Analytical Technologies in Biotechnology
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Module - 7
PCR, DNA Sequencing and ELISA
Lecture - 1
Polymerase Chain Reaction (PCR)

In this section, we are going to discuss a few more advanced techniques, which are widely utilized in the field of biotechnology, in different areas. These will include polymerize chain reaction, we call it PCR, automated DNA sequencing methods, and ELISA, that is Enzyme Linked Immuno Sorbent Assay.

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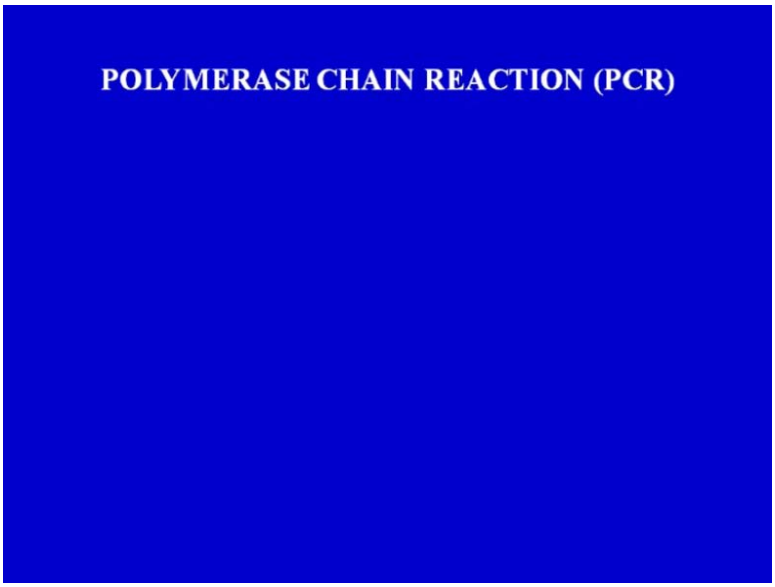

We will begin our discussion with polymerase chain reaction, now this technique was developed in 1983 by Kary Mullis for which he received the noble prize, in chemistry in 93. Now, PCR is a technique that takes specific sequence of DNA of small amounts and amplifies it to be used for further testing.

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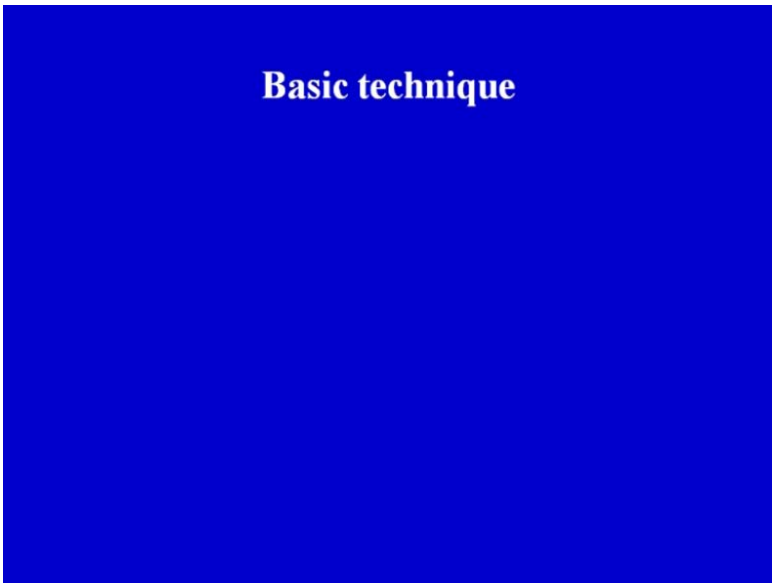

Now, basic technique here in very simple case is PCR amplification requires a small amount of nucleotide sequence at each end of the region to be amplified. So, whichever sequence you are going to amplify, you require knowledge of nucleotide sequence at each end of this region. Now, oligonucleotides complementary to that sequences are synthesized, and it is typically around 20 or so nucleotide long, these oligonucleotides are used as primers for enzymatic amplification.

Now, here a reaction mixture is setup containing a sample of DNA that includes the region to be amplified, the primers in large molar axis deoxynucleoside triphosphate or we call it DNTP's and a heat stable DNA polymerase, for the enzymatic synthesis. So, the mixture is heated to a temperature sufficient to melt or separate the strand of the sample DNA, remember that in cells this function is performed by an enzyme, that is helicase. But, here outside the two DNA strands needs to be separated, and that is called a DNA melting at particular temperature, they will separate from each other.

Now, after they separated they are cooled to a temperature, which is low enough for the primers to anneal to the DNA, that is at that two ends where in a particular region, which needs to be amplified. Then the mixtures incubated at a temperature sufficient for the polymerase to synthesize complementary strand to each piece of sample DNA. Now, starting with the primers and using the DNTP's provided, so the number of copies of the target region is doubled after this round of DNA synthesis.

The mixture is heated for strand separation, again cooled again for primer annealing and again incubated for another round of DNA synthesis. And likewise many cycles will be performed, so each time the cycle is carried out, there is a doubling of the number of copies of the target region between the primer annealing sites, after say 25 or 30 cycles there is theoretically amplification of over 10 raise to power 9 fold after 30 cycles. Now, applied even to a single DNA molecule of one kilobase pair that would generate one nano gram of DNA, so it is about sufficient to see in a gel or for any other application thereafter.

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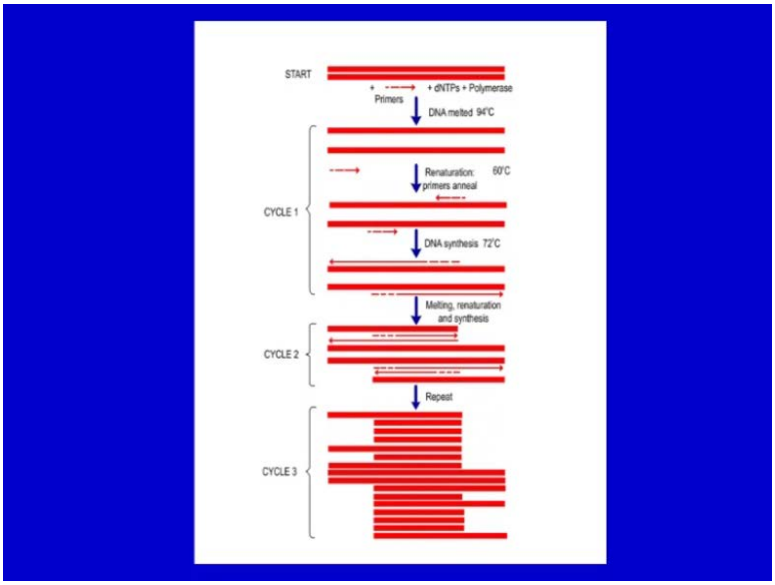

So, in very simple terms this figure explains the technique here, the simple technique, so in starting what you have is, you have a piece of DNA that is double stranded DNA and there are primers DNTP's and polymerase, which are mixed with in a tube. Now, the first step is the DNA melting or denaturation step, so the two DNA strands are separated here. Once they are separated then temperature is lowered to say around 60 degree Celsius, now this temperature will depend upon the melting temperature of the primers.

So, the primer will anneal at this temperature, which is little lower than the melting temperature of primer, and they will anneal at the right complementary place, so DNA synthesis then temperature again heated to 72 degree Celsius. And the DNA synthesis takes place, now at 72 degree around DNA polymerase will synthesize the complementary strands. Then again the cycle will be repeated many times and many

double stranded DNA amplified fragments will be obtained, through these three steps the DNA amplification or polymerase chain reaction will take place or at.

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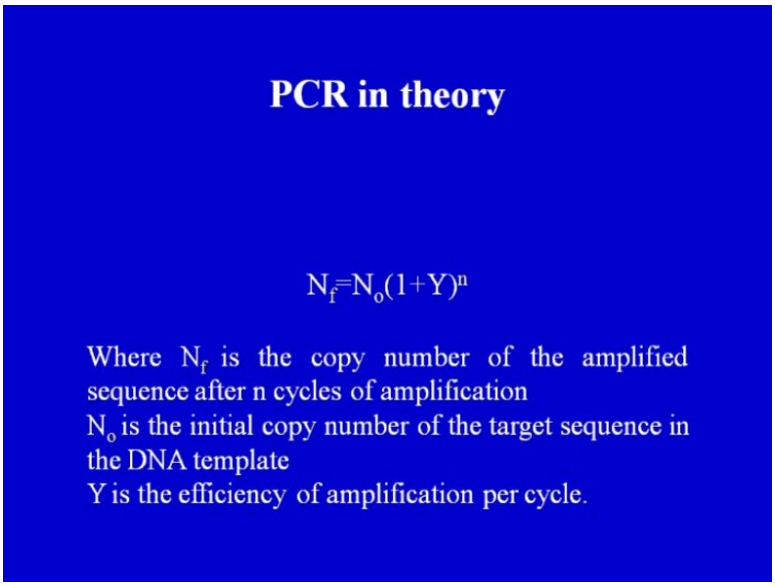

So, let us little bit get into details of the PCR technique, when amplifying a small segment of a large double stranded DNA template, the desired fragments first appear in the third cycle of PCR, as we have seen in the figure. The amplified products then accumulate exponentially as described in this equation, where you can see N f equals N 0 one plus y raise to power N. Where, N f is the copy number of the amplified sequence after N cycles of amplification, and N 0 is the initial copy number of the target sequence in the DNA template, y is the efficiency of the amplification per cycle.

