Discipline: Botany

Paper: Plant Biotechnology

Lesson: Polymerase Chain Reaction (PCR)

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Table of Contents

Chapter: Polymerase Chain Reaction

- **Summary**
- **Exercises**
- **Glossary**
- **References**

Introduction

Polymerase Chain Reaction (PCR) is one of the principal techniques used in molecular biology. It is a simple, rapid and inexpensive tool used to a generate millions of copies of DNA from fairly small amount of DNA. This technique was developed in 1983 by Kary B. Mullis. In recognition of their contribution to science, Mullis along with Michael Smith was awarded Nobel Prize (1993) in Chemistry.

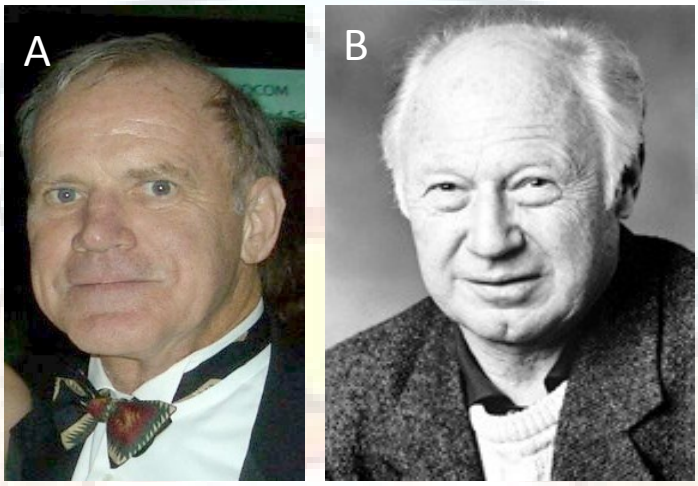

Figure: A. Kary Mullis; B Michael Smith

Source:http://en.wikipedia.org/wiki/Kary_Mullis, http://en.wikipedia.org/wiki/Michael_Smith_%28chemist%29 (CC)

The technique has wide range of applications in biological research, like gene cloning, sequencing, forensic sciences and diagnosis of different diseases.

Video:https://www.youtube.com/watch?v=2KoLnIwoZKU&list=PL854F3A5D92AFC121 (CC)

Discovery of PCR

In 1983, Mullis was working for one of the biotechnology companies, Cetus Corporation as a chemist. One day he had an idea that a pair of primers can be used to copy the DNA sequence using DNA polymerase. Mullis succeeded in demonstrating PCR in December 16, 1983. Other Cetus scientists, Randall Saiki and Henry Erlich, also worked on the PCR project to test whether PCR could amplify a specific human gene (betaglobin) from genomic DNA. Saiki and Erlich published first paper on utilization of the technique. At the same time, Mullis was still working on a paper that would describe PCR itself. The polymerase that was used for the amplification destroyed at high temperature. In 1986, Saiki started to use Thermophilus aquaticus (Taq) DNA polymerase to amplify segments of DNA. The Taq polymerase was heat resistant and would only need to be added once, thus making the technique dramatically more affordable and subject to automation. This has created revolution in biochemistry,molecular biology, genetics, medicine and forensics.

Source: https://en.wikipedia.org/wiki/Kary_Mullis

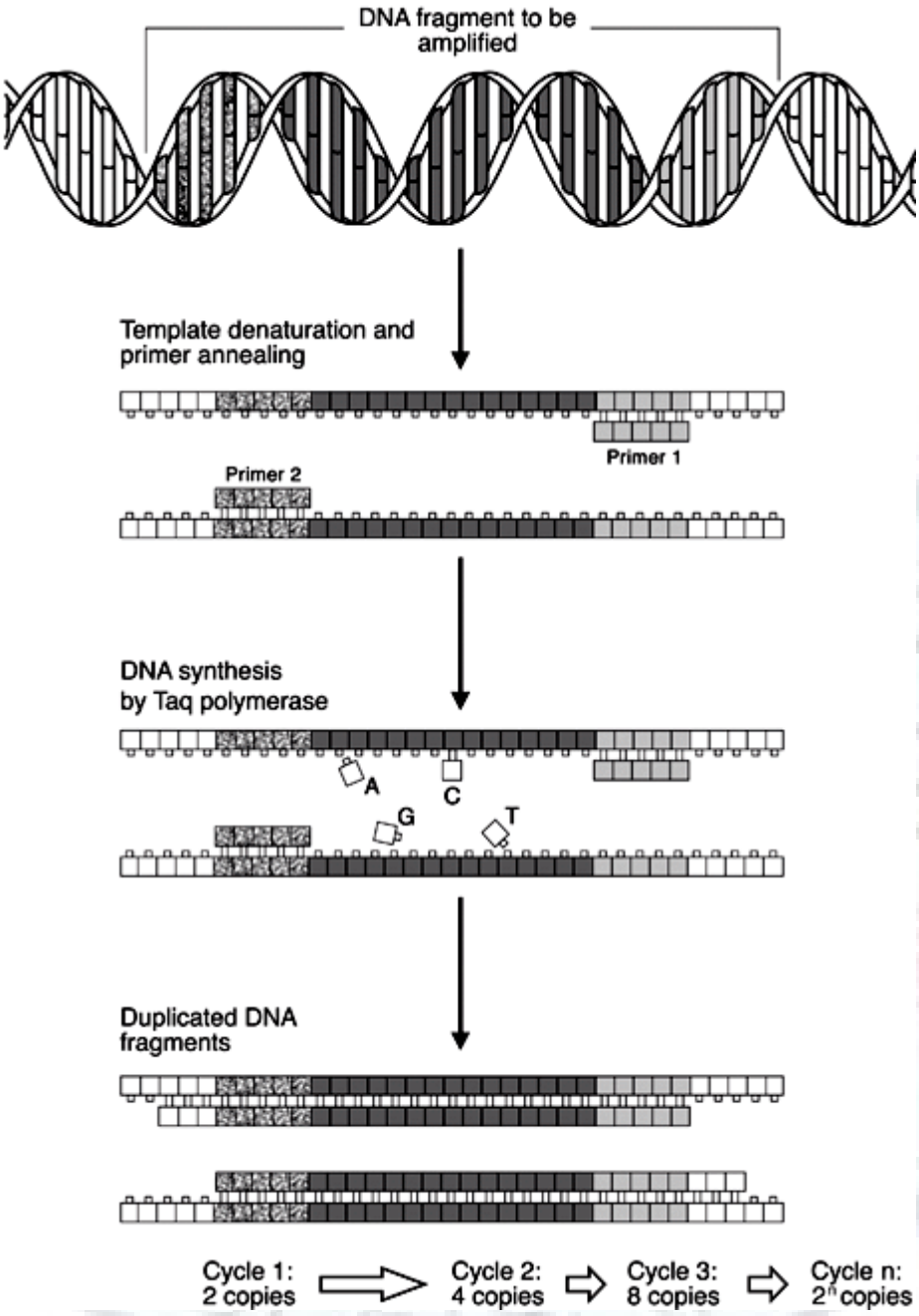

Figure: A schematic illustration showing PCR steps. Each cycle consists of three steps – denaturation of DNA, annealing of primers and extension by polymerase. Number of copies of the target sequences increases exponentially during the reaction.

http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1678-77572004000100002

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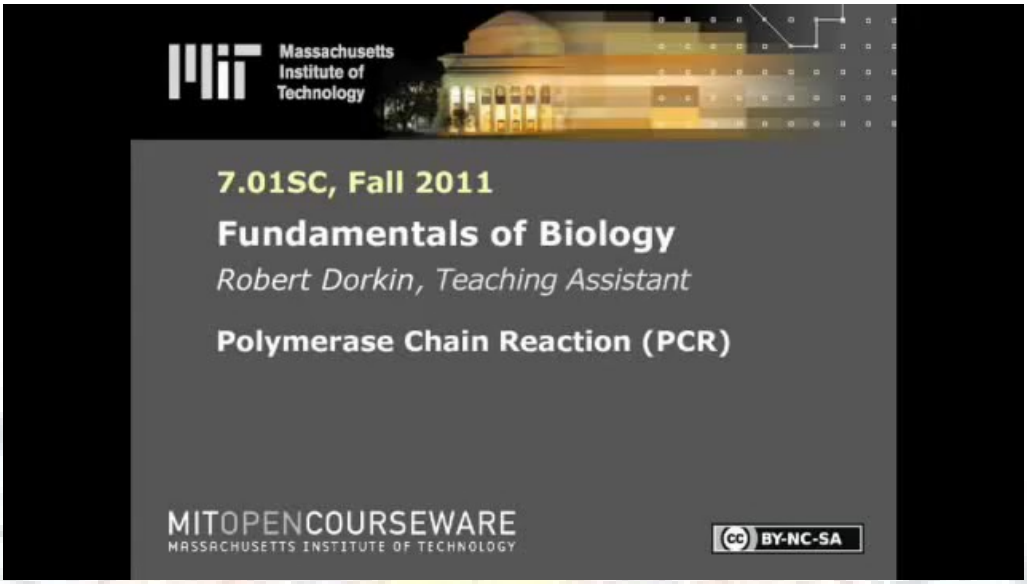


Source:https://www.youtube.com/watch?v=OK7_ReXhVaQ (CC)

Video: https://www.youtube.com/watch?v=0rQFnbcEsog (CC)

Components of PCR

The PCR requires various components like DNA template, primers, DNA polymerases, nucleotides and buffer.

