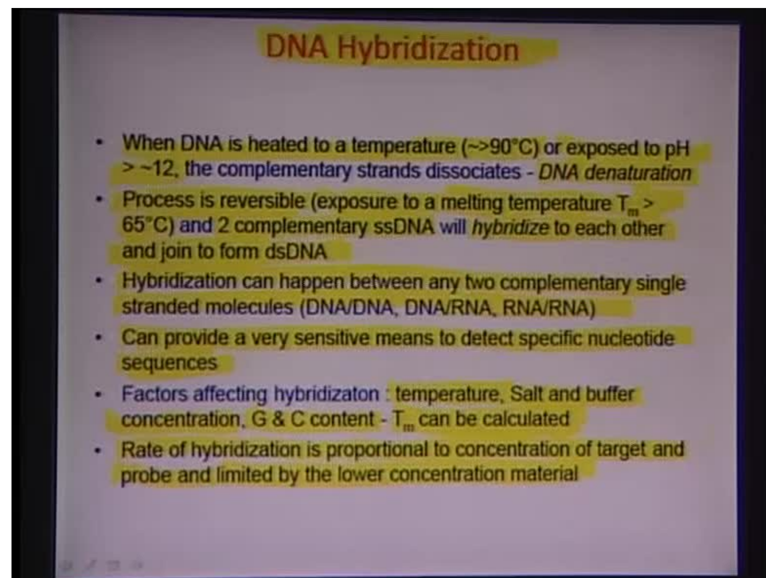

The flank which is there to capture is also known as the capture pro and the other portion which kind of comes and binds to this capture pro is the target DNA. So it is a very sensitive, very specific means of detecting sequences on nucleotide not so extremely rapid. There are several factors which affect this hybridization process: variation in temperature, salt and buffer concentrations and essentially the guanine cytosine content that is all the things which would really vary in this hybridization property.

Logically that make sense because more is the 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. In this particular illustration here, it joins with three hydrogen bonds. Therefore, more is the GC content are more is the guanine cytosine content in DNA, more is the bonded essentially between both the strands and so hybridization can be a little more difficult and the melting temperature or the melting characteristic may change because of the presence of extra hydrogen bond in a more abundant manner in such a molecular sequence.

Therefore, temperature, salt and buffer concentration and also the GC content can really be critical for finding out the hybridization rate and also the melting characteristics of the particular target molecule. One more issue here is that the rate of hybridization has been found to be proportional to the concentration of the target and probe. It is essentially

limited by the construction of the lower material or other lower abundant material - the one which has a lower concentration is limiting concentration.

Therefore, the hybridization can be characterized effectively proportional to the concentrations of the both the species which have binding the target molecule as well as the capture pro. The limitations imposed by the species with the lower concentration if there is more a number of targets in the capture probe, hybridization will be limited by the capture probes or if there is more number of capture probes in the target then hybridization essentially will be limited by the target. So, they would not be any binding after a while because target is unavailable.

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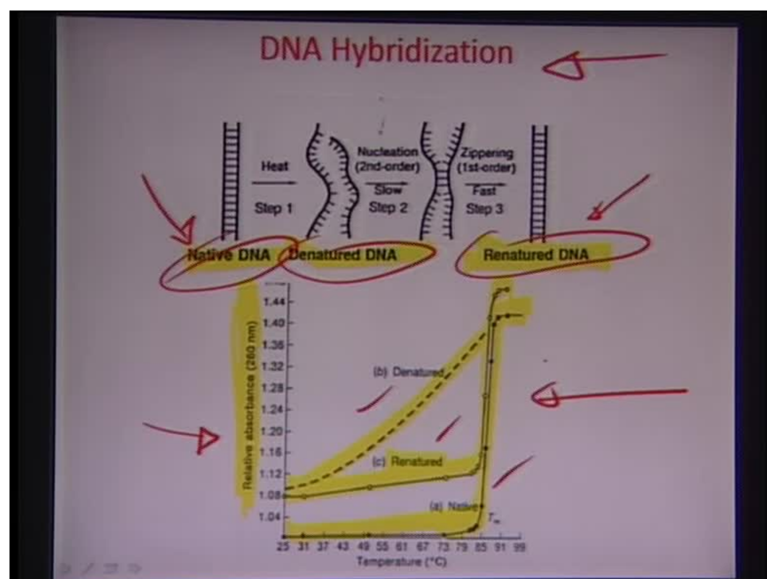

So that is what this process of hybridization is, so if you really look at some of these kinds of relative absorbance trends at 260 nanometers, you can see the way that DNA would kind to go from its negative state into the denatured state and then again get denatured, thus change the relative absorbance of light at 260 nanometers. So this is the denatured case, where you are just heeding it and as you see here at with the rise in temperature there is a sudden change in the rate of rise of relative absorbance around 90 degrees, which is also effectively the denaturation temperature.

The case of renatured DNA there is no absorption or almost a very limited absorption after the temperature reaches let us say about 80 degrees or so beyond which there is a sudden rise in the relative absorbance. The way that the absorbance to light behaves for

the different processes is also to an extent different. For a native DNA this would happen somewhere along again about 90 degrees or so that the absorption criteria would change but it would happen quite towards the end of the spectrum almost around 80 degrees or so there would be a sudden rise in the absorption.