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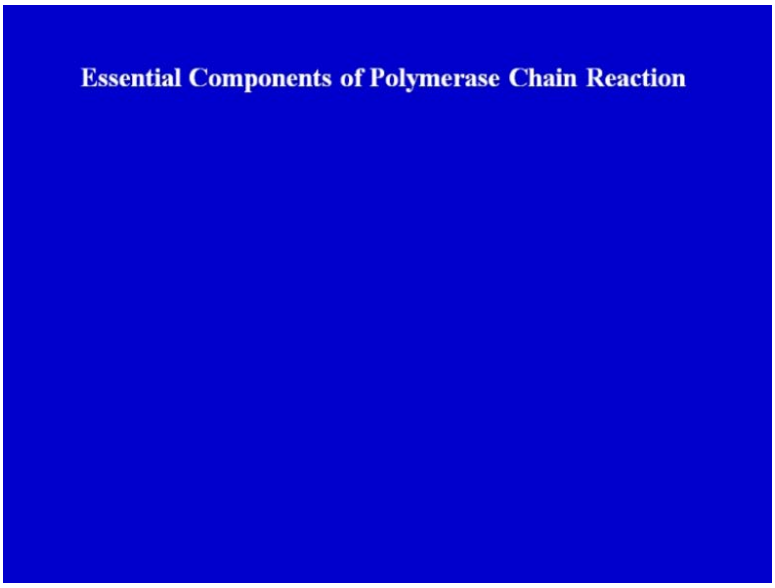

Now, what are the essential components of polymerase chain reaction, as we have seen one is thermostable DNA polymerase to catalyze the template dependant synthesis of DNA. Then a pair of synthetic oligonucleotides that is primers, then careful design of primers is required to obtain the desired products in high yield, and to suppress amplification of unwanted sequences, and to facilitate subsequent manipulation of the amplified product.

Then DNTP's which are required and standard PCR contains equimolar amounts of the 4 DNTP's DATP that is DTP, DCTP and DGTP, then there is a requirement of divalent cations, because all thermostable DNA polymerases require free divalent cations, you will usually magnesium for activity. They are kept in a buffer to maintain the PH like tris-c l adjusted to PH between 8.3 and 8.8 at room temperature is included in a standard PCR's at a concentration of around 10 millimolar.

Monovalent cations can are also included in standard PCR, PCR buffer like KCl, which is like works well for amplification of segments of DNA greater than 500 base spheres in length. Raising the concentration to 70 to 100 millimolar improves the yield of shorter DNA fragments, then you require template DNA, the template DNA containing the target sequence can be added in single or double stranded form, closed circular DNA templates are amplified slightly less efficiently than linear DNA's.

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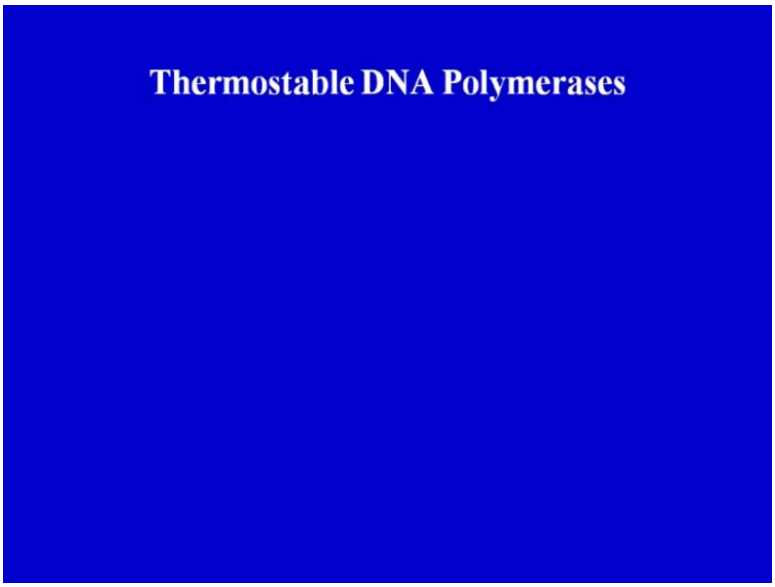

Now, let us discuss about thermostable DNA polymerase, the most common enzyme which is utilized in PCR is taq polymerase, it is a DNA polymerase isolated from thermophilic bacterium, thermus aquaticus. And this enzyme which the bacterium uses for cellular DNA synthesis has a temperature optimum of at least up to 80 degree Celsius, and is not readily denatured by the repeating, heating and cooling cycles. That are needed in the amplification process, it is generally produced by expression of the gene in e coli, and enzyme has a 5 prime to 3 prime DNA polymerase and 5 prime to 3 prime exonuclease activities.

So, it will polymerase about 50 to 60 nucleotides per second, how about the enzyme has a number of properties that may be disadvantageous, like taq polymerase has no proof reading that is three prime to 5 prime exonuclease activity. If a mutation happens in an early cycle then a large fraction of PCR products will have the altered sequence, then taq polymerase has relatively low processability, which places a maximum size limit of molecules that can be amplified using this enzyme, which is typically 2 to 4 kilobase pair or may be little lower actually.

Now, taq polymerase is not fully heat stable, it has the half life of about 40 minutes at 95 degree Celsius, which means that there will be significant loss of activity over the 30 cycles used in a typical PCR experiment. Then taq polymerase incorporates an extra a

residue, and this is incorporated on the three prime end of the molecule synthesized and is not template encoded.

So, this over hang residue may help in cloning the products of PCR, but it depends on what strategy one is utilizing, now apart from taq polymerase they are number of other polymerases, which are available from other thermus species. Like TF 1 and TTH enzymes from thermus flavus, and thermus thermophiles respectively are available which have 3 prime to 5 prime proof reading activity also. Polymerases are also available from other bacteriums, like RK bacterium also and many of these enzymes have proof reading activity.

These include like TLI from thermococcus litoralis, PFU from pyrococcus furiosus, and deep went from pyrococcus species these are enzymes, which are available and these are sometimes more stable than the taq enzyme.

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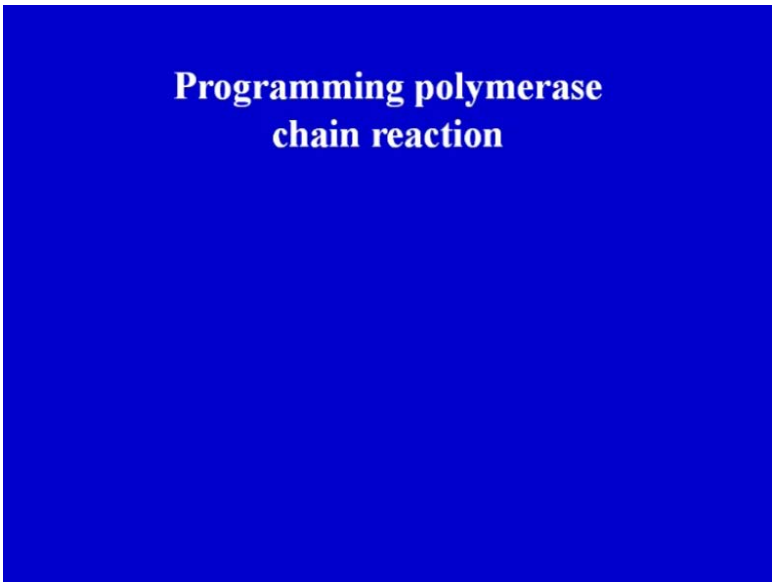

So, when you are performing one is performing polymerase chain reaction, it is performed on a thermocycler and programming can be done like PCR, it is a iterative process which involves three steps. And these three steps can be programmed, so that machine can run for particular number of cycles, now these three steps one like we have discussed.

First is denaturation, we have double stranded DNA template denatures at temperatures of around 95 degree Celsius, which is the highest temperature that taq polymerase can really endure for around 30's or odd cycles.So, in the first cycle of PCR denaturation is carried out for 5 minutes, to increase the probability that long molecules of template DNA are fully denatured.