Template

In order to amplify the target DNA sequence, whole genomic DNA or plasmid DNA can be used as a template for carrying out PCR.

Primers

Primers are essentially nothing but short pieces of DNA which are synthesized artificially in laboratories. They are synthesized in pairs – a forward primer, which is complimentary to a target DNA sequence and a reverse primer which is reverse compliment of the same sequence towards the other end. Through complimentary binding, forward primer binds to one end of separated single stranded DNA and the reverse primer to the other end of the complimentary strand, to the synthesize new DNA.

Properties of primers

For the primer design the following conditions have to be followed:

Length

Primers are usually 18 to 25 bases in length.

Meting temperature

Primers have a definite Tm i.e. the melting temperature. It is generally kept between 60°C and 65°C, and the difference between the forward and reverse primers should not exceed 5°C. Primers above 65°C have a tendency for secondary annealing.

The Tm is calculated using the formula: Tm = (4 × [G + C]) + (2 × [A + T])°C, in which [G + C] is the number of G and C nucleotides in the primer sequence and [A + T] is the number of A and T nucleotides. Generally the annealing temperature is kept 5 ºC less the Tm of the primers. Thus the Tm of the forward and reverse primers should be nearly the same.

Primer dimers

The primers should not be self-complementary. If they bind to each other they form self dimers or primer dimers. This prevents DNA amplification as instead of annealing to the desired DNA sequences the primer anneals to themselves.

GC content

The content of G and C bases together should be 40% to 60%. The 3' of a primer should be C or G to which promotes binding and specificity.

Secondary structures

Any regions capable of forming secondary structures, like runs of 4 or more of one base, should be avoided. The dinucleotide repeats are also avoided while designing the primers.

Using these criteria, primers can be designed manually by analyzing the template DNA sequence carefully, or various softwares can be used, which are freely available. For example,

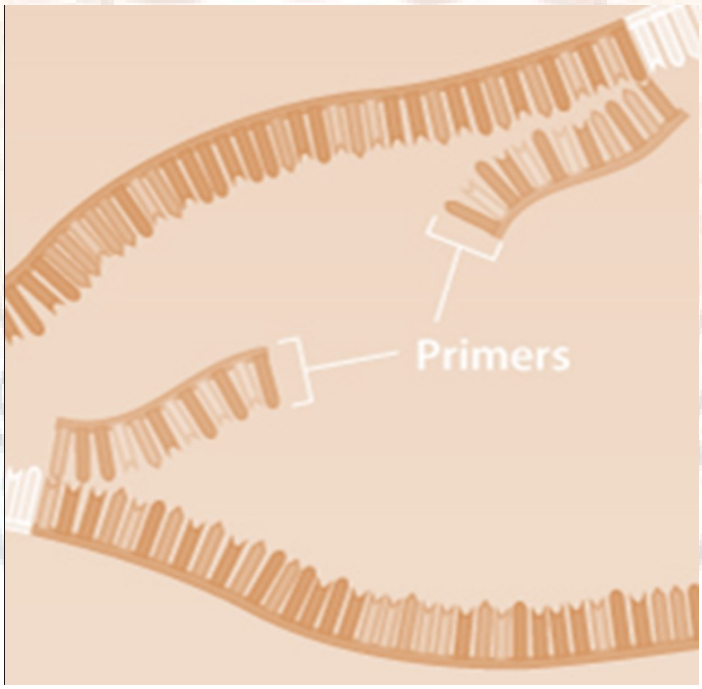


Figure: A representative diagram of a denatured DNA molecule showing how binding of forward and reverse primers takes place on the opposite strands.
Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

Protein coding region 1..324bp

ATGGTATCTC TAAAGTCCCT TGCTGCTATT CTCGTTGCCA TGTTTCTTGC CACCGGACCT

Forward primer FP

ACGGTTCTAG CCCAGCAGTG CAGAGACGAA CTGAGCAATG TGCAGGTGTG CGCGCCGCTG
CTTCTGCCCG GTGCGGTCAA TCCTGCCGCG AACTCAAATT GCTGCGCTGC CCTCCAAGCA
ACTAACAAAG ATTGTCTATG TAACCGTCTT CGAGCAGCCA CCACACTTAC CTCTCTTTGT
AACCTCCCCT CTTTTGATTG TGGTAAGATG ATCCATCGAT TAAAACCTTT TTTACTAGAT
TTTTATAAAT TATTCCATCA ATAGTGTTTG TTTTATATTT GTTCTCATGA TTTTTTGGAC

reverse primer RP

TTATGTTTTG TGAACTGTGC AGGCATAAGT GCCTAGTTGA

Primer name	Sequence	Tm (^{0}C)	%GC
FP	5′-GGT ATC TCT AAA GTC CCT TGC-3′	62	50
RP	5′-CAC TTA TGC CTG CAC AGT TCA-3′	62	45

Figure: The figure shows a pair of primers designed for a particular protein coding region. FP and RP are highlighted. The sequence of the respective primer along with Tm and GC content is shown below.

Source: Dr. Parul Agarwal, Department of Genetics, University of Delhi, South Campus.

Calculating the Tm of the primer:

An arbitrary example of the primers sequence: ATGCATGCAGGCCTGAGCT

Number of G+C=11 and A+T= 8

Tm = 11*4 +2*8 = 44+16 = 60°C

Source: Dr. Parul Agarwal, Department of Genetics, University of Delhi, South Campus.

DNA polymerase

DNA polymerase is an enzyme that synthesizes the new strand of DNA complementary to target DNA. The DNA polymerase such as *Taq* polymerases isolated from heat resistant (thermophilic) bacteria *Thermu saquaticus* is the most commonly used enzyme.*Taq* DNA polymerase withstand at high temperature for synthesis of target DNA. Apart from *Taq* DNA polymerase, there are various other polymerases that can be used, for example, *Pfu* polymerase isolated from single celled archaeon *Pyrococcus furiosus,* Vent polymerase isolated from *Thermococcus litoralis* etc.

dNTPs (deoxynucleotide triphosphates)

Nucleotide bases (A,T,G and C)are building blocks of new DNA strand synthesis. DNA polymerases binds the nucleotide bases floating in the reaction mixture and uses them for synthesis.

Buffer

Any enzymatic reaction requires a suitable medium in which the enzymes can function. There are buffers available, which provide optimal pH and optimum concentration of various other ions which are required in order to stabilize the reaction.

Divalent cations

All the thermostable DNA polemerases need free divalent cations for their activity. Usually, Mg^{++} ions at an optimum concentration are used.

Thermal cycler

PCR is carried out in a machine called a thermal cycler. It works on the principle of Peltier effect. It increases and lowers the temperature in a pre-programmed manner. It has a thermal block with holes where the PCR tubes with reaction mixtures can be inserted.

Visit link: http://www.wiley.com/college/boyer/0470003790/animations/pcr/pcr.htm

Figure: Image of a PCR thermocycler (BioRad Company). The yellow arrow indicates the block where PCR tubes are inserted.

Source: http://commons.wikimedia.org/wiki/File:PCR_machine.jpg (CC)