You can actually have differential characteristics as you can see here of optical absorbance between these three states denature, renatured and native state of hybridized DNA. These characterized are important for essentially determining the hybridization process. You have to understand here that the DNA molecule being extremely small about 20 to 30 nanometers Armstrong's in radius about a few microns length.

You have to rely on the optical data or like data have given here this illustration for finding out the signatures and that can give some information about the state that the DNA is in essentially.

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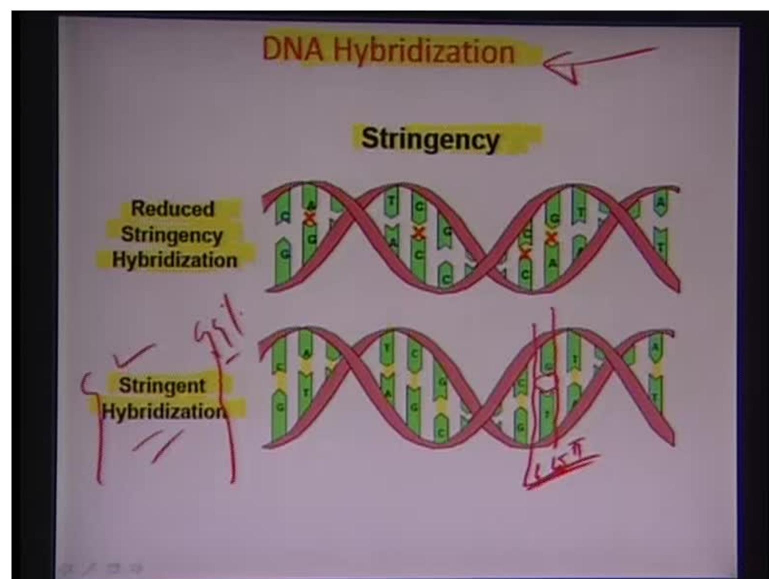

For DNA the hybridization arrays it is critical that these characterizations are done in advance and the calibration so obtained can be compared to the binding characteristics later on with any unknown target inside of capture probes to determine, what the target can possibly be in terms of sequences. There are several other issues that I would like to discuss here about hybridization.

Hybridization essentially is not a full proof process; there may be illustrations where there is a stringent binding like for example here, if you look at this particular figure here. You have almost complimentary base pairs a post to each other like for example C and G are binding (Refer Slide Time: 14:48) A and T are binding here, again T and A are binding this is CG, this is GC, this is CG, this is a G and T so they would not bind.

Essentially, this is something that is there in the molecule by virtue of and it is present were it does not bind. Here they are essentially will not be any bond which is developed. Similarly, the A and T here would again be something which is binding, so here there would be bond let me just erase this for convenience, so there is no bond in the GT case. You have another A and T here where they would be bond. Essentially as you are seeing here are most of the base pairs facing each other complimentary and they would do the binding; whereas, there is only one base pair here, which is a non complimentary and they would not be any characteristic binding in this particular region, as guanine to thymine binding does not exist.

Therefore, still it is a very stringent and very perfect stitching process of hybridization process. As post to this other case here on the top, which shows about a reduced 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 in this region which is again a mismatch, again C and C and again A and G.

There are so many mismatches in such a particular situation, DNA still binds because of the number of bonds which are holding together more probably and the number of bonds which are not getting formulated because of a non complimentary base pairs facing each other; but again, the force is given by the binding pairs are probably much more, so that the DNA can still be help together; but, the problem here is that it is not as stringently bonded to both the flanks are not very stringently the bonded to each other as in the case of the highly stringent case hybridization.

So it is desirable that in a specially for the readout aspect as well when you are trying to decide for what is there on a target by looking at the capture pro, that we go for a stringent hybridization case, where almost 99 percent of the molecules to bind to each other because they are exactly commentary of each other.

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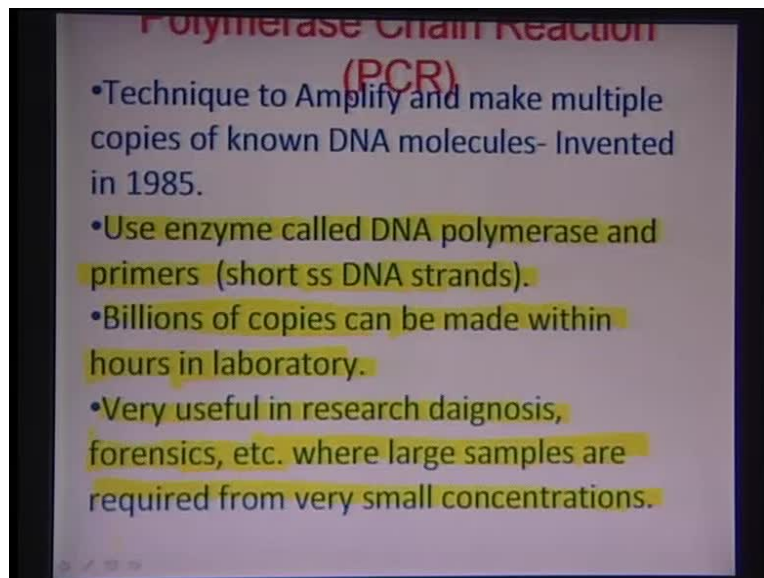

Therefore, this is something which is very important in designing hybridization arrays the focus of the target of the capture pro really should be able to have a highly stringent hybridization process for the sake of clarity and false readouts. There is another kind of very interesting phenomenon with a DNA which was invented way back in about 1985 and this is the known as the polymerase chain reaction.