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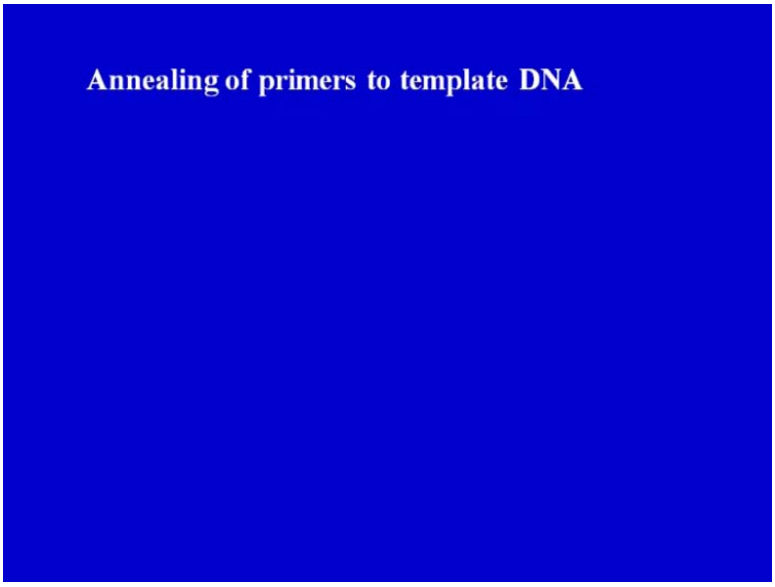

The second step is annealing of primers, so what is done for annealing of primers temperature is brought down, and this is a very critical step. Like if the annealing temperature is too high, then oligonucleotide primer anneal poorly, and yield of amplified DNA is low. If the annealing temperature is too low, non specific annealing of primers may occur resulting in the amplification of unwanted segment of DNA. So, annealing is usually carried out at 3 to 5 degree Celsius below the calculated melting temperature of the oligonucleotides that is primers.

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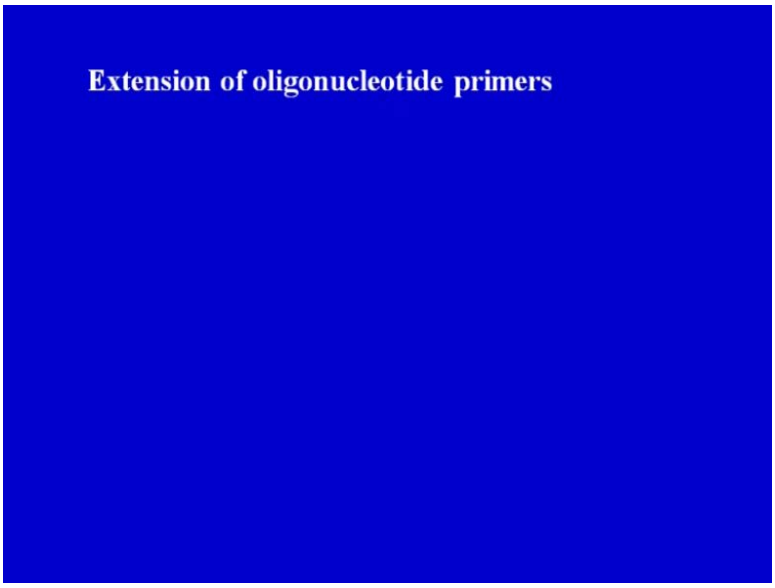

So, after annealing that is when primers are annealed at a particular specific sites, third step is extension that is extension of oligonucleotide primers. Now, this is carried out at the optimal temperature for DNA synthesis catalyzed by the particular thermostable polymerase, like in case of taq polymerase it is around 72 degree Celsius. Now, in the first two cycles extension from one primer proceeds beyond the sequence complementary to the binding site of other primer.

Then in the next cycle the first molecules are produced, whose length is equal to the segment of DNA delimited by the binding sides of the primer. So, from third cycle onwards thus particular segment of DNA amplifies geometrically whereas, longer amplification products accumulate arithmetically. So, extension is carried out for one minute for every 1000 base pair of product, that is the kind of particular efficiency. For the last cycle of PCR extension is carried out 3 times longer than the previous cycles to allow completion of all amplified products.

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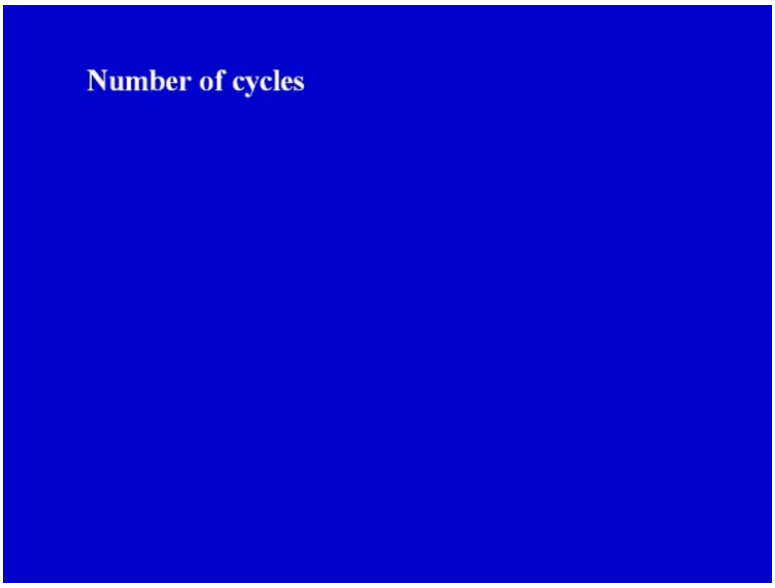

So, the last extension step is little longer, now number of cycles required for amplification depends on number of many factors, like say number of copies of template DNA present at the beginning of the reaction, that is important. Then efficiency of primer extension and amplification, so once established in the geometric phase the reaction proceeds until one of the components becomes limiting. So, at this point the yield of specific amplification product should be maximal whereas, non specific amplification product should be barely detectable.

Now, this is the case after 30 cycles of PCR containing almost like ten raise to power 5 copies of target DNA, at least 25 cycles are required to achieve acceptable labels of amplification of single copy target sequences in mammalian DNA templates. A typical set of reactions might have like in initial melting carrying carried out for 5 minutes, at say 94 degree Celsius, then 30 cycles each comprising like it is a repetitive like melting for 1 minute at 94 degree Celsius. Renaturation for 1 minute at 60 degree Celsius and then DNA synthesis for say one and half minute at 72 degree Celsius, and finally final extended round of DNA synthesis for 5 to 10 minutes.

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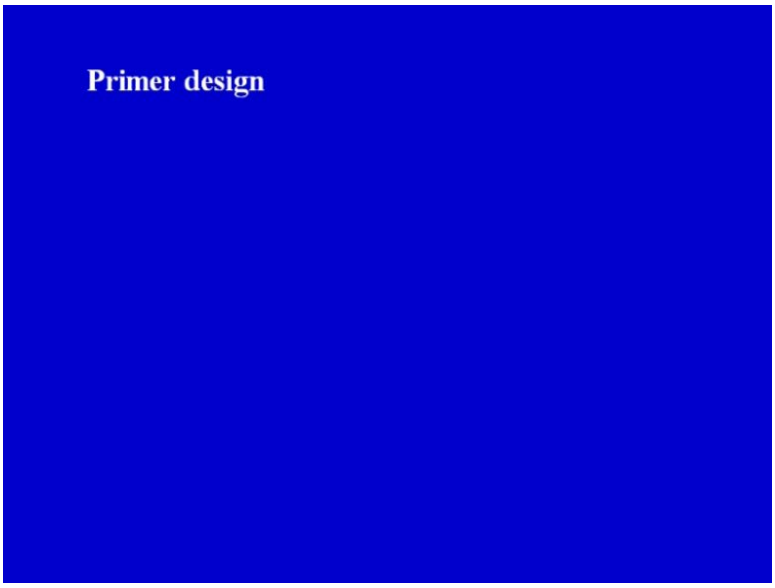

Now, primer design is an important part of PCR experiment, and there are several computer programs available, now to suggest suitable primers and general guidelines is one length. A short primer may offer sufficient specificity, while amplifying using a simple template such as a small plasmid, a long primer may be required, when using say eukaryotic genomic DNA as template. So, in practice like we said around 20 to 30 nucleotides are satisfactory, now members of a primer pair should not differ in length too much like greater than 3 pairs bases.

And base composition also has to be looked in, like if you have higher g plus c content that will be a problem, so it should be around 40 to 60 percent with an even distribution of all 4 bases along the length of the primer. If there is very low G C content then also there is a problem, because the melting temperatures will be lower actually. Then mismatches, where primers do not need to match the template completely, although the three prime end of the primer should be correctly base pair to the template rather than 5 prime.

It is often beneficial to have c or g as their three prime terminal nucleotide, this makes the binding of the three prime end of the primer to the template more effective. Some mismatches in the body of the primer sequences may be allowed particularly during mutation, like when somebody is trying to mutate single base or try to introduce say restriction sides. So, that way sequences can be incorporated at these ends that contain

restriction endonuclease recognition sites to facilitate subsequent cloning and manipulation of the PCR product.

Melting temperature is the temperature at which the two primers can associate with the template and should be relatively similar, so that they both can bind at the same time. As, temperatures are being lower during annealing if the temperatures of both primers are not same, then they will not bind at the same time and there will be problem in extension of both the strands. Now, strand internal secondary structure formation should be avoided, primer sometimes may fold back on itself, and not be available to bind to the template and then proper precautions or has to be taken to avoid this.

There could be cases where primer, primer annealing could take place, and to avoid the primers being able to anneal to each other extension by DNA polymerase of two self annealed primer leads to the formation of primer dimer. And this will be very efficient templates for amplication in subsequent rounds of PCR as they are like small actually, so primer primer annealing has to be avoided.