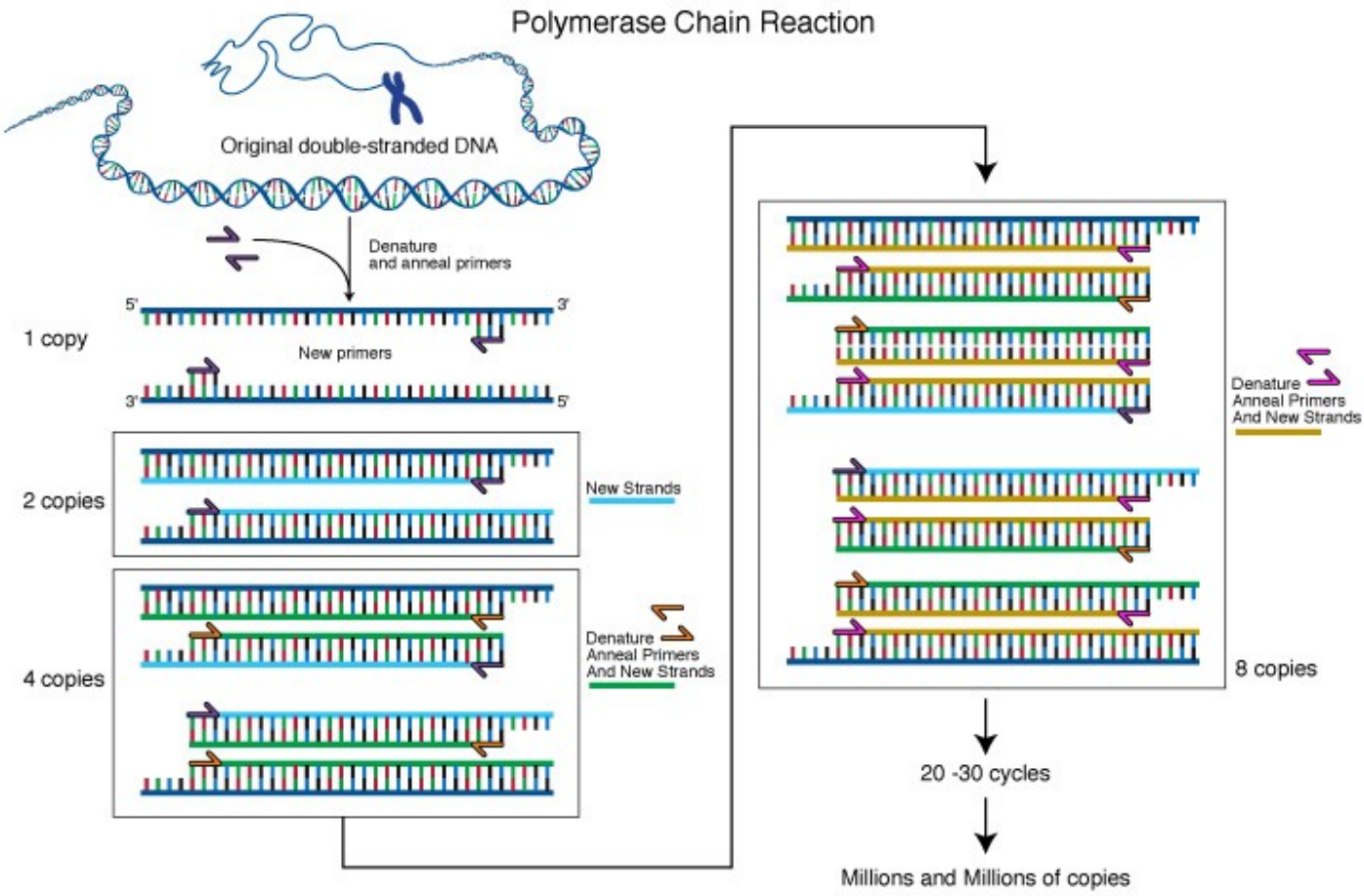


Figure: Millions of copy of a desired sequence can be generated using a single DNA molecule as a template.

Source: http://www.genome.gov/glossary/resources/polymerase_chain_reaction_lg.jpg (CC)

Procedure

PCR is based on three major steps called denaturation, annealing and extension. All three steps are repeated 30 to 40 cycles to amplify the target DNA in large quantity.

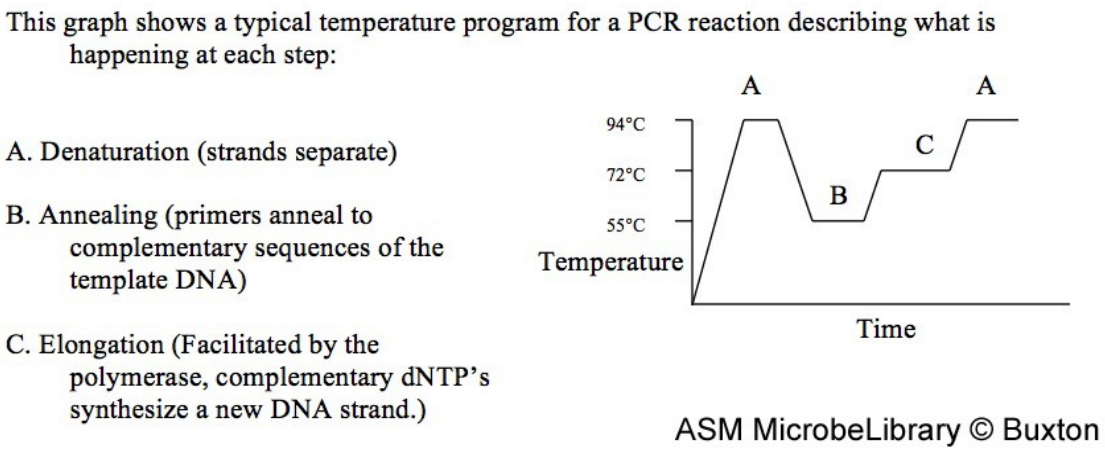


Figure: Steps in a typical PCR program

Source: http://www.microbelibrary.org/library/laboratory-test/3656-polymerase-chain-reaction (CC)

Denaturation

In the first step, the double stranded DNA is denatured to produce a single straded template. This is achieved by raising the temperature of the reaction mixture to 94–95 °C. The double stranded DNA is separated because the hydrogen bonds are broken at high temperature leading to the formation of single stranded DNA. This reaction is usually carried out for around 45 seconds for about 30 to 40 cycles. In the first cycle denaturation may be carried out for 5 minutes to increase the probability of long DNA molecules to get denatured. This step is called 'Initial denaturation' and it precedes denaturation, and is perform only once in the beginning of the reaction.

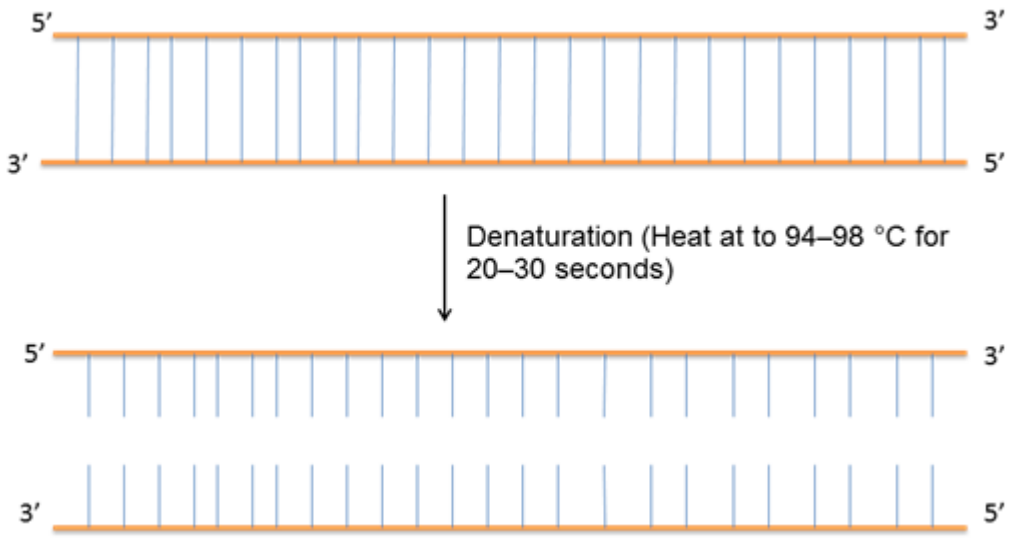


Figure: During denaturation, double stranded DNA is converted into single stranded DNA
Source: http://nptel.ac.in/courses/102103013/12 (cc)

Annealing

In the second step, the temperature is reduced to low nearly 50°C to 65 °C, depending upon the Tm of the primers, for 35 to 40 seconds, so that the primers can anneal to the single stranded DNA. The annealing temperature is very critical because if it is very high primer does not bind to the template and the primers and templates remain dissociated. And if the temperatureis too low nonspecific bindings occur. Annealing step is usually carried out at a temperature 3 to 5°C lower than the T_m of the primers.

In cases where proper annealing temperature for a particular primer pair needs to be standardized, a 'Gradient PCR' may be done i.e. a series of annealing temperatures can be checked in different reactions for deciding the optimum annealing temperature.

Figure: Annealing of primers
Source: http://nptel.ac.in/courses/102103013/12
(cc)

Extension

In the third step, the temperature is reduced to 72°C. At this temperature DNA polymerase starts adding the nucleotides onto the annealed primers to extend target DNA in 5' to 3' direction.

This step is followed by an additional step called 'Final elongation' in which the temperature is kept at 72°C for 7-9 minutes so that any remaining single-stranded DNA can be fully extended.

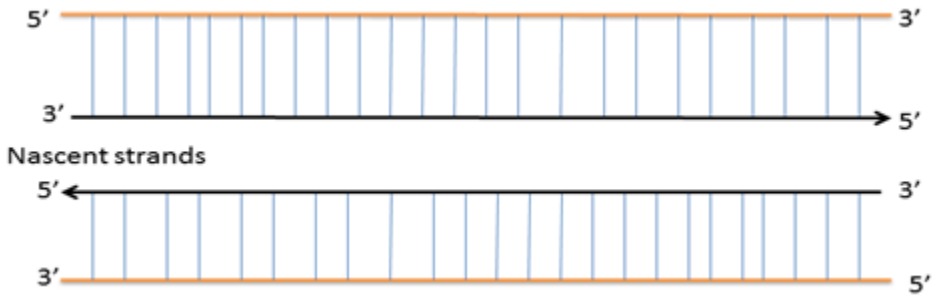


Figure: Extension

Source: http://nptel.ac.in/courses/102103013/12

(cc)

Number of cycles

Generally, the three steps of PCR are carried out for 30 to 40 cycles. The number of cycles required usually depends upon the initial number of copies of template DNA present, the concentration and specificity of the primers and the efficiency of annealing and extension. When the reaction starts the number of copies synthesized gradually increases in three stages as follows:

- Exponential phase: the amount of product is doubled at every cycle (assuming 100% reaction efficiency).
- Linear phase: the reaction slows down as the substrates like dNTPs and primers are being consumed and become limiting for the enzymatic reaction.
- Plateau: no more products are being made. The reaction is exhausted and saturated.

