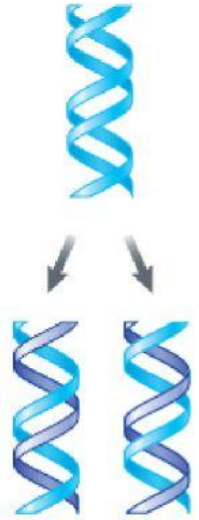
Polymerase Chain Reaction

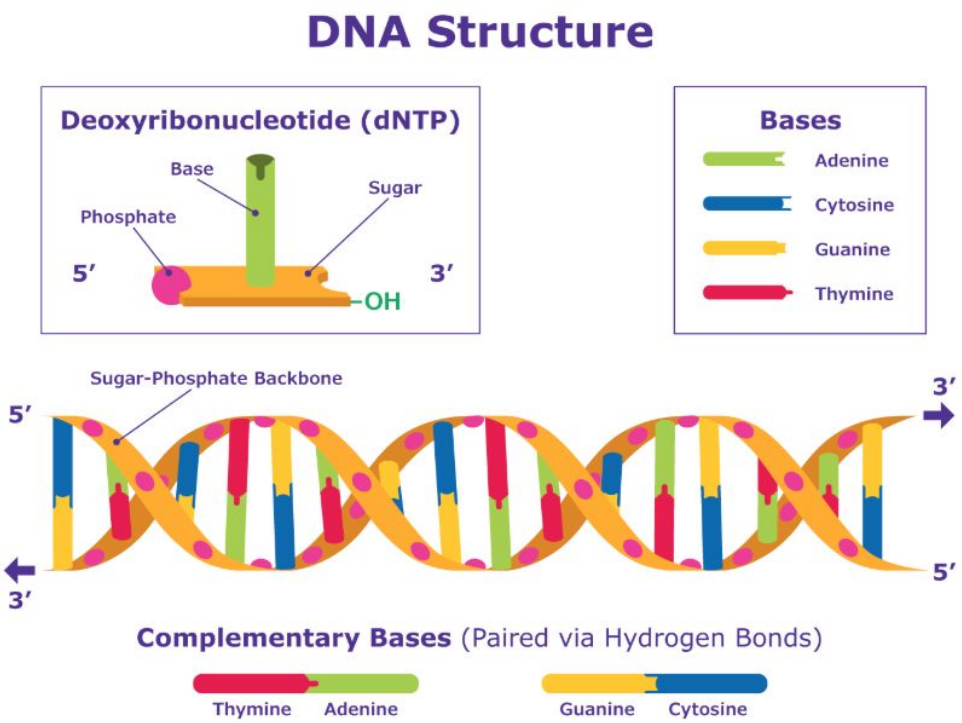
(make many copies of small sections of DNA? or a gene in vitro)



Dharmendra Pratap

Assistant Professor (Stage II)
Dept of Genetics & Plant Breeding
Ch. Charan Singh University, Meerut

Thermocycler



DNA (**A polynucleotide chain**) is a molecule composed of two strands that coil around each other to form a double helix. **Each strand is made up of a string of molecules called deoxyribonucleotides (dNTPs).**

Each dNTP contains a **phosphate group**, a pentose **sugar group (deoxyribose)**, and **one of four nitrogenous bases** [adenine (A),thymine (T), guanine (G), or cytosine (C)].

The dNTPs are strung together in a linear fashion by phosphodiester covalent bonds between the sugar of one dNTP and the phosphate group of the next; this **repeated sugar-phosphate pattern makes up the sugar-phosphate backbone.**

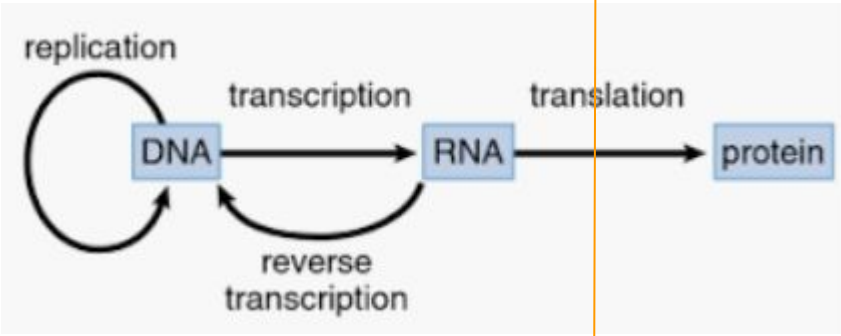
The **nitrogenous bases of the two separate strands are bound together by hydrogen bonds** between complementary bases to form the double-stranded DNA helix.