Now, this was the basic technique of PCR polymerase chain reaction, with three main steps here and many like enzymes particularly, it lead started with taq polymerase, but other enzymes could be also utilized. And PCR has number of applications in various areas of biotechnology, and other areas as well now why it has, so many applications one is it is a very convenient technique, amplifying a DNA segment was a very difficult before this thermocycle technique came in or PCR technique came in.

So, PCR is automated, a typical set of cycles can be carried out on many separate samples simultaneously in a few hours. And starting amounts of DNA can be very very small like in forensic science it it is like, when you get a blood sample or hair sample from that also amplification can occur. So, there are, so many applications of PCR technique or this particular method, and we are listing we just discussing some of them here, one is DNA sequencing.

Now, DNA this PCR in the presence of dideoxynucleotide tri phosphate or DDN TP's, like we will be discussing about that in the next section, it is a chain termination reaction or method. Where, DDN TP's are utilized for chain terminating termination for sequencing in sanger's method, so this allows DNA sequencing reactions to run successfully with very small amounts of template. This is also this is known as cycle

sequencing it requires a specially developed enzyme that combines the properties needed for sequencing with thermo stability.

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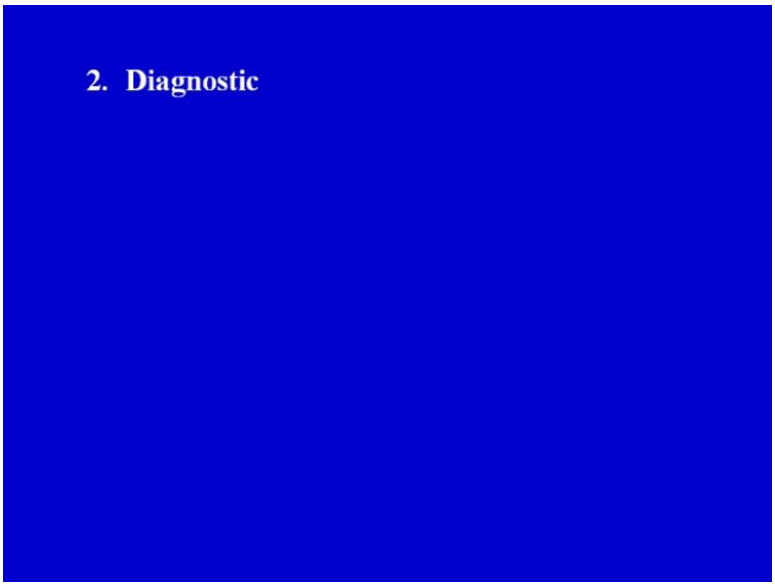

So, one application a is DNA sequencing, and this could be done by done like by different methods including the sanger's method, then PCR technique is widely utilized in diagnostic. It is a diagnostic tool actually like for example, in the identification of specific genetic traits or for the detection of pathogens or food contaminants, it could be utilized. One of the first applications of PCR was to genetical diagnosis for sickel cell anemia actually, the sickel cell anemia in the beta globin gene detroys the restriction site.

And the test involved PCR amplification of this region of the genome, and analysis of the PCR product for the presence or absence of this restriction site. PCR products can be analyzed by use of restriction enzymes, although it is rare for a mutation to create or destroy a restriction site, but this could be done. Determining, whether an oligonucleotide probe specific for a particular allele is able to hybridize to PCR products, then electrophoresis could be utilized like a screening for mobility changes caused by sequenced differences between a PCR product, carrying a mutant allele and wild type molecule can be done.

Then also sequencing can be done like PCR products can be directly sequenced by like DNA sequencing methods, so the test for the presence of a particular pathogen here, like in diagnostics can be made using primers specific for the genomes of those organisms.

And this permits detection at extremely low levels, like infections could be detected at a very initial stages, rather than advanced stages, a similar approach can be used to detect DNA from contaminating sources in food.

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So, then the PCR is also widely utilized in forensic science, the ability to amplify DNA from regions of the genome that are highly polymorphic, and therefore which are variable between the individuals. This could be starting with sample containing a very small amount of DNA, like single hair or traces of body fluids, like say blood or semen this could lead to the applications in forensic work.

Number of polymorphic regions have been utilized as targets for amplication, including like D loop of mytochondrial DNA which is variable between individuals. Then tendemly repeated mini satellites also known as variable number of tandem repeats VNTR’s then micro satellites used in conventional, genetic finger printing human leucocyte antigen sequences they could be utilized for these applications.

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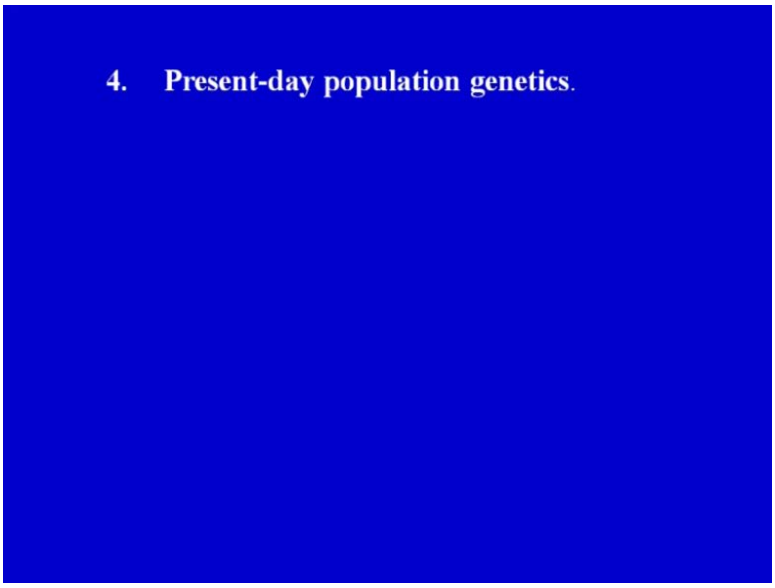

Now, PCR can also be utilized in present day population genetics, the ability to amplify material rapidly from a large number of DNA preparations leads to the application in population genetics. Allowing for example, the determining of frequencies of a particular allele in a large collection of individuals, there are number of PCR techniques that will provide information from many parts of the genome simultaneously.

Now, one such is random amplification of polymorphic DNA or we call it RAPD, so this analysis utilises relatively short primers that will anneal too many different sites in the genome under study, producing many different bands when the PCR products are analyzed on electrophoresis. Now, the similarities and differences between the band patterns generated from genomic DNA of different organisms provide information on their genetic relatedness.

Another technique is there which involves amplification of large set of restriction fragments of genomic DNA, whose sizes can be compared on electrophoresis, and this is termed as amplified fragment length polymorphism or a FLP. So, PCR is also used in population genetic studies like, we said of micro satellites or other like tandemly repeated sequences, single nucleotide polymorphism and transpozones could be studied.

A particular advantage of using PCR in population genetic study is that with appropriately designed specific primers, it may be possible to amplify DNA from one organism, that cannot be separated from others. So, such as particular bacterial strain in a

mixed population, that is very difficult to separate, but through PCR it could be done. So, this approach, therefore can be used to study genetics of bacteria or other other organisms that cannot be cultured axenically.

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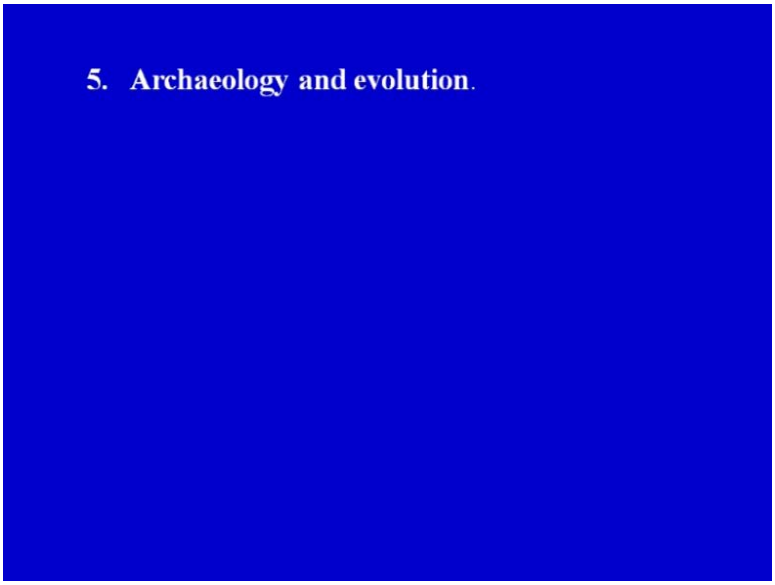

Now, another application of PCR is in archaeology and evolution, PCR can be used with old material as well as more recent samples, and it is often possible to amplify ancient DNA from museum specimens and archaeological remains. Multiple copy sequences such as mytochondrial DNA or chloroplast DNA are particularly useful target, and nuclear DNA could also be retrievable in certain cases.

So, comparison of polymorphic sequences from the ancient DNA with the sequences observed today, allows inferences to be made about the origins of a particular population of species, so these were some of the applications which makes PCR a widely used technique.