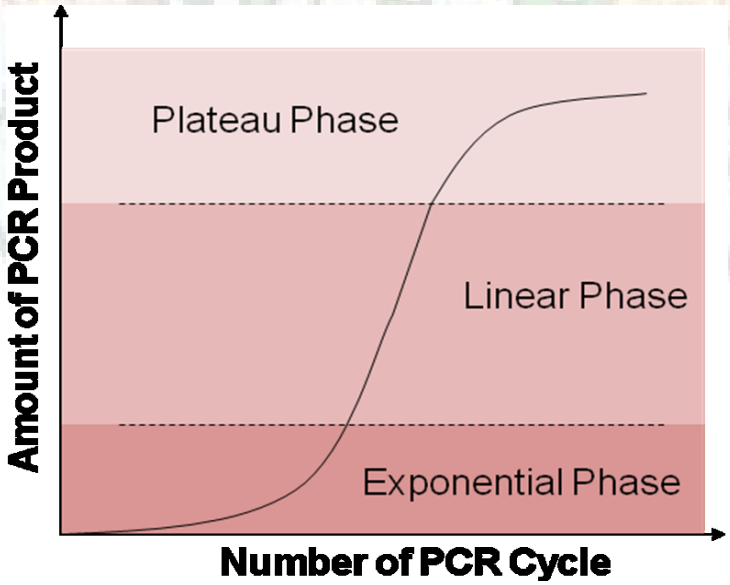
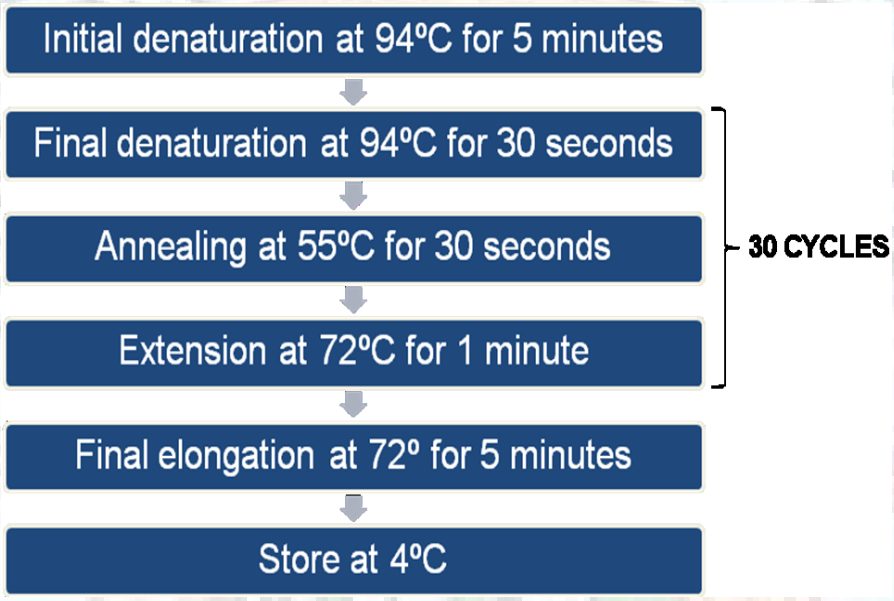


Figure: A theoretical plot showing increase in the amount of PCR product with increase in the number of cycles.
Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

Thus, the number of cycle depends upon how many cycles it takes to reach the plateau phase. This number is usually after ~30 cycles if ~10^5 number of template DNA molecules are available at the beginning of reaction (which is usually the case).

A typical PCR program involving all these steps may look like as follows;



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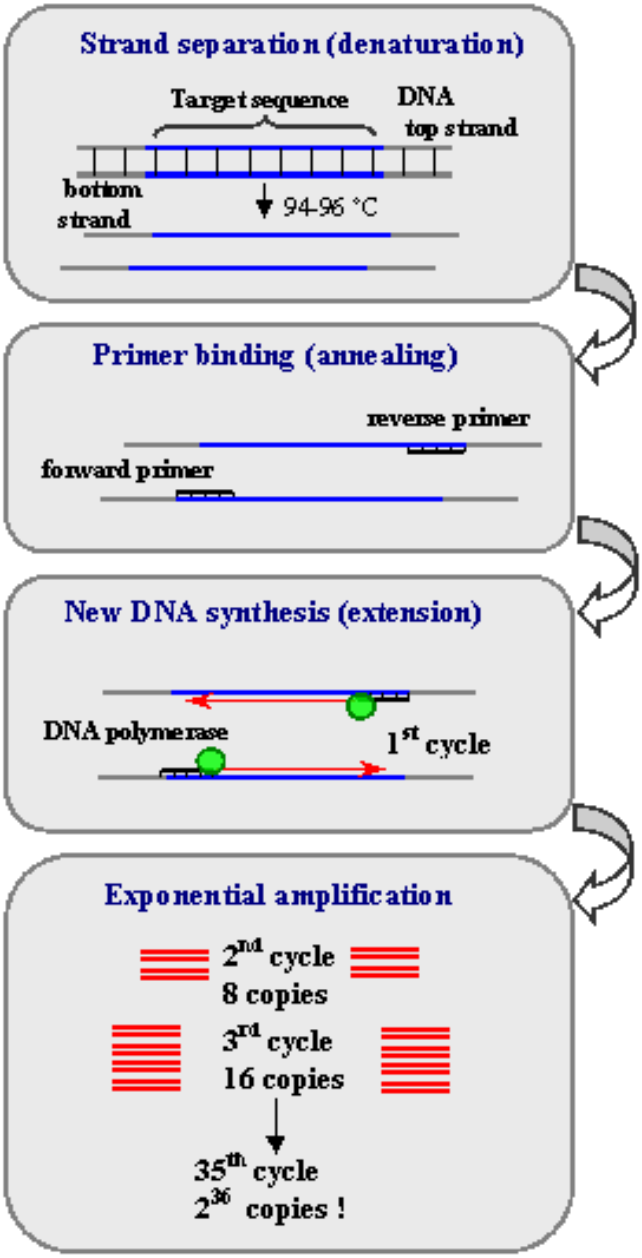

Figure: Different steps in PCR leading to exponential increase in the number of amplicons.