Essentially, it is also a process which was invented by scientist by the name of Robe Mullis and he was essentially awarded the Nobel Prize for this discovery. This reaction is really something which is able to give a breakthrough, I would say in the field of molecular biology and identification especially in diagnostics. We look at what this reaction is but before starting it let me just retreat some concepts of the sensing mechanisms and sensors. So one of the interesting aspects of the sensor is how to amplify a weak signal?

When we are talking about a certain sequence of bases in DNA it is essentially a bunch of few molecules and if we are attaching normal probes or actually by probes, what I mean is fluorescent probes or we are attaching a detection mechanism which is very normalized without really very high sensitivity then there is really a problem of identification of the signal.

Therefore, it is important to enhance the signal, so that we really get a very detailed good data for confirming whether a DNA with certain base pair sequences present on a sample

of interest. So it is almost in the interest of the proper sensing to develop amplification protocols for the signal. One way of amplifying would be just to take the photons generated by something like fluorescence using a probe and then trying to kind of collect this and amplify this and go ahead 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 that can be a great advantage because essentially we are copying certain size or certain region of the DNA sequence which is unique to a particular organism or a particular cell of interest. Therefore, the idea would be that you take a particular sequence and try to chemically use certain steps so that we can exactly copy identical sequences and make multiple copies that way the signal can be increased.

So that is what the PCR reaction is, if you look into the way that cells replicate and divide. Essentially this is what happens, that the DNA zips down or the zips open and then there is the replication of the individual data strands, which actually get double the number and they are exactly split into two data cells during the cell division process. So the same chemistry or the same mechanism can be used in virtual as well.

Robert Mullis the first guide kind of propose this PCR 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. There are also identification sequences called primers, which are short single stranded DNA strands and you put that in the solution at the very beginning before starting the chemical reaction.

I will, in just about a minute show schematic which would give you a better understanding of what these primers are and what they do really and some characteristics of a PCR process is that billions of copies of DNA molecules can be made within about hours in the laboratory. Essentially, it is very useful in research diagnosis forensics, where large samples are required from small concentrations.

Another interesting and this I forgot to mention is the area forensics. During investigation protocols from just blood stains of a patient or the person who is actually conducted the crime, it is important from that little stain to decide for some information about the fingerprinting aspect of the person, it gives an idea of who the person is essentially. So, if you look at that aspect PCR is essentially immensely vital because how

do you take a stain which is probably there for the next the last few hours after any such criminal event has happened and how do you detect from that small amount of samples which is dried of the finger prints of essentially the people who are there on the site of the crime or about the identity of the person who has suffered who is the victim.

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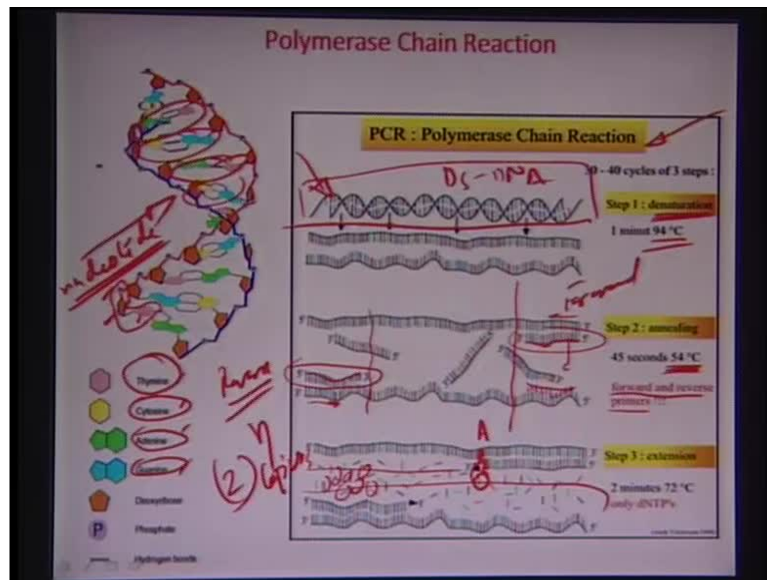

Forensic science really relies a lot on PCR as well or from diagnostic science for this technique. Let us look at what this interesting technique is really. Essentially it is a three step thermal cycling process which does the following. In the first step if you look at so again, I would just like to reiterate this is what the DNA structure really looks like; it is like a double helical structure with base pairs which are connected in a complimentary manner to each other.