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Now, there are certain modifications in the technique for improving specificity, one of the most frequently encountered problem is the annealing of the primers, at the wrong location or to generate the wrong and this will generate wrong PCR product. So, it may be possible to reduce this problem by raising the annealing temperature in the cycle conditions, or altering the ionic concentration in the reaction mixture. But, there are number of other approaches also, which we are going to discuss one approach could be which is called hot start PCR.

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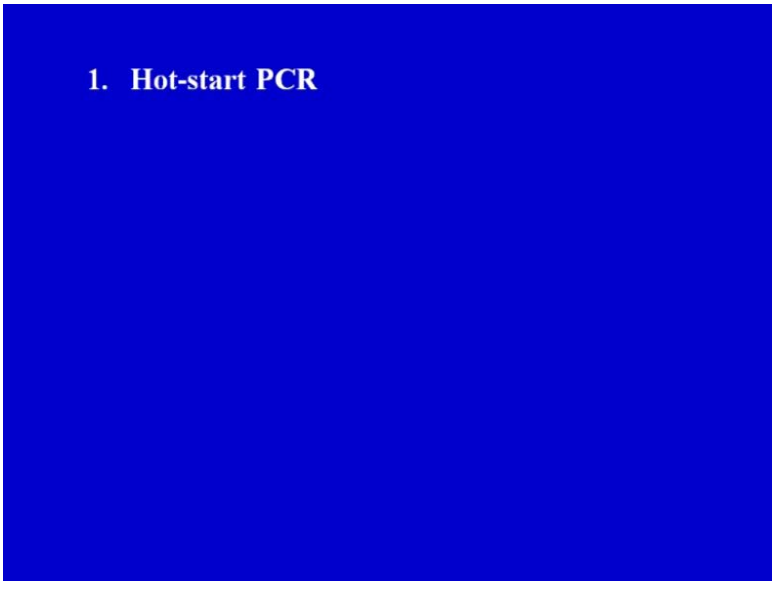

So, as soon as the PCR reagents have all been mixed up together, it is possible for the DNA polymerase to start synthesis, although the optimum temperature is very high and this may happen while the reaction mixture is being heated for the first time. And is at a temperature which is low enough to allow non specific annealing of primer to template generating a range of non specific products. So, this problem could be prevented if DNA synthesis could not is takes place or doesnot take place, until the first cycle had reached it is maximum temperature. So, this is the basis of hot start PCR that the to prevent the DNA synthesis to occur until it reaches the highest or maximum temperature.

So, in the simplest form the DNA polymerase is not added to the reaction tubes until they have reached the DNA melting temperature of the first first cycle, but this is like not possible. Though you can do it, but it is like then you have to mix again and again, more convenient approach would be to have one of the reaction components, in a form that is unavailable and until that particular appropriate temperature has been reached actually.

And this can be achieved by incorporating the polymerase or say the magnesium salt in wax or some capsule like structure, and which these beads or this particular material melts at the appropriate temperature allowing the reaction to start. A different approach could be to have the polymerase inactivated, at the start by complexing it with an antibody and then antibody denatures at high temperature, so that polymerase could be functional.

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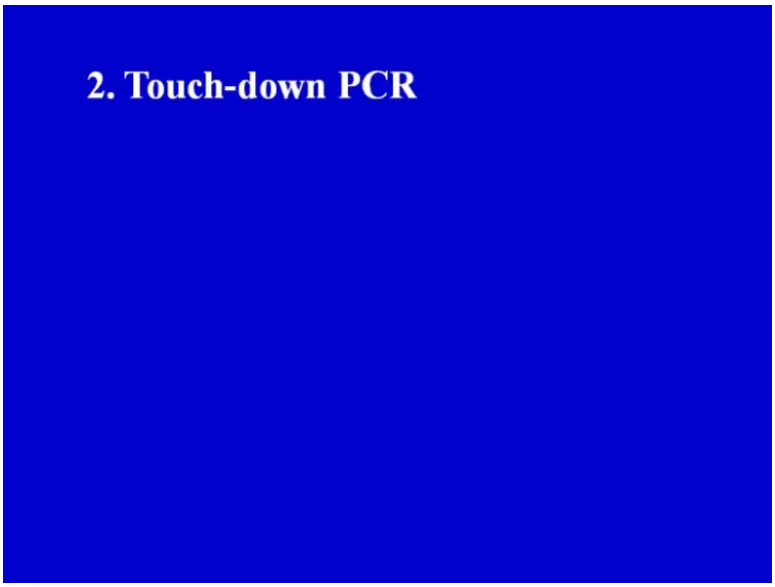

Another approach could be touch down PCR, now touch down PCR here the annealing temperatures used in conventional PCR is usually several degrees below the maximum at which primers can remain bound to template, to ensure stable binding. However, this use of lower temperature permits a small amount of mismatching between primers, and template which may allow primers to bind to incorrect sites, and generate the non specific or spurious products.

Now, the effects of this can be reduced with touch down PCR, what is done here, in this the high annealing temperature is used initially at which even correct binding may not be possible. The annealing temperature is reduced in subsequent rounds actually, so there will come certain point at which correctly matched primer template annealing will be possible, but incorrect matching is not, so DNA synthesis can, therefore start. Now, although later cycles may be under less stringent conditions, the earlier cycles will be will have been carried out under the most stringent condition, and the desired products will be the most abundant.

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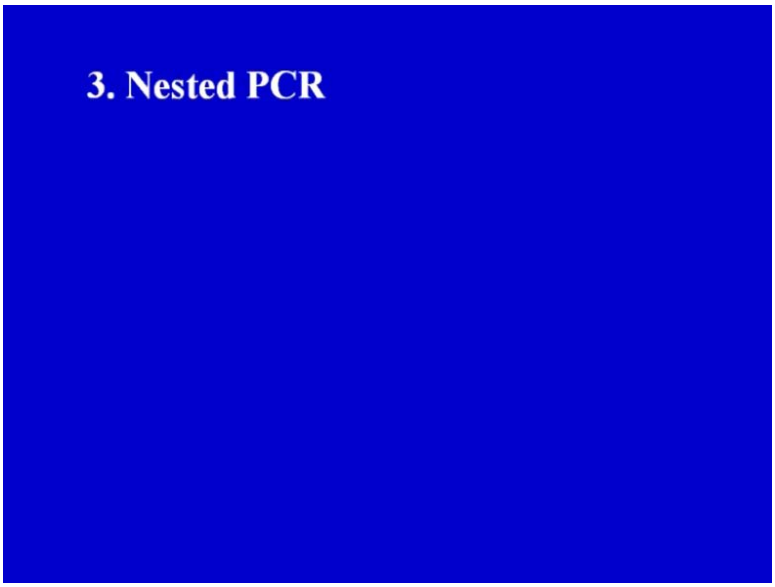

Another approach could be nested PCR, now here two successive PCR's are carried out, now in first PCR it utilises conventional template, the product of the first PCR are then used as the template for the second PCR. With, primers that are designed to anneal within the desired product of the first PCR, now although the first PCR may generate some non specific products, in addition to the desired product. It is unlikely that the non

specific product will also contain annealing sites for both the primers used in the second PCR, thus only the desired product from the first PCR are likely to be suitable templates templates for the second.

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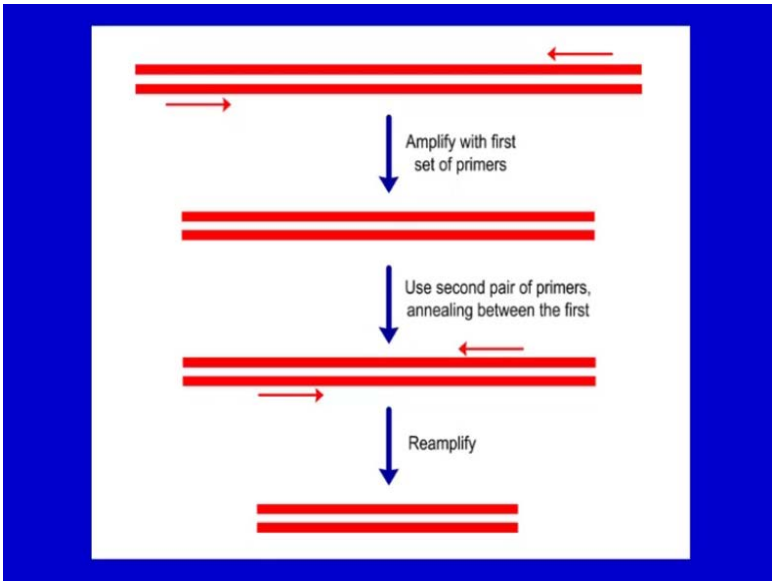

So, here it is shown here in this figure you have a DNA template and you will amplify with first set of primers here, and amplification will occur, now what is done. The second pair of primers are annealing between the like product of the first PCR, and then it is reamplified. So, that only that those which sites, because sites is within the first product, so that will be very specific.

Now, there is another term called making hard copies actually, so having amplified as specific piece of DNA it may be convenient to insert a copy of the product,s into a vector for subsequent cloning and maintenance by conventional means, this is sometimes called making a hard copy actually. So, the PCR product can be treated as a blunt ended DNA molecules for cloning purpose actually, another approach is to digest the PCR products with restriction enzymes prior to cloning.