Source: http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechPCR.shtml

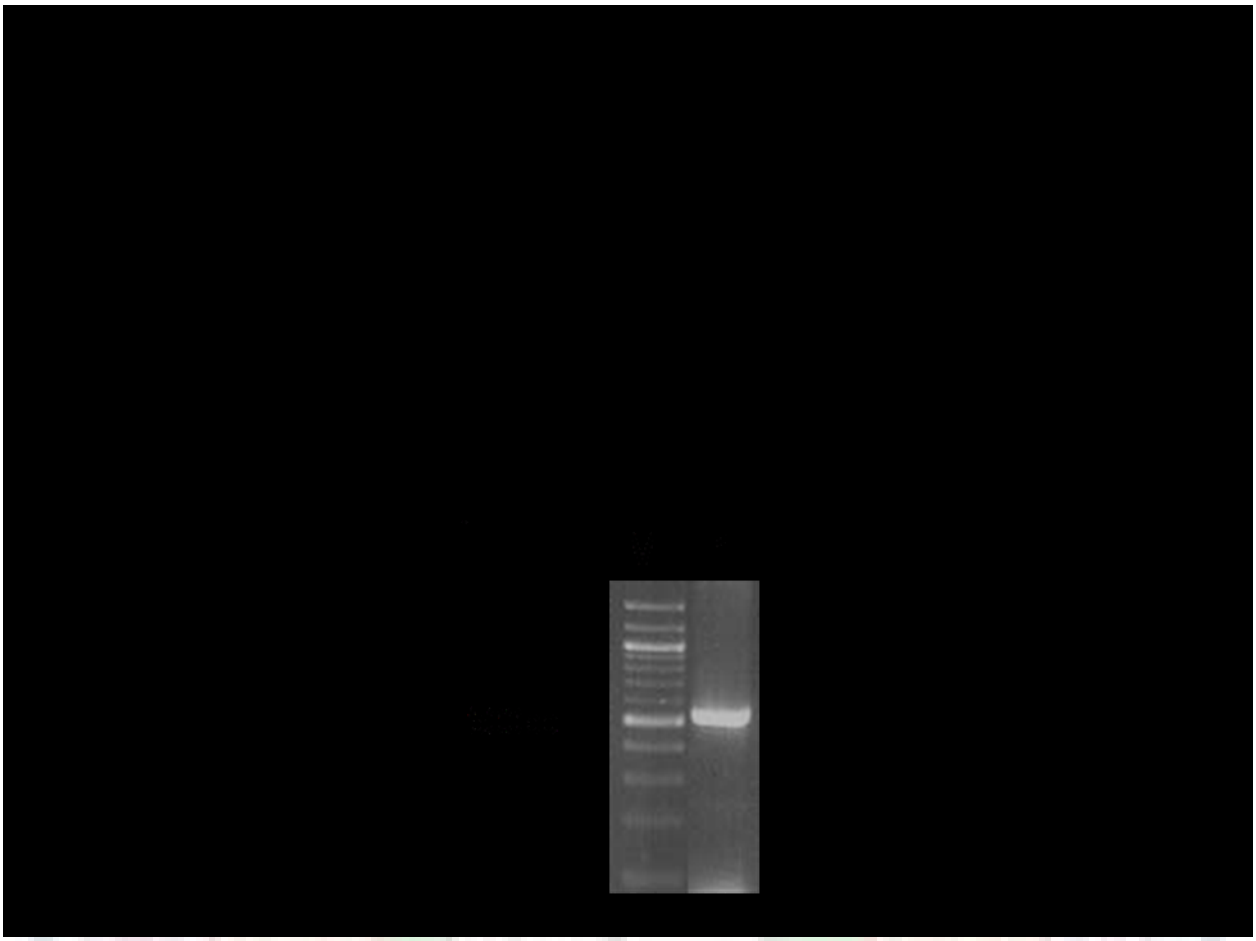

Figure: A representation for setting up the PCR for amplification of the desired gene which is nearly 500 bp long. The agarose gel shows M which is the 100bp ladder and 1, is the sample which shows amplification of the 500 bp band. The template here was genomic DNA from *Arabidopsis thaliana.*

Source: Dr. Parul Agarwal, Department of Genetics, University of Delhi, South Campus.

Agarose Gel Electrophoresis

Agarose gel electrophoresis can resolve DNA or RNA by size. DNA/RNA is visible in the gel when ethidium bromide is added during the gel casting process. EtBr intercalates between dsDNA or dsRNA and absorbs invisible UV light and emits visible orange light. SYBR Green absorbs blue light (λmax = 488 nm) and emits green light (λmax = 522 nm) may be used in place of EtBr, although more commonly this dye is used for qPCR.

- 0.7% gel will show good separation (resolution) of large DNA fragments (5–10kb)
- 2% gel will show good resolution for small fragments (0.4–1kb)
- 3% gel or vertical polyacrylamide gel for separating smaller fragments (the higher the % gel, the more brittle the gel)

Gel Casting

The volume of agarose required for a minigel is around 30–50mL.

- Weigh out 0.5g of agarose into a 250mL conical flask. Add 50mL of 0.5xTBE, swirl to mix.
- Microwave for about 1 minute to dissolve the agarose. Use gloves to handle and do not let it over boil; molten agarose is very hot.
- Let cool for 5 minutes or warm to touch with bare hands.
- Add 1μL of ethidium bromide (10mg/mL) and swirl to mix.
- Pour the gel into the resevoir. Push any bubbles away to the side using a disposable tip. Insert the comb. Wait at least 30-60 min.
- Add 0.5x TBE buffer into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer.
- Load samples and run the gel no greater than 5 Volt/cm.

2L of 10xTBE

- 218g Tris base
- 110g Boric acid
- 9.3g EDTA

Dissolve the ingredients in 1.9L of distilled water. Set the pH to about 8.3 using NaOH and make up to 2L.

Loading buffer

- 25mg bromophenol blue or xylene cyanol (Bromophenol blue migrates @ ~200–400bp. Xylene cyanol migrates ~4kb)
- 4g sucrose
- H2O to 10mL

Store at 4°C or -20

Source: http://openwetware.org/wiki/Griffin:Nested_RT-PCR

Variants of PCR

Most commonly used PCR variants are:

Long accurate- PCR (LA-PCR)

This method is used to amplify very long stretches of DNA efficiently. Under normal conditions *Taq* polymerase can amplify efficiently up to 3 kb of DNA. The *Taq* polymerase have low processivity and also lack proof reading activity. By modifying temperature and pH conditions along with use of two DNA polymerases, one with normal polymerase activity and the other with proof reading activity (3'–5' exonuclease) enables us to amplify up to 10-12kb or more depending upon the enzyme used for amplification.

The principle of Long and accurate PCR
In 1992, investigators working on mixing and fusing various domains of different DNA polymerases showed that, when combined with the robust reliability of *Taq* DNA polymerase, these enzymes produced longer amplicons. Long and accurate polymerase chain reaction (LA PCR) refers to the production of amplified product longer than approximately3 kilobases (kb) with high fidelity. Long PCR mixtures typically yield PCR products with some tenfold fewer mutations than those observed in products resulting from conventional PCR. The mixture of DNA polymerases typically included either Taq or Klentaq1 (which have no 3'-exonuclease proofreading activity) as the major component and, as the minor component, an archaebacterial DNA polymerase (with proofreading activity) such as Deep Vent, Vent or Pfu1. Among other factors that improve LA PCR are the enzyme deoxyuridinetriphosphatase (dUTPase)2, which prevents the incorporation of dUTP, the deaminated form of deoxycytosine triphosphate (dCTP) into DNA, and the chemical betaine. Although introduced for amplification of high G-C content targets3, betaine, when included at surprisingly high concentrations, usually helps to promote long PCR up to at least 20 kb. Since the introduction of mixtures of DNA polymerases, most PCRs of any length have improved in reliability and in yield of product.
For detailed protocol visit:http://www.nature.com/nmeth/journal/v2/n11/full/nmeth1105-885.htmlv

Inverse PCR

This method is used to amplify DNA surrounding an insert sequence.This is used when only one internal sequence of the target DNA is known. It is therefore very useful in identifying flanking DNA sequences of genomic inserts.

One limitation of conventional PCR is that it requires primers complementary to both termini of the target DNA, but this method allows PCR to be carried out even if only one sequence is available from which primers may be designed.

Inverse PCR is especially useful for the determination of insert locations. For example, various retroviruses and transposons randomly integrate into genomic DNA. To identify the sites where they have entered, the known, "internal" viral or transposon sequences can be used to design primers that will amplify a small portion of the flanking, "external" genomic DNA. The amplified product can then be sequenced and compared with DNA databases to locate the sequence which has been disrupted.

The inverse PCR method involves a series of restriction digests and ligation, resulting in a looped fragment that can be primed for PCR from a single section of known sequence. Then, like other polymerase chain reaction processes, the DNA is amplified by the temperature-sensitive DNA polymerase. The steps may be summarized as below:

- A target region with an internal section of known sequence and unknown flanking regions is identified.
- Genomic DNA is digested into fragments of a few kilobases by a usually low-moderate frequency (6-8 base) cutting restriction enzyme.
- Under low DNA concentrations, self-ligation is induced to give a circular DNA product.
- PCR is carried out as usual, with primers complementary to sections of the known internal sequence.

Finally the sequence is compared with the sequence available in the data base.