Let us think that this is double helical structure here which is kept in the solution with a bunch of different other chemicals. The first step that the PCR reaction would execute is that it is basically denature the DNA by heating for about 1 minute or so to temperature of about more than 90 degree celsius. Essentially you are breaking open the hydrogen bonds and you are not doing that the cost of breaking the sugar frustrate backbone, you just simple splitting two hydrogen bonds or the two base pairs apart and the process separating the strands; the strands individually still remaining intact but, getting separated from each other that is denaturation.

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

The PCR is an effective process because without looking anyway into this particular flank and by just developing some identification complementary strands 20 base pairs, 30 base pairs, may be about 50 base pairs long, which kind of binds to the sequence which are there in both flanks of this DNA; one in the let us say one strand, another in the complimentary strand in the reverse direction we have done. So this identification is good enough for giving us an idea of what the sequences are on a particular DNA molecule.

The primers again a kind of go and kneel to the data strands which have been formulated by denaturation and that happens at a certain temperature; this temperature is about 54 degree celsius. Another very interesting factor here is that why is it needed to have two side of primers forward and reverse? What would happen really if we start doing this synthesis from the same flank on both ends of the DNA molecule?

So this is very interesting aspect, if there are two complementary strands at just the opposite to each other, the primers which are exact complementary of each other would bind to each other rather than getting bounded to the strands. Therefore, you have to forward and reverse sequence and why both sequences are needed is that you have to really copy the molecules. The idea is to be able to exactly duplicate copy into two folds, the number of molecules that are generally present in solution, so this is the kneeling step.

So once the primer binds, you have other moieties and other chemical species within the chemical reaction or the parent solution which does the remaining job and the species which are there include an enzyme, which is also contact polymerase there are these individual sequences called DNT piece or the essentially nucleotides. What nucleotide is really is one of these base pairs ATC or G bonded to the first carbon of this sugar molecule and a part of this phosphate. Essentially the base pairs, the sugar, and the phosphate all together this is essentially what a nucleotide means.

So, there are four nucleotides with this base pairs that in between adenine, thymine, cytosine and guanine. So there is the sugar phosphate thymine, there is the sugar phosphate cytosine, there is the sugar phosphate adenine and then there is a guanine; these four base pairs are present in the solution and then there are these enzymes which would essentially be intelligent molecules by a molecules which would look at, what is present on the DNA flank to be sutured?

So if it finds out that here we have an A, the enzyme would try to pick up a T molecule and a fixed this here (Refer Slide Time: 27:27) and 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 particular base pair, the phosphate sugar on the base pair and suit use it to the complementary base pair on the DNA but also suit use the phosphate connecting both the sugars; the other sugar is fixed on the sugar, which is mobile is on the particular DNTP or the nucleotide.

In that way, you can actually get a whole coverage very rapid manner and enzyme can suit you quickly by adding the base pair, you know joining it to the dater 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 solution by the help of the enzyme.

The tuck polymer is also goes up to an extent of doing proof reading. So there is by a mistake when there is something, which has been wrongly sutured or there is not good enough match between the base pair which is there on the dater strands and the particularly DNTP there has been picked up. So, the tuck polymerase always has this tendency of kind of proof reading and cleaving back the incomplete or the wrongly sutured base pair back into the solution.

So it is also self proofreading mechanism and it is very stringent process because the errors happen while copying DNA inside cells of the raid about 1 in 10 to the power 9 such events, so it is such as highly stringent, such highly accurate error free process. If it were to be a little more intensive, we will have a lot more mutations and they would not be a stability in the way we have evolved over the past, so just because of the sturdiness of the DNA molecule we have been able to evolve in a manner with minimum possible

mutations because of this error rate while copying is extremely loads about 1 in 10 to the power 9 such suturing events.

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 double stranded DNA that had been opened up here or denatured here. Now, we repeat this cycle for n times you should idle get about 2 to the power n copies of the pair in number of molecules, which where there in the solution and this is exactly the replica of one other; there exactly copying the sequence that was there on the parent molecule. Thus, you have amplified the chemical signal in terms of creating identical molecules throughout the particular chemical reaction process.

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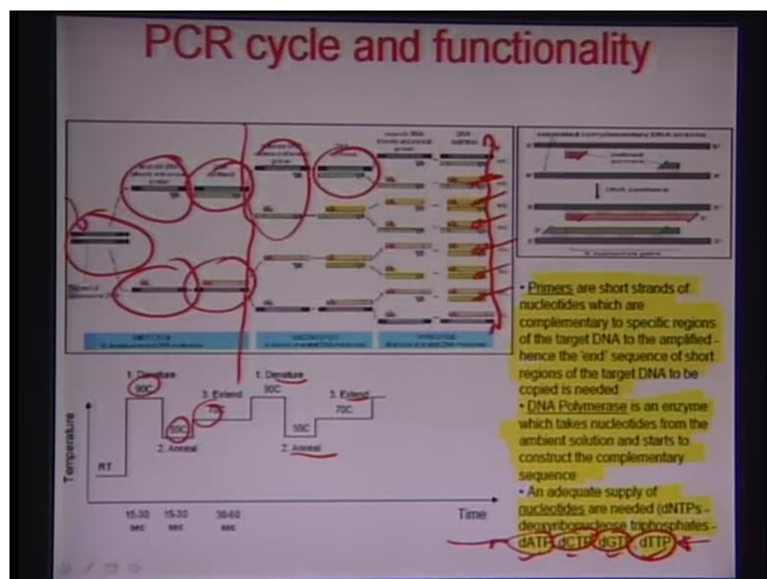