Now, if suitable restriction sites are not present within the DNA sequences to be amplified then it is possible to incorporate, so either it could be done by blunt end or there could be other like restriction sites, which can provide for the cloning. So, then if they are incorporated this can be done by inclusion of an appropriate sequence containing a restriction enzyme recognition site, at the ends of the primers.

And when they are synthesized, they will the new new DNA will have those restriction sites at the ends. So, the primers annealed to the target DNA with the restriction site sequences unbound, and the intermediate molecules that are formed will have the restriction sites present, at one end and all the target length DNA molecules will have to have the sites at both ends.

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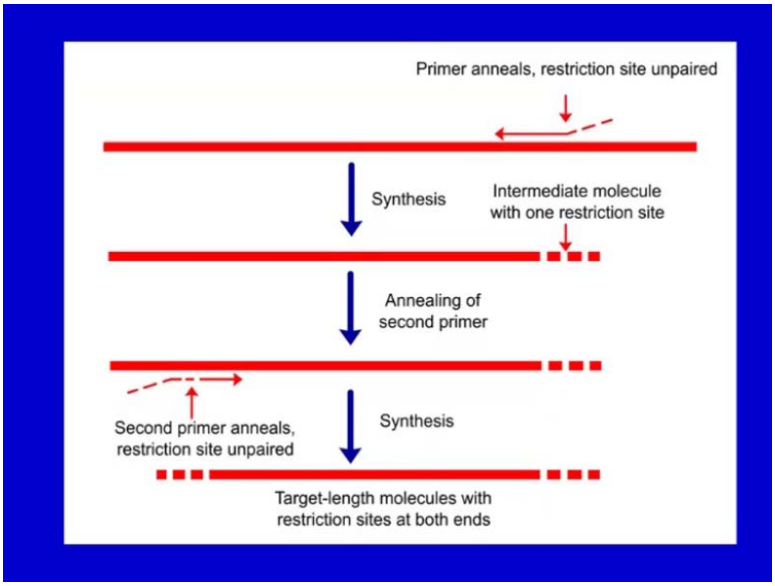

So, the amplified molecules can then be cut at their ends, with the appropriate restriction enzymes and then fragments can be cloned easily, so here like it is shown here the if you can see these are not really pairing here, but these contains the restriction sites. And finally, what the product will have the two restriction sites here, due to the primers containing the restriction sites actually, so the target length molecule with restriction site at both ends is obtained, and which can easily be cloned.

A third approach to making hard copies of PCR products relies on the fact that polymerases, such as the taq enzyme they that lack proof reading activity and they add an extra unpaired a residue or at the three prime and of the molecule, they have synthesized. So, a PCR product can made using such an enzyme will therefore, have unpaired over hanging a residue, so at each end and this allow the molecule to be ligated to a linearised plasmid molecule that has unpaired t residues.

Remember, a will pair with t, and c will pair with g, so another, so this could be another approach where taq polymerase, if you are utilizing and there are a overhangs, the

plasmid can contain t overhangs and there could be cloned in their. Another approach is to incorporate topoisomerase one recognition site into the PCR primer, and factors with covalently attached topoisomerase is then added and enzyme cuts the PCR product at the recognition sites and joints it to the vector.

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Then, there is another term called inverse PCR, result of the standard approach outlined, so far as we have discussed is the amplification of sequence between the primer annealing sites, that is you have two primers at the ends and in between that the particular segment of DNA is amplified. However, it is also possible to arrange for the amplification of sequences outside the primers, and this technique is called inverse PCR.

Now, the sample DNA's first cut with an enzyme outside, the region whose sequence is already known, now the resulting linear molecule are then circularised by ligation under conditions, that favor intramolecular reactions rather than intermolecular reactions. Second restriction digestion is then done, using an enzyme cutting within the region of the known sequence, the result is now that the first fragment containing this sequence has been turned actually inside out, leaving known sequence on the outside and the material that had previously, we will flanking it within.

So, primers complementary to the known sequences on the outside of the molecule, now be used to amplify the region of interest between them. This technique is specially powerful, when combined with transphozone tagging actually. If a previously

characterized transphozone has inserted into a gene of interest, the gene can be amplified using the inverse PCR and primers from transphozone sequences could be utilized.

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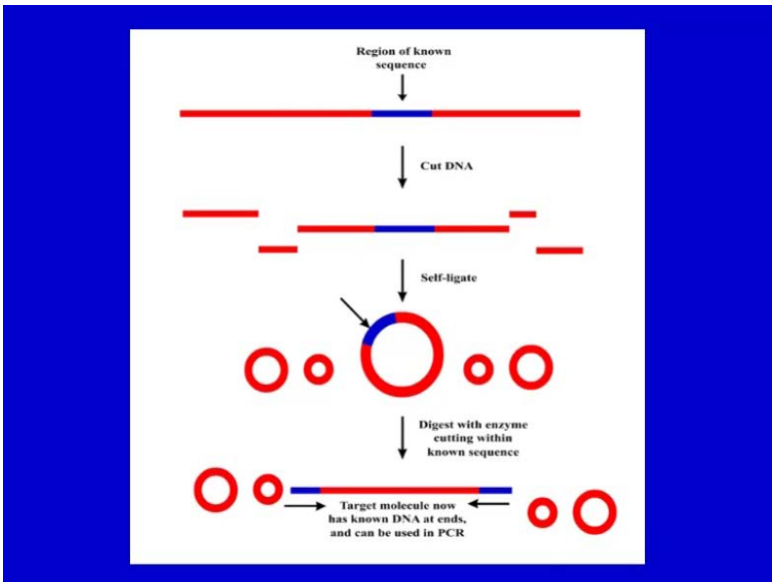

Now, if you see here this this figure on a screen explains this, so there is a region of known sequence here in the middle, and when you cut the DNA this region is in here and this is self ligated and what you get is that known region is in here. Now, what is done is it is digested with enzyme, which is cutting within the known sequence, so what you have done is first you have circularised it and then there is a known region is in this particular place, which was at the center earlier.

And it is being, when you cut it the within the known sequence and linearises it this known sequence, which was at the center is now at the ends. And then primers against this could be utilized, since you have known DNA ends and this could be utilized for PCR amplification, this was about inverse PCR. Now, there are other types of PCR like in situ PCR, now it is possible to carry out PCR using permeabilized tissues actually, say thin sections on a microscopy slide.

So, what it requires is specially adopted PCR machine to accommodate these slides, actually if the PCR product can be detected then this allows one to identify, where in the tissue the target nucleic acid is located. Although, this can be used to identify the location of particular DNA species in tissues, it is very often combined with reverse transcriptase to identify the location of particular transcript actually.

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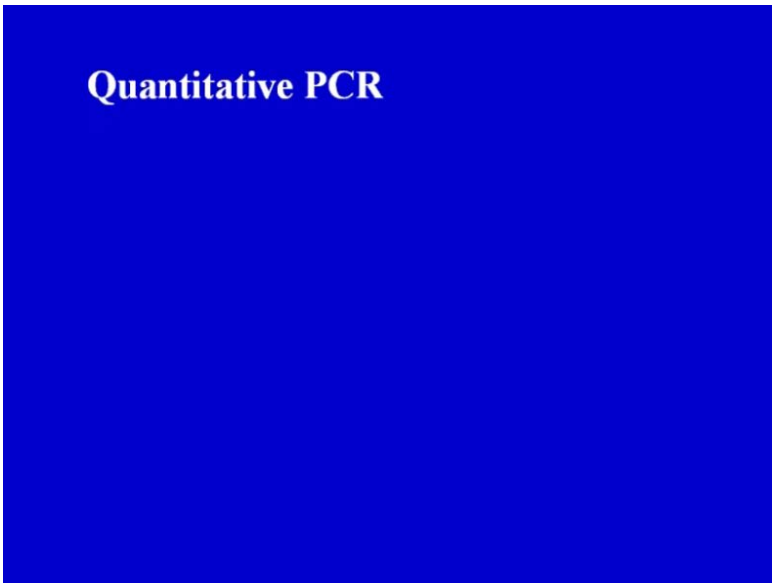

Quantitative PCR could also be done, and it is possible to use PCR to estimate the abundance of a particular nucleic acid molecule in a sample, say we are interested in an RNA molecule it will be necessary to start with the reverse transcriptase PCR or RT PCR to make AC DNA copy. Now, there are two main approaches here for estimating the abundance of a molecule, one is to use a standard PCR and visualize the amount of the product of interest by electrophoresis technique, like comparing it with suitable controls or standards.