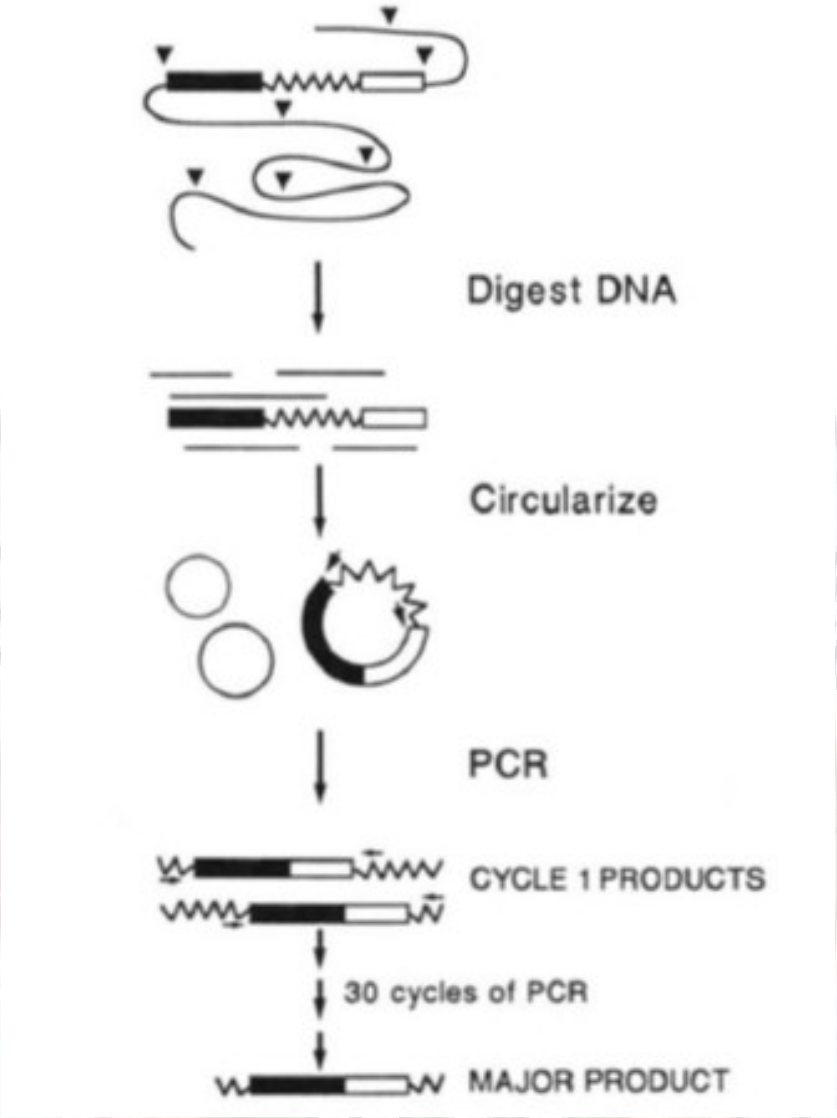

Figure: Diagrammatic representation of the method of inverse PCR

Source: Ochman, Howard, Anne S. Gerber, and Daniel L. Hartl. "Genetic applications of an inverse polymerase chain reaction." *Genetics* 120.3 (1988): 621-623. http://www.genetics.org/content/120/3/621.short (OLDER THAN 20 YEARS)

Hot Start PCR

Hot start PCR is modified form of Polymerase Chain Reaction. In this method the *Taq-polymerase* is added after the denaturation step. Specific antibodies are used to block this Taq-polymerase at initial stages. During the extension phase of PCR due to high

temperature, the antibodies detach and the activity of polymerase begins. This reduces amplification of non-specific products.

For details visit:http://nar.oxfordjournals.org/content/19/13/3749.extract

Nested PCR

In this method two sets of primers are used where in the first set amplifies a stretch of DNA and the second set of primers is used to amplify the desired DNA sequence within the first amplified product.

http://genome.cshlp.org/content/3/6/332.full.pdf?origin=publicationDetail

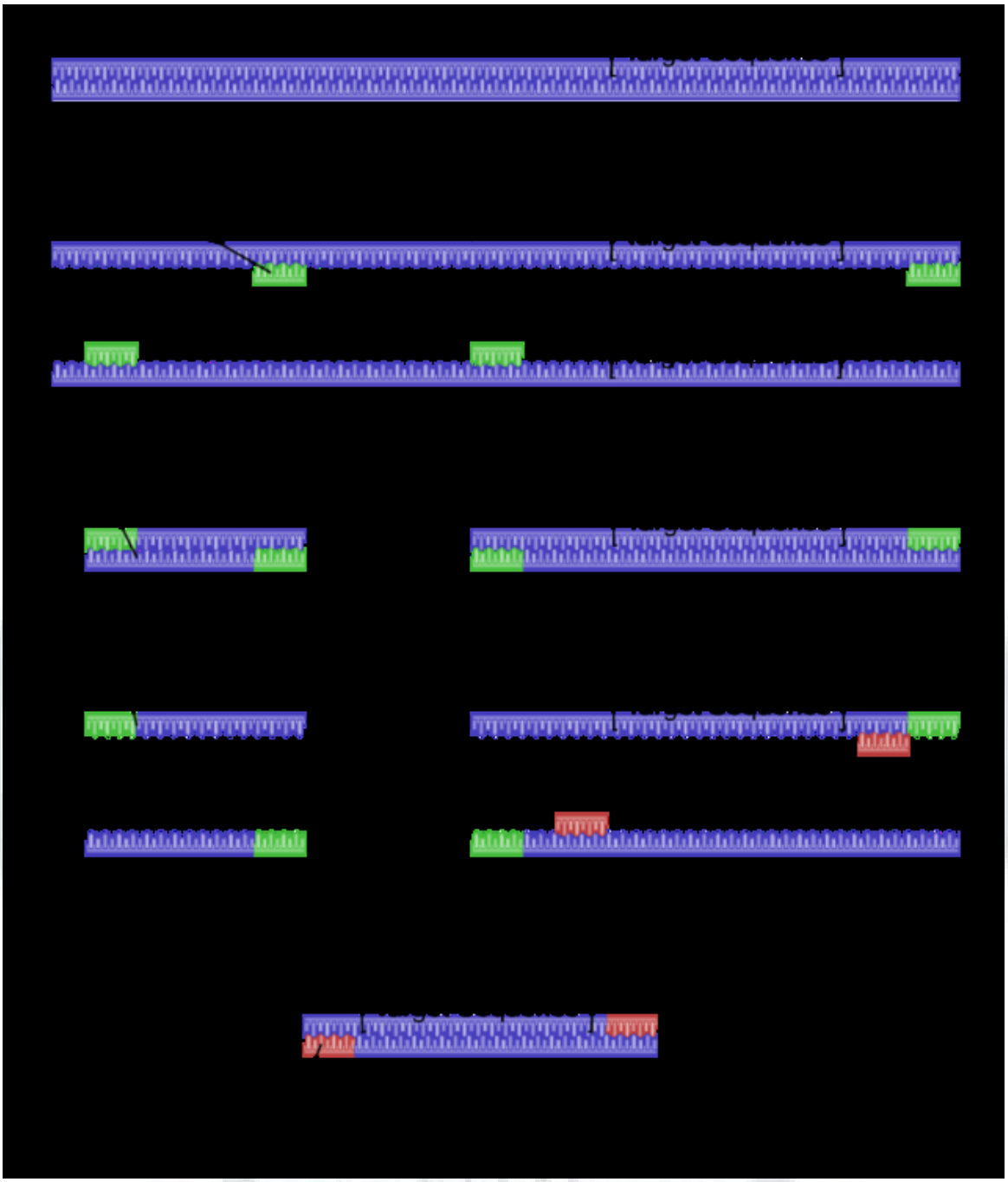

Figure: A diagrammatic representation of the Nested PCR.

Source:http://en.wikipedia.org/wiki/Nested_polymerase_chain_reaction#mediaviewer/File:Nested_PCR.png

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RT-PCR (Reverse Transcription PCR)

This method is used to amplify the DNA from RNA. In this method, the RNA is first reverse-transcribed to cDNA to provide the necessary DNA template for the DNA polymerase. Usually, Avian myeloblastosis virus (AMV) or Moloney murine leukaemia virus (MuLV) reverse transcriptases are used to make DNA copies from RNA template. RT-PCR is a sensitive method to find out the expression of RNA.

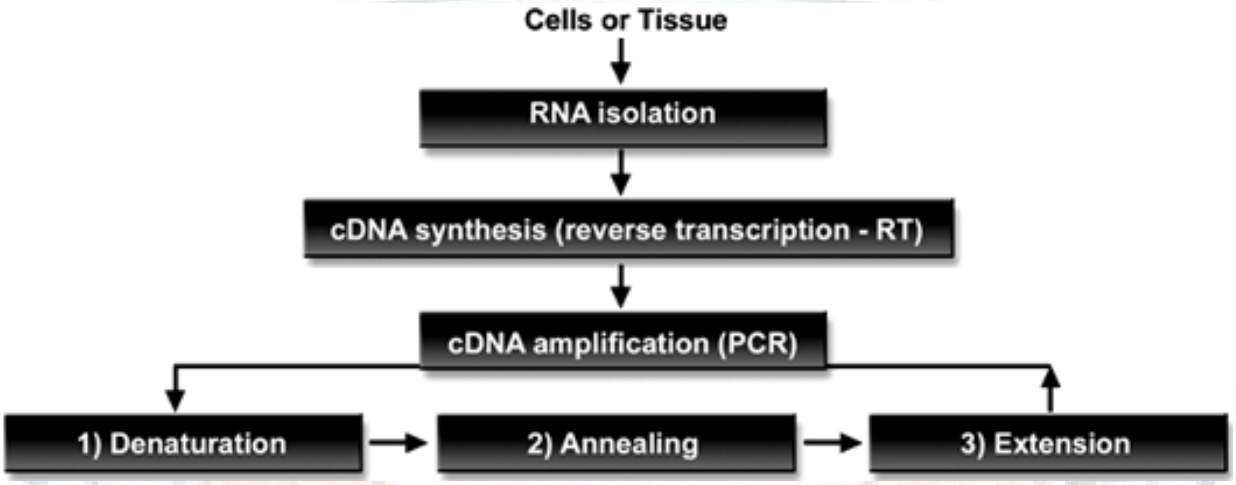

Figure: Steps invoved in RT-PCR.
Source:http://www.scielo.br/scielo.php?script=sci_arttext&pid=S16787572004000100002 (CC)