What is a DNA Polymerase ?

The DNA polymerase is an important biological molecule of the central dogma specifically, **in replication**. **It synthesises new DNA strand from the existing strand by adding dNTPs to the growing DNA.** The enzyme is discovered by Arthur Kornberg in 1956 (awarded Nobel prize for that in 1959).

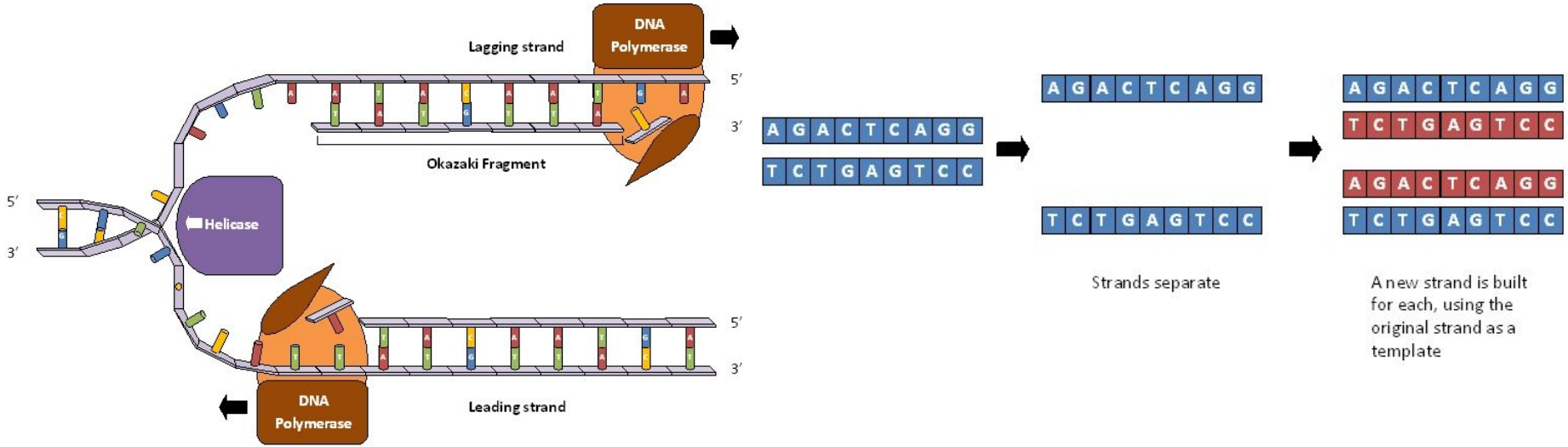
- The DNA polymerase has two important activity during replication: 5' to 3' polymerase activity (Klenow Fragment) and 3' to 5' exonuclease proofreading activity. As the polymerase binds to DNA, it adds nucleotide in a direction of 5' to 3' according to the Watson–Crick base pairing rule, i.e., A pairs with T (or U) and C with G.
- However, the polymerase incorporates some wrong non complementary nucleotides during replication results in a an "error." The error frequency or fidelity (accuracy) is an important characteristic of a polymerase.
- The 3' to 5' proofreading activity helps in removing this mismatch nucleotides. The polymerase moves back to one nucleotide, removes the mismatched nucleotide and reinsert the exact nucleotide match. The mechanism is called base excision repair.
- The polarity of the newly synthesized chain is antiparallel or opposite to that of the template.

CENTRAL DOGMA



NUCLEUS CYTOPLASM

Replication of DNA: By **DNA dependent DNA Polymerase** or simply DNA Polymerase
Replication of RNA: found in RNA viruses, Occurs through Viral RNA Polymerase
Transcription: By RNA Polymerase
Reverse Transcription: found in RNA viruses, Occurs through Reverse Transcriptase or **RNA dependent DNA Polymerase** leads to formation of complementary DNA (cDNA)
Translation: In cytoplasm with the help of Ribosomes.



DNA polymerase while performing DNA replication in cell

All prokaryotic and eukaryotic cells contain several DNA polymerases, which are used to replicate and safeguard the genetic material.

DNA Polymerase types (on the basis of template used)

a. DNA dependent DNA Polymerase e.g. DNA polymerase found in most cells for DNA Replication.
b. RNA dependent DNA Polymerase (RdDp) e.g. Reverse Transcriptase found in retroviruses