Now, let us look at some of the other aspects. Essentially, you have for here you see a double stranded structure you are splitting it open and bonding it to primers and then you are completing it through an enzyme. These are on different temperature scales 90 degrees, 55 degrees, 70 degree celsius and then from here (Refer Slide Time: 30:30) again, you do repeat the cycle by heating it and denaturing, bonding it to the primers again and then you know trying to extend it.

You have essentially again another denatured anneal on extend step and this continues as you see here there is a slow growth in the number of sequences. These are all the chemical signals which amplified products are made from sutured DNA out of the

template, which was given by the original DNA. It essentially contains information about the sequence and exactly copies the sequence in the following manner.

So, some they come here and the primary essentially is short stands of nucleotides which are complementary to specific regions of the target DNA, which is to be amplified. Hence, the end sequence of short regions of the target DNA to be copied is needed. Now, how it is important that diagnostics is again a very critical question. If I am able to somehow sequence a particular area, definitive area, in a certain region of the gene of cell of interest and if that particular region is able to address a particular entity or cell of interest.

So, I may 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 is addressing the different entities. You have a certain bacterial cell which has a certain region of the DNA which is unique to that particular cell. If you have another cell y, you have another region of the DNA or another sequence in the DNA which is very unique to y so it addresses the x y so on etcetera.

Now the whole idea is that, if I want to explore a particular sample for a target of interest I want to find out whether the food sample I have a certain bacteria x. How do I do that? I essentially try to read if there is a sequence which was addressing x, which has been found out to address x in that particular sample.

For doing that you develop primers which can go and attach to the end flanks - one in the forward direction, one in the reverse direction and start copying DNA. So, if I put such primers there with exact complementariness on the first 20 sequences may be on either side of the known DNA sequence or the target DNA sequence. Then, we essentially put the primer and run the PCR process and there is a signal growth because of the growth in the chemical signal or growth in the number of copies.

I would be able to assertively say that the bacteria x or the other pathogenic x that I was looking at or looking for is present in the surface because the primer was design for that particular pathogen; it went there and it search to that particular sequence of DNA and it was successfully amplified.

How that can be possible without that sequence being present here? So these kind of yes no answer about the presence or absence of the particular pathogens. There are other protocols which develops substantially as there has been a lot of research in this area where there are real time fluorescence based detection techniques, which also talks about not only in yes no type of answer about the presence of absence of a species but, also about the intensity or the concentration, or the availability of that species of interest.

Therefore, if a signal is stronger than definitely, it is appropriate to assume that the parent concentration of the DNA strand that we were looking at is a huge number. So, there is proportionality in the concentration of what you are detecting to the signal that you are getting from system. So that in a nutshell is what primers do.

DNA polymer is enzymes which takes nucleotides from ambient solution and starts to construct the complementary sequences and also you do need an adequate supply of the nucleotides are so called the DNTPs –Deoxyribo Nucleotide Triphosphates. It can be a DATP that means with the adenine group, the CTP with this cytosine group similarly, with the guanine group and with the thymine groups.

So these all four DATP, DCTP, DGTP and DTTP are mixed in a certain ratio and actually certain equivalent ratio and this is fixed based on how many molecules you supposed to copy and also on an idea as to they should never fall short. The reaction should not be limited by the unavailability of these nucleotides; nucleotides are given in access almost always in the particular reaction. Therefore, we are more or less now kind of quintet to this PCR process. Now, there are certain considerations that we need to investigate for designing any PCR process and we will be actually doing the design here subsequent slides.

Essentially again, the denaturation is the process of cleaving the molecule by a DNA loses its secondary structure. Hybridization on the other hand, cools the single stranded DNA molecule to lower temperature about 60 degrees or so and hybridizes the primer. Extension is essentially when the incomplete double stranded molecules are extended with the help of an enzyme tuck polymerase.