Video link:
http://www.bio.davidson.edu/courses/immunology/flash/rt_pcr.html

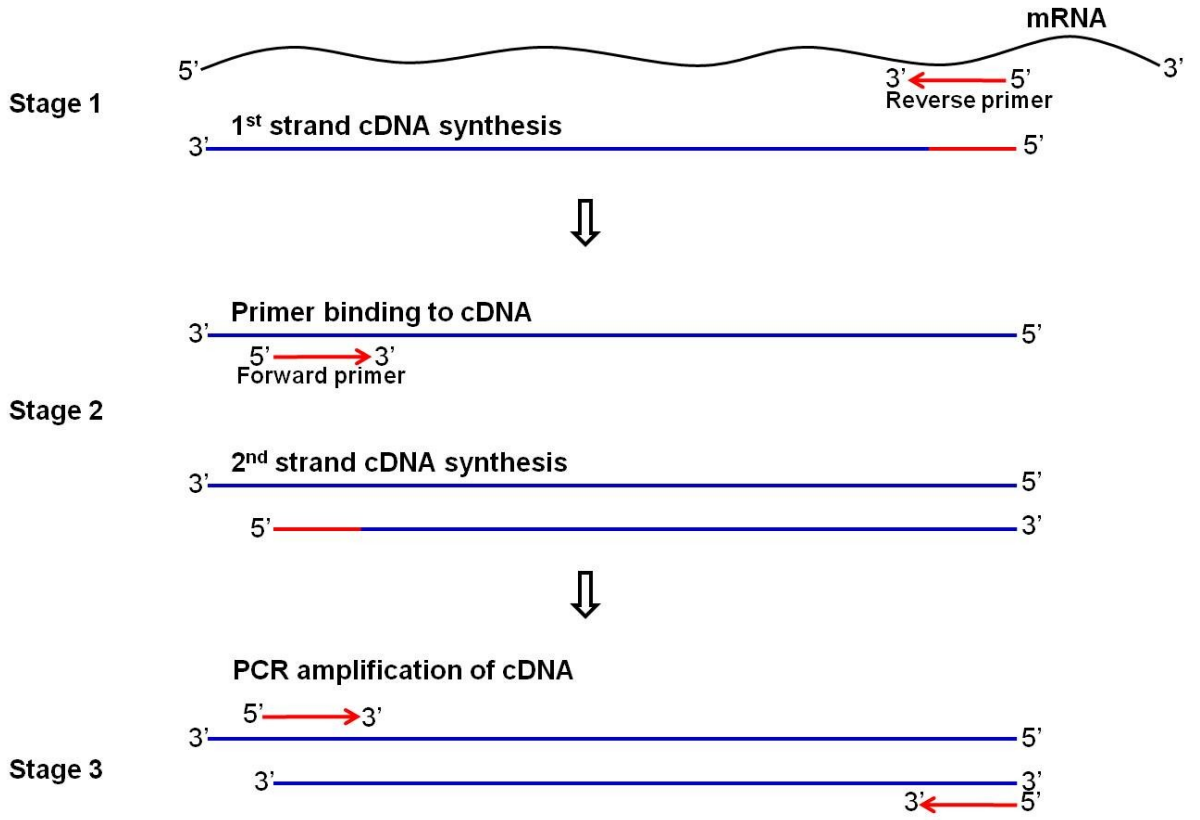

Figure: Schematic illustration showing steps involved in RT-PCR.

Source: http://cdn.intechopen.com/pdfs-wm/17719.pdf(CC)

Real time-qPCR (Quantitative PCR)

It measures the quantity of DNA amplified at each cycle. It gives the absolute quantification. It is also used to determine the copy number present in the sample. Two methods are generally used for detection

1. SYBR green: SYBR Green is a dye that binds the minor Groove of double stranded DNA. When SYBR Green dye binds to double stranded DNA, the intensity of fluorescent emission increases. As more double stranded amplicons are produced, SYBR Green dye signal increases

Visit page:

http://www.sigmaaldrich.com/life-science/molecular-biology/pcr/quantitative-pcr/sybr-green-based-qpcr.html

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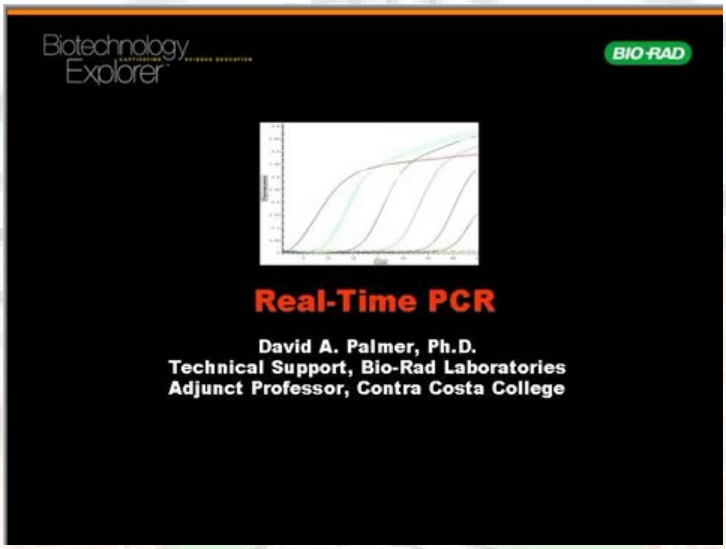
2. Taq man probes: This method uses a DNA-based probe which has a fluorescent reporter at one end and a quencher of fluorescence at the other. If the reporter and the quencher

come close to each other, the detection of its fluorescence is prevented. When the 5' to 3' exonuclease activity of Taq polymerase breaks the proximity of reporter-quencher proximity, fluorescence is emitted and can be detected after laser excitation. An increase in the amplified product, targeted by the probe after each PCR cycle results in a proportional increase in fluorescence due to release of the reporter caused by the breakdown of the probe.



Source:https://www.youtube.com/watch?v=h0yRrDWtdA4&index=9&list=PL110959E78716EA5D

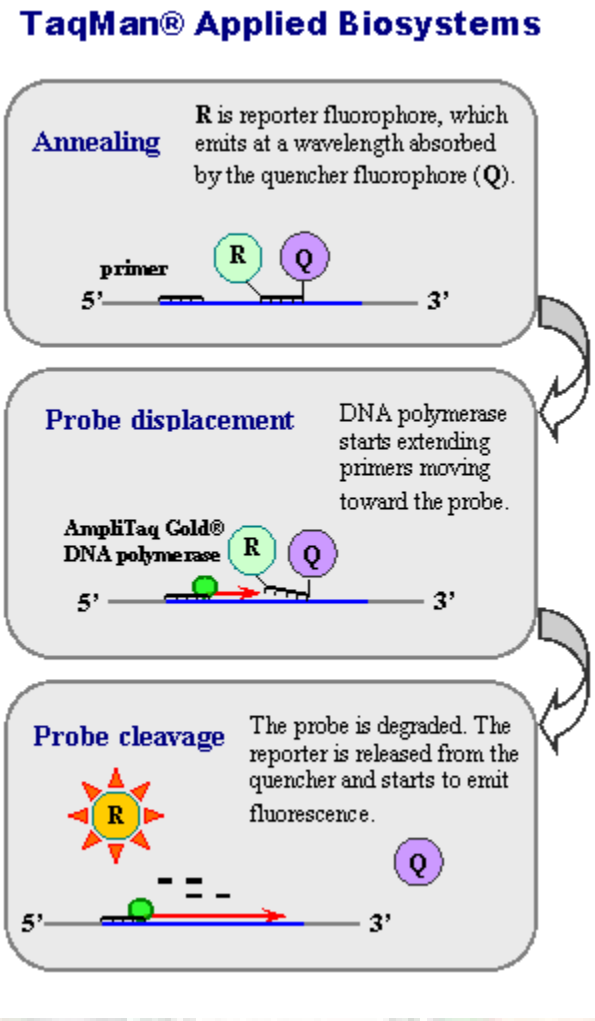


Figure: Steps involved in Real time RT-PCR using Taqman probe

Source: http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml

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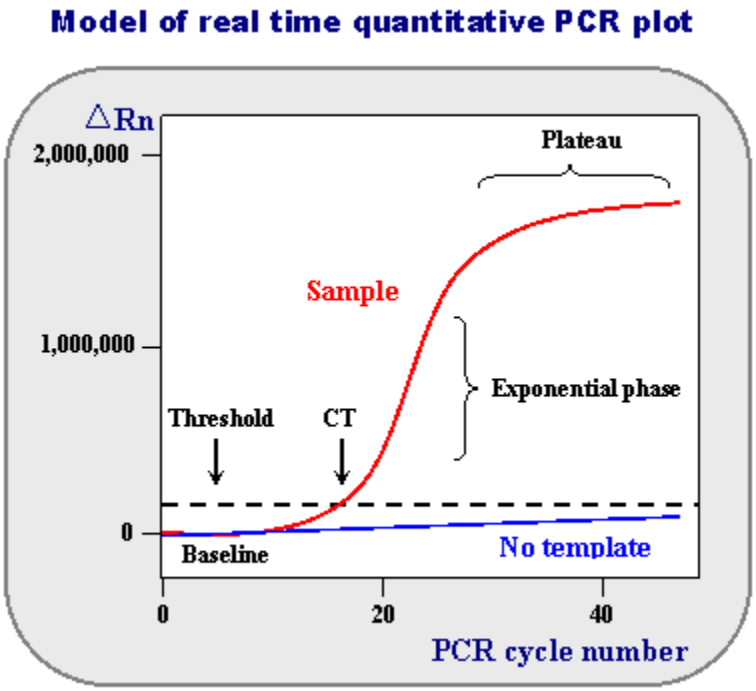

Figure: A model of real time RT-PCR plot.