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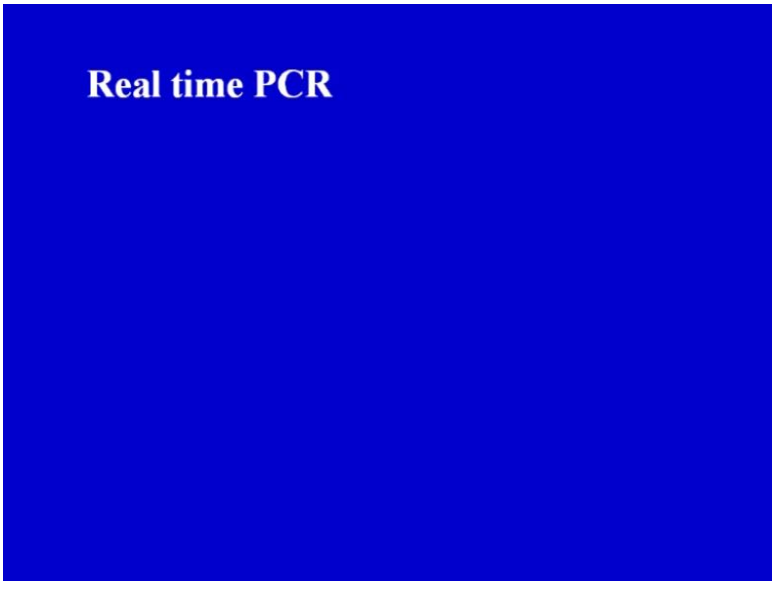

So, this is with like in at the end of the experiment you are measuring the amount of target sequence, now second approach could be to quantify the reaction in real time that is while the PCR is progress. So, the real time PCR is a very, very important technique in terms of giving an information about like in terms of quantitative PCR, and this can be done in two ways one is flourescent double stranded DNA binding dye like say sybr green is present in the PCR. And as the double stranded DNA product accumulates the amount of flourescence from the dye increases, and this can be detected.

Now, the experiment requires PCR machine that is also equipped with a flourescence measurement facility, so this approach is adequate if the PCR generates the products of interest very specifically. Now, because the method simply detects double stranded DNA it measures the amount of PCR product, at a given time regardless of whether it is from the correct region or not actually.

So, here like through fluorescent probes like double stranded DNA binding dye could be utilized as one approach, and the second approach to real time PCR, it allows detection of a specific product rather than double stranded DNA in general here. And what it does is it utilizes a spatially synthesized probe oligonucleotide, and this probe is designed to anneal within the region to be amplified, and carries the flourescent reporter dye at one end, and a quencher at the other end of the molecule.

So, what happens is if the quencher and the reporter are in close proximity, that is attached to the same oligonucleotide, then the quencher stops the reporter from fluorescing. Now, during PCR the probe will anneal to single stranded DNA within the target region, and when the polymerase meets the annealed probe the 5 prime to three prime exonuclease activity of the enzyme degrades the probe. Liberating the reporter from the quencher and thus the flourescent reporter accumulates during the course of PCR.

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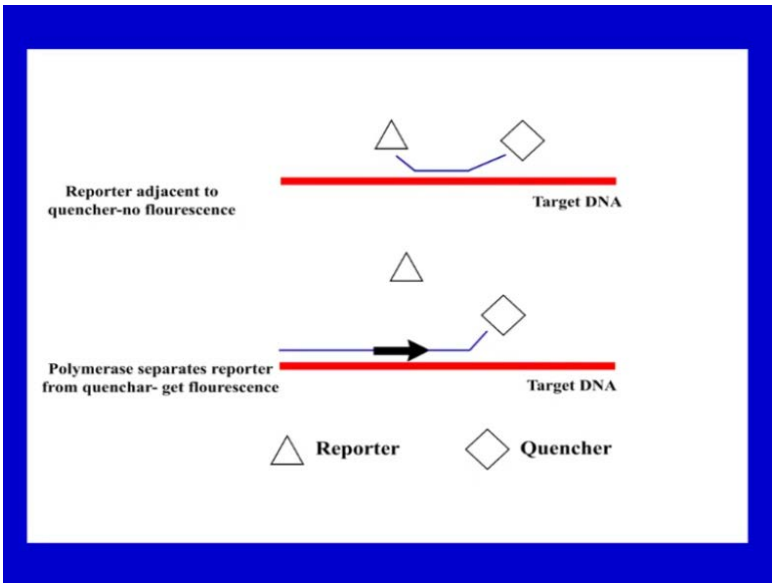

So, this could be then detected, so here is very simple depiction of that, what you have is if you can see on your screen there is a reporter adjacent to quencher. So, when this reporter, this quencher is there the no flourescence will be obtained or seen, but as soon as DNA polymerase comes and it kind of removes this. Then this is freed here, and a polymerase will separate the reporter from quencher, and the flourescence could be detected actually, so this is the approach here for real time PCR.

Now, in alternative method for specific products detection in real time PCR, utilizes two oligonucleotide probes that anneal to adjustance sides in the target DNA. One oligonucleotide is tagged with a molecule that absorbs light, and the second with a molecule that is able to accept energy from the first, and remit energy at a different wavelength. Now, if the two oligonucleotides can both anneal to the target DNA, then the energy absorbing and energy emitting molecules are brought into close proximity, and energy transfer is possible.

Now, if the oligonucleotides are not brought together in this way, then the little energy transfer can take place, thus measurement of the amount of flourescence from the second tag provides a measure of the amount of the target DNA.

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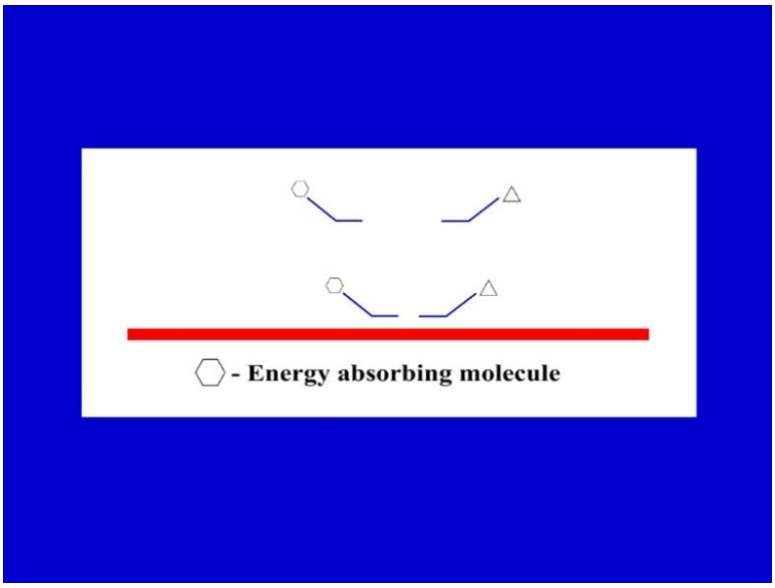

So, here it is like as you see these two targets, when they come together that is they are brought into the proximity, then only the particular like flourescence measurement of the amount of flourescence is provided actually. Mutagenesis could also be like in PCR reactions, a mutagenesis can also be performed like, because perfect matching between the primer and the target DNA is not necessary.

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So, this could also be one of the methods to make like sequences with certain mutations, now provided that this allowance is made for this in determining the condition for primer

annealing. So, primer annealing should not be affected, if you are putting in a primer with mismatches PCR can therefore, be used to engineer specific mutations in the amplified sequences, by constructing a primer that corresponds to the mutated sequences rather than the original.

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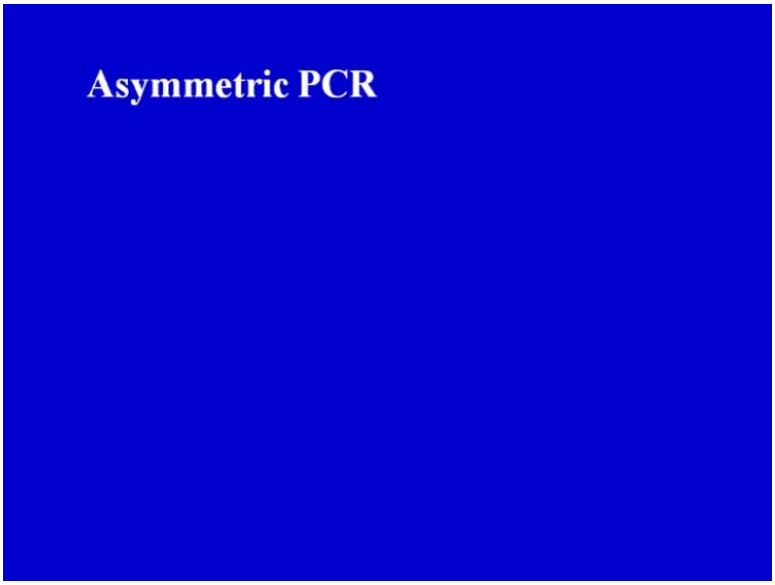

There are other forms of PCR like asymmetric PCR by reducing the amount of one of the two primers, it is possible to arrange for preferential application of the one of the two strands. Resulting in a preparation of single stranded DNA, which has a number of uses in molecular biology, so asymmetric PCR can be another form, where single stranded DNA could be there then anchored PCR.