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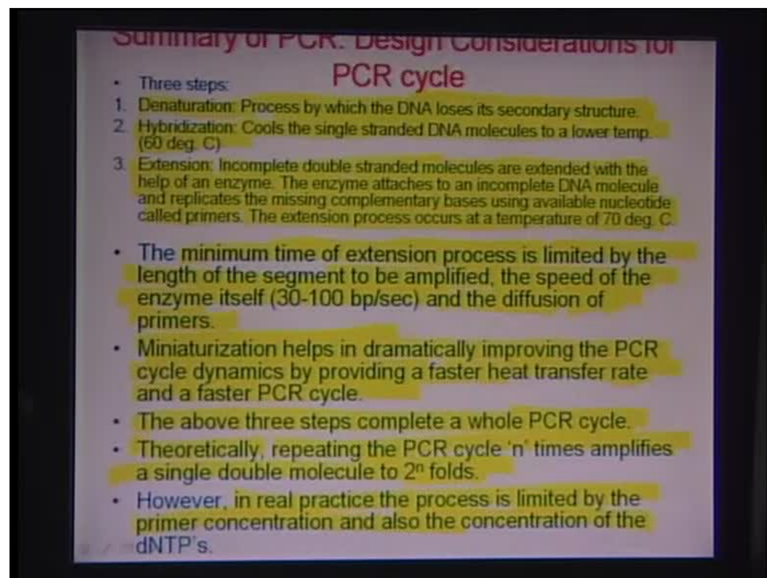

The enzyme attached to the incomplete DNA molecules and replicates the missing complementary bases using available nucleotide called primers. The extension process occurs at temperature of about 70 degrees or so, these three temperature states are very critical. This essentially can be achieved in a thermal cycler, which is conventionally designed in a manner, where there has a huge aluminum block with various through holes and essentially this is used as a thermal cycling modality for holding together some vial with the PCR fluid on it. Then what happens is that the block is circulated a different temperature points by using the paltrier cooler and resistance heater.

One of the earliest forms of PCR were really PCR is such a rigid process done using just dipping into different solutions or different temperatures. Let us say, you have water at 90 degrees, water at again 70 and 60 degrees. You keep on dipping this vial in different solutions and PCR would be successfully executed but, rapidity of diagnostics kind of first the technology to change towards an automated temperature control system.

Now with all this view, one important aspect that the micro scale devices have to offer as we have talked earlier is also the low thermal mass and also the greater amount of surface area to volume ratio. Heat transfer essentially is again a surface area dependent property. Therefore, it is the most amenable to assume that in such a case when there is a thin film let us say, a fluid in a certain chamber and we are trying to oscillate the temperature or vary the temperature of that particular film of the fluid.

You basically get a better efficiency than rather is thermally cycling volume. Here surface area is very high over the volume. We are taking a droplet which was earlier in a vial on the top of a chamber and spreading it into a thin film. In the process the molecules of that film would be subjected to more amount of heat, then all clumping together in a droplet form in a certain vial. Therefore, microchips have been designed often for doing this activity of polymerase chain reaction and there have been many successful models, which can generate amplification at great speed, and with great rapidity and accuracy by exploring the micro scale, micro systems the design in a architecture.

Some design considerations for the PCR reaction itself is that if you look at timescales of the various cycles the denaturation, hybridization or kneeling cycles. Then the process is really rate limited by the way that the enzyme would be able to stitch the different base pairs and make completely DNA strand. So extension process is really the slowest step of the whole PCR process. Denaturation almost always occurs, the primary binding also does not take much time and extension does take a lot of time because it is actually enzyme, which goes over a certain base pairs tries to read; it tries to see if there is complementary around it and then tries to make hydrogen bond or formulate the bonds. So, it is a catalyst and then suit use the phosphate group and sugar on the last sugar.

Therefore, it kind of zips, first of all make the zip and slowly starts zipping at the DNA. So that is why, it is the slowest step of the process. Normally, minimum time of extension process is limited by the length of the segment to be amplified. The speed of the enzyme itself is about 30 to 100 base pair per second and also the diffusion of primers is important aspect in a PCR reaction.

Therefore, the enzyme can suit only at a rate of about 30 to a maximum about 100 base pairs per second. The maximum speed there the enzyme can actually go kind of a do the securing action is about 100 base pairs in 1 second. The way that the primers would defuse and essentially all chemical reactions are to a major extent limited by the diffusion step of the diffusion rate.

Here, we have to ensure that the primer really goes and hits the right molecule which is just being denatured and it is just ready for reading the primer on to it. The primer has to really diffuse through the whole mass and just because it is a thermal recycling process

and there are convective currents which are there in the medium which would help or aid this process of diffusion; but still it does have some rate limitations, so that is another aspect. There are two aspects - one is the enzyme, the rate at which it suit use and then another aspect is the diffusion of primers on the other moieties are given the DNTPs or the ionic transport across the solution which maintains a certain pH so on so forth.

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

Essentially repeating the PCR cycle n times amplifies a single double molecule to 2 to the power n folds of the same molecule of the same sequence identical copies. Now the question is does it really happen this way? So, if you look into the way the diffusion kinetics works that is a major problem. Therefore, reaction is slower at the onset at the initial phase because the primers are not really diffuse to the point where they are supposed to the DNTP is also are not very well diffused. Therefore, there is a question of delivery rates of these different moieties to the exact site of their suiturement and because that is a limitation in real practice the PCR process is slow at the very outset. It picks up than an after a while because of exhausting of the concentration of one or more species in the solution it falls down back again.

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

Therefore, real practice somehow the process is limited by the primary concentration also the concentration of the DNTP's and the diffusion rate, which the molecules transport within solution. There is an important factor is here called amplification factor gamma, which is of some importance in practice; there exists an application factor which is also dependent on the cycle number.