Source: http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml

(cc)

Multiplex PCR

Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of different sizes that are specific to different DNA sequences. This PCR method can use to identify the multiple genes at once, which would otherwise require several different reactions and more time to perform. Annealing temperatures for each of the primer sets must be optimized to amplify within a single reaction, and the expected amplicon sizes should be different from each other so as to be able to be resolved by gel electrophoresis. Multiplex-PCR is for analysis of microsatellites and SNPs and forensic laboratories.

The primers to be used in multiplex PCR should be short in length (18-22 bases long)they should have similar melting temperature (55 $^{\circ}$C -60 $^{\circ}$C), high GC content, specificity and avoid primer dimers should not be formed in the entire pool of primers.

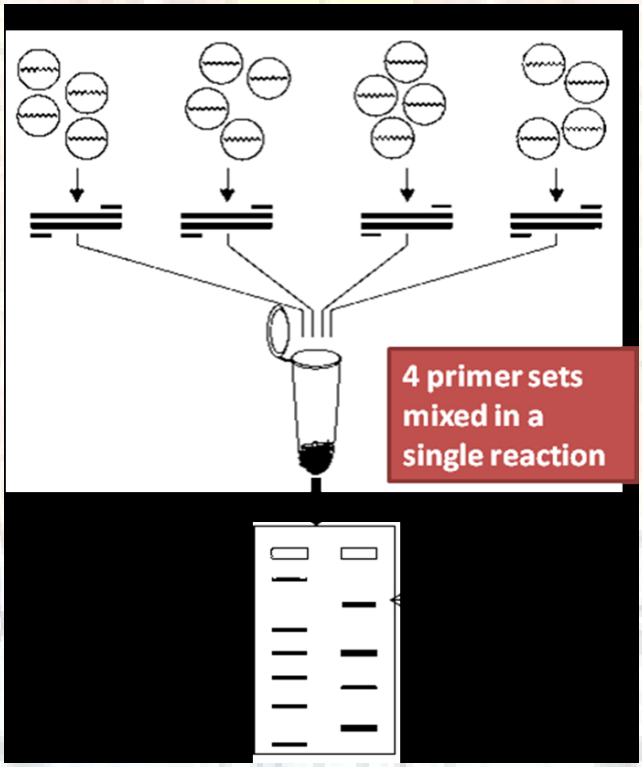

Figure: An example showing multiplex PCR

Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

Applications

Diagnosis of Genetic diseases

One of the important applications to diagnose the mutations that occurs in many genetic diseases like cystic fibrosis, phenylketonuria, muscular dystrophy, sickle cell anemia etc. The blood or cells are taken from the diseased patients and the DNA is extracted to carry out the PCR to identify the mutations causing the disease. It can be determined whether the individual is homozygous or heterozygous for a particular mutation. This information is especially useful for genetic counseling where infected or carrier parents of any particular disease can be identified and appropriately counseled on the basis of the results.

Detection of the bacterial and viral infections

The clinical specimens can be analyzed for the presence of DNA or RNA of pathogens. These days, PCR – based kits are available for testing of many disease causing bacteria and virus including HIV, Hepatitis, malaria and anthrax (*Bacillus anthracis*) and Human Papillomavirus. This method is advantageous over serologoical tests based on detection of antibodies because PCR – based can show presence of pathogen at a very early stage of infection while serological tests can only be done after some time of infection when a patient's body starts producing antibodies against the antigens.

Forensic studies

The method 'DNA fingerprinting' or 'DNA profiling' can be used by investigators to identify individuals as the DNA fingerprint of each individual differs from another. DNA fingerprinting can be easily carried out using DNA isolated from tissue samples like hair, blood, bone, semen etc. and PCR – based molecular markers, like VNTRs (Variable Number of Tandem Repeats) can be used to visualize the DNA profiles.

Research laboratories

PCR is so important for routine molecular biology work that today; it is difficult to imagine a lab without a PCR machine! PCR is used for various purposes in labs; some of them are listed below:

Gene cloning

Genomic DNA can be used to amplify a desired gene and further PCR can be used to add nucleotides forming restriction sites for particular restriction sites to a portion of amplified

DNA to enable its cloning into a vector. These days, commercial kits are available for high – efficiency PCR based cloning which are easy to use and require less time for cloning.
Visit page -
https://www.neb.com/applications/cloning-and-synthetic-biology/pcr-cloning

Identification of transgenics

PCR is used to identify the individuals which have been successfully transformed with foreign DNA in Recombinant DNA Technology experiments. Primers specific to the gene of interest are used to test the integration of the transgene into the host genome.

Gene expression studies

Real time RT – PCR can be used to study the level of expression of any gene during different developmental stages of an organism. RT-PCR can be used to study tissue specific gene expression.

DNA Sequencing

PCR is integral to all DNA sequencing methods. (Refer Chapter on DNA Sequencing for details.)

Marker-assisted plant breeding

Unlike the conventional methods relying on the phenotype of the plant, modern plant breeding methods use molecular markers to identify desirable traits in plants. Genotyping through molecular markers is done through PCR – based methods. (Refer Chapter on Molecular Markers).

Summary

Polymerase chain reaction is a technique used to generate millions of copies of a targeted stretch of DNA from a small amount of template DNA. It involves three main steps – the double stranded DNA is first denatured, followed by annealing of short stretches of oligonucleotides called primers, which are complimentary to the two opposite termini of the target sequence and lastly, the extension of the primers by a thermostable DNA polymerase to amplify the complete target region. The designing of primers is a crucial step in PCR. They should be 18 to 25 nucleotide long, have 40 – 60% of GC content and their melting

temperature should be around 55 to 65ºC. Another important factor in PCR is annealing temperature, which needs to be standardized for a new set of primers. If all the PCR conditions are optimum, a large number of copies of target DNA sequence can be generated.

There are numerous variations of PCR designed to suit various purposes, like inverse PCR, nested PCR, RT-PCR, Real time RT-PCR, multiplex PCR etc. There are various applications of PCR. In diagnostics, PCR can be used to test various genetic diseases and other diseases caused by different kinds of pathogens. In forensic science PCR can be used to identify criminals or can be used in paternity cases if DNA sample of the individuals in question is available, using DNA fingerprinting. Molecular biology laboratories use PCR for a variety of objectives like Recombinant DNA technology experiments, marker assisted breeding, gene expression studies and sequencing of DNA.

Exercises

- What is the principle of Polymerase Chain Reaction?
- What is meant by 'primer'?
- How does PCR lead to increase the copy number of target DNA sequence? Explain with the help of a diagram.
- What are the different steps in PCR?
- Why are divalent cations required in a PCR reaction?
- What is the need of buffer in PCR reaction?
- How would you decide upon which polymerase to use in a PCR reaction?
- Why does concentration of DNA reach a plateau phase after a certain number of cycles?
- Why would a researcher use RT-PCR?
- Differentiate between the inverse PCR and a normal PCR?
- Calculate the volume of reaction components to be added according to the given concentrations for a 25 µl reaction:

Reaction components	Stock concentration	Working concentration/Amount
DNA	10 ng/µl	30 ng
Taq Buffer	10 X	1 X

dNTPs	10 mM	800 µM
MgCl2	10 mM	800 µM
Primers	10 pmol/µl	10 pmol
Taq Polymerase	5 Units/µl	1 Unit
Water	-	To make up the volume

- Define Tm and how is it calculated?
- Elaborate: a Nested PCR; b Colony PCR; c Hot start PCR ; d Multiplex PCR.
- How is Pfu polymerase different from the Taq polymerase?
- What are the different applications of PCR?

Glossary

Agarose gel: A polysachharide matrix that is used to separate nucleic acid molecules according to their size.

Amplicon: A region of nucleic acid defined by two oligonucleotide primer annealing sites on the opposite strands.

Amplification: Increase in the copy number of a particular stretch of nucleic acid after undergoing an *in vitro* reaction.

Base: A cyclic nitrogen containing molecule which is the building block of nucleic acid molecule of DNA or RNA.

DNA Fingerprinting: A technique for generating a pattern of DNA showing DNA polymorphism. RFLP, microsatellites or minisatellites (VNTR) are generally used to generate a DNA fingerprint.

DNA sequencing: Methods to determine the sequence of bases in a DNA molecule.

Electrophoresis: A method to separate molecules in an electric field according to their net charge, shape and size.

Gene: The physical and functional unit of inheritance. Usually it is defined as a stretch of DNA that is responsible for synthesis of a protein.

Kilobase (kb): One thousand base pairs.

Primer: A short oligonucleotide complementary to a sequence in a larger nucleic acid molecule(template), which serves as a substrate for DNA polymerase in an *in vitro* reaction.

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