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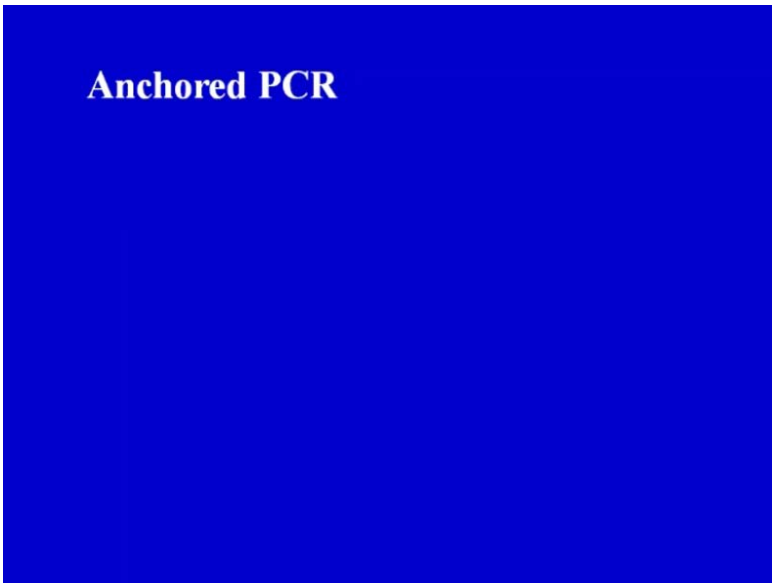

Anchored PCR is applied, when only one piece of sequence for the reason of interest is known, the aim is to attach the region to be amplified to a piece of known sequence. And then to use this that as the second priming site, and there are two types or two ways in which these can be most easily done. One is to fragment the sample DNA and ligate it two molecules of the known sequences, such as vector and this known sequence is used as the basis for designing, one of the two main PCR's.

And second could be to add tails enzymatically to the sample DNA or the molecules, produced after the first round of synthesis for example, a run of say g residues can be added by treatment with a terminal deoxynucleotidyl transferase or some other can be used. And this could be combined with RT PCR this forms the basis of rapid amplification of C DNA end, so what is done is say through an enzyme you have put in G. And 2 prime deoxyguanosine 5 prime triphosphate or oligo DC can then be used as a primer, because it will anneal to the oligo DC combined with RT PCR this forms, basis of rapid amplification of C DNA ends or it is called race.

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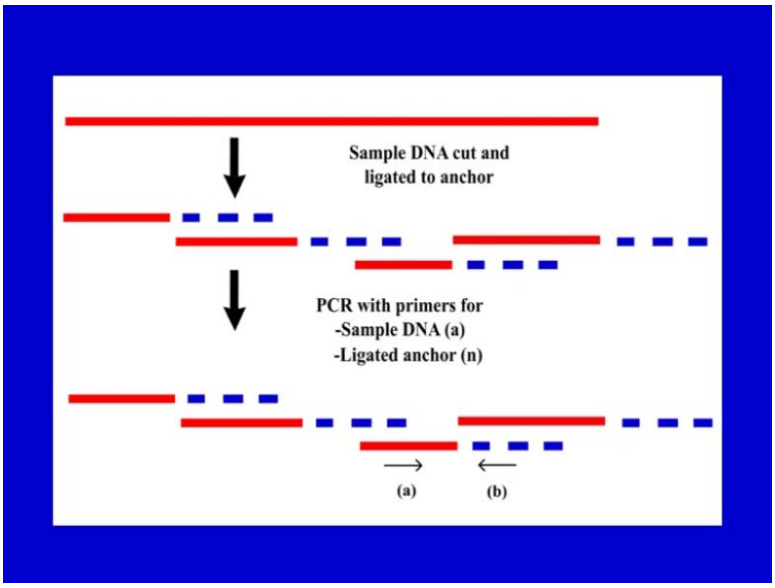

So, this picture kind of explains this, there is a single sample DNA cut and ligated to anchor, and then PCR with primers for sample DNA and ligated anchor is performed and this way you can get the full sequences actually, so this is called anchored PCR.

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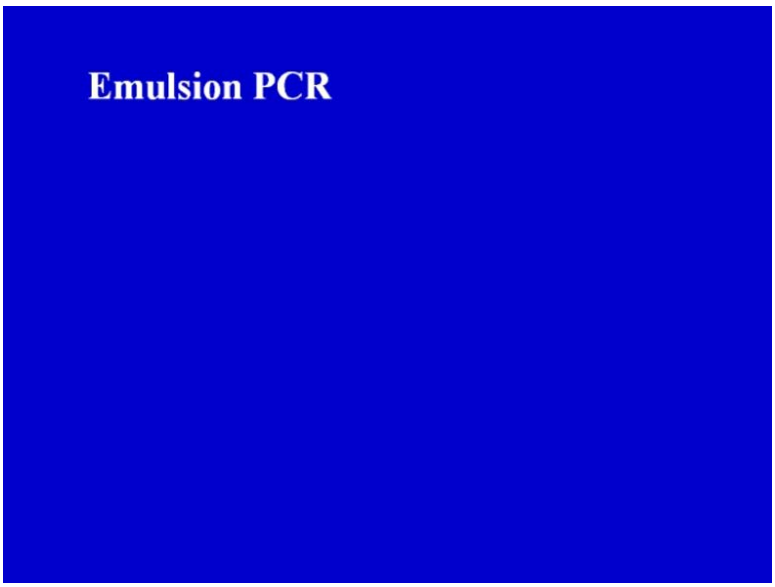

There are other forms, like emulsion PCR can be another form, in this technique all the reagents are incorporated inside the lipid droplets, and PCR is carried out on a much smaller scale. Advantages, could be to it is possible to increase and decrease the temperature of small droplets very quickly.

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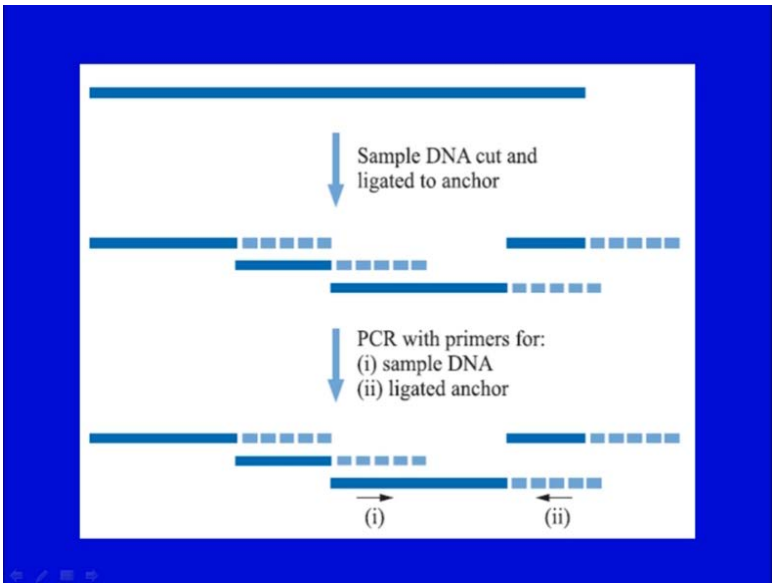

If each droplets contains a single template molecule at the start, then all the products in an individual droplets results from the amplification of single template molecule, this is also called droplet PCR.

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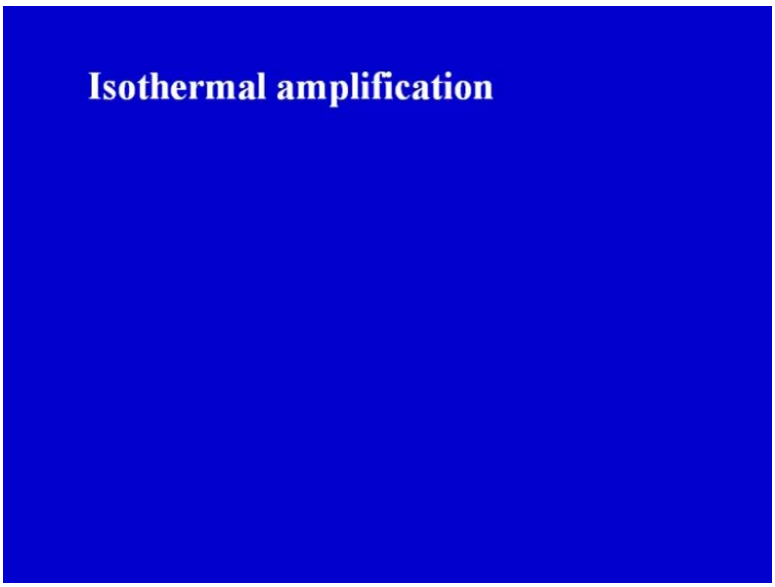

Then there could be another form could be isothermal amplification, the repeated heating and cooling required by PCR limits, how quickly the process can be carried out in addition to this the cost of PCR machine is not low. So, loop mediated isothermal

amplification has been developed, which allows templates to be assembled amplified templates to be amplified at a constant temperature or say around 65 degree Celsius.

It uses a DNA polymerase with strand displacing activity, and allows the and avoids the need for heating to high temperature. There is a particular interest in the use of this method for the detection of pathogens, outside of specilaist laboratories as it is a rapid and avoids the need for expensive PCR machines. So, these were some of the different modified techniques and different approaches, which have been developed from the simple basic techniques of PCR.

So, in this lecture we have discussed about polymerase chain reaction, basic technique and modified versions of this technique. Also, we have discussd about the applications of various applications of PCR technique, in the next lwcture we are going to discuss about two more techniques here that is automated DNA sequencing methods, and ELISA.

Thank you.