Normally, 2 to the power n so amplification efficiency should be 1 so E PCR n here should be 1 that is not what happens; so therefore, this is also is a function of the number of cycles. Gamma is equal to 1 plus E PCR 10 to the power n, where n is cycle number. E PCR is the PCR efficiency, which is also a function of the cycle number. Therefore we see that for the initial few cycles, let us say for n less than 30 cycles the E PCR is 1. After assumption that the diffusion has taken place in the molecules are distributed all throughout the matrix of the chemical reaction. For a number of cycles more than 30 cycles the E PCR drastically drops down and this kind of plateaus the growth rate for the DNA of a particular sequence.

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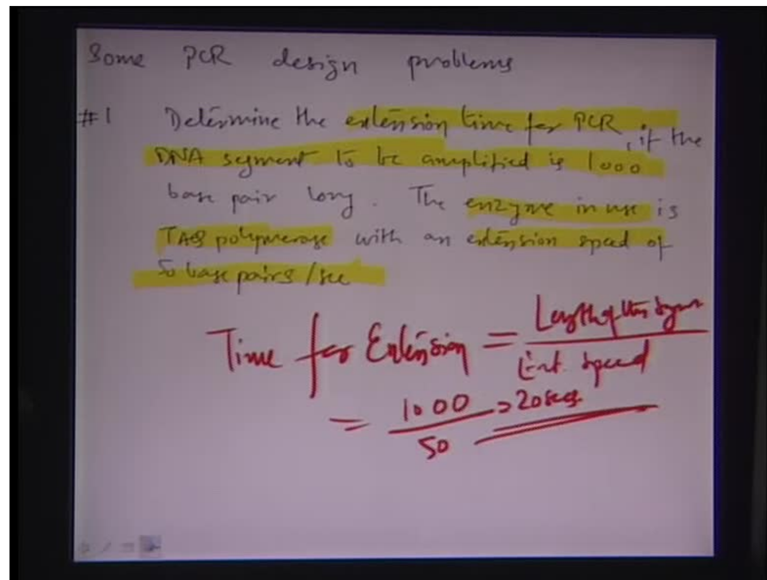

Therefore, gamma here 1 plus E PCR efficiency PCR function of power n would tends to 2 to the power n for n less than 30 and will be much smaller than this 2 to the power n for a n value; so it will be much smaller 2 to the power n, for n value more than 30 cycles. So that is what the dynamics of a PCR processes. Let us physically look at some examples.

Let us say, we want to determine of extension time for PCR process. What is essentially given is that the DNA segment of the amplifier is about 1000 base pairs long and the enzyme in use is TAQ polymerase, which is a suiturement rate and extension speed of about 50 base pairs per second. So, the time that it would take really for the extension is around 20 seconds.

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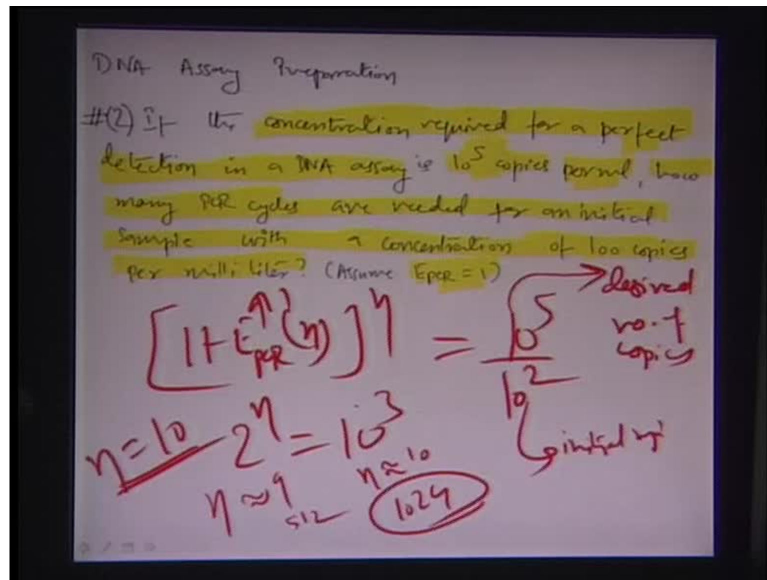

Time for extension equals the length of the segment divided by the extension speed that is about 1000 by 50 which is 20 seconds. Now, let us do another example wherein we are suggested that the concentration required for a perfect detection in a DNA says about 10 to the power 5 copies. So, this is what the final concentration of the DNA say the PCR reaction should be and that is per mille of a sample, we need to certain how many PCR cycles are needed for an initial sample with a concentration of 100 copies per mille, we are assume E PCR of 1.

We really write down this equation 1 plus E PCR is the function of n to the power n is essentially 10 to the power 5 by 10 to the power 2; 10 to the power 5 is the desired number of copies at the end of the PCR process and this is the initial sample in the PCR being one. This happens to be about 1000 and is therefore, the n can be accordingly determined, so here therefore n; if n is equal to or if n were 9 then, 2 to the power n would be about 512, if n where n is about 1024. Essentially, it is somewhere between 9 and 10. You can say that the number cycles here are about 10, so this consolation would reach about 10 cycles or 10 thermal cycles for this particular reaction.

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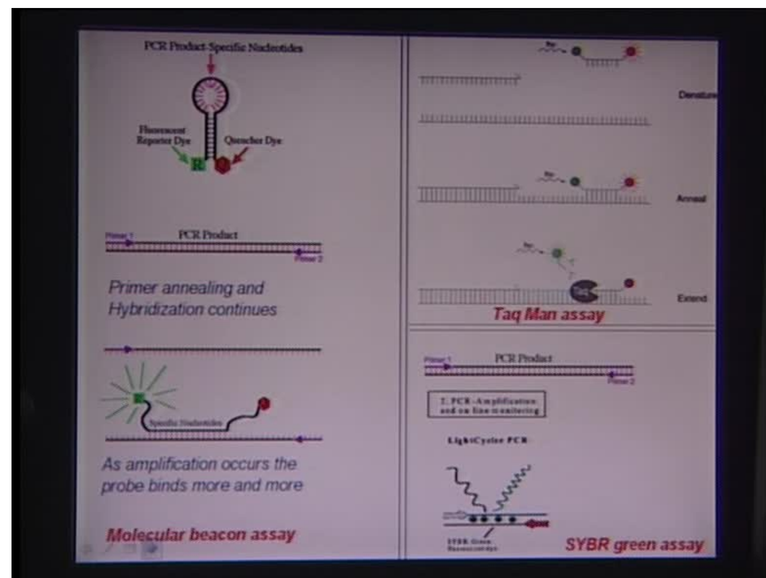

So, I would like to also introduce another very interesting area that once the DNA is being copied into several different copies. How do you read the signal really on the DNA? How do you ascertain that the DNA is amplifying it is in the molecules state? Either, you have to get characteristic spectra or you have to get some kind of an imprint or a signature, which can show you in terms of intensity growth in terms of some other factor that the number of copies of DNA is increasing.

There many detection methods to establish this increase in signal, one of them being fluorescence. Essentially fluorescence is again a physical phenomenon wherein there is a tendency of getting a certain orbital configuration in a manner, where there is a transition of electrons which takes place from ground to an excited state but, then the electron comes back in a step, so it actually comes back to a layer excited state first and then a bunch of different electrons all together come back onto the ground state and the fact that it goes from a higher to lower excited state is nothing but an energy loss and it is called phononing loss. It is essentially a vibration loss because of the bond vibrations in a particular molecule but the same time, what is interesting to know if there is such orbital state is developed to there is a continued high quantum yields emission from particular agent which would develop essentially those states.

In the case or the DNA as you know the DNA structures 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 helix on the DNA; so about 2 to 3 nanometer 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 because of the presence of so many SP2, SP3 orbital especially in the base pair regions and there is a tendency of formulation of this ground excited states and suddenly there is an increase in the quantum yield in terms of emission. Such a configuration is excited using excitation frequency of the particular dye of interest, if emit at a very large rate high quantum yield and that would also determine the number of copies of the DNA molecules, which are there within the solution.

Fluorescence essentially is the means of determining the signal from which we can also interpret how many DNA molecules have been amplified or copied. What is also important here to mention for me to tell you that the fluorescence is definitely a method of ascertaining the real time kinetics of the reaction. So, as the PCR process happens and it goes on and then there is the growth in amplification of the molecules. On a real time basis of the fluorescence spectra does report what is going on: if there is a slow growth in the molecules, it reports slow growth in the fluorescence; if there is a rapid growth in the molecules, it reports exponential growth in the fluorescence.

If there is some kind of a plateauing action happening because one or more of the reactants are depleted in the overall reaction and other copies are not getting formulated any more, the fluorescence signal would also report the same. So, it gives kind of real time snapshot from time to time of the whole PCR reactions; this is also known as real time PCR or QPCR quantitative PCR process.

Let us look at some of these different dyes or how innovatively we can make a combination of the dyes in the primers, so that the RTPCR process can be successfully executed. There are several techniques which have been sided of and on literature and a lot of researchers existing or is going on this area are where develop different dyes and also different configuration of this dyes to different constituents of the PCR reaction. What I can recapitulate, what we can actually tell at this time that at this point of time there are about to close to four different techniques through which forces can be reported.

The first technique is a just an intercalation, it is essentially a case where there is a molecule which would just going to a DNA structure and start fluorescing. The second case is combination of an essentially quencher and dye molecule and these are bound two flags of polymer. This technology is also known as the taqman PCR or taqman.

The third case is a molecule beacon, were we talk about hair pin loop DNA which has a floor for the quencher is exactly doing the same thing by separating the two and getting signal. In the fourth case, there is again some kind of a affect technique of the fluorescence resonance energy transfer technique where in two florofluores of different wavelengths of excitation an emission and with some connection between the two are brought close by so bring them part would change their emission characteristics. So in the next lecture, we would like to discuss some of these aspects and then do some devices design for PCR microchips.

Thank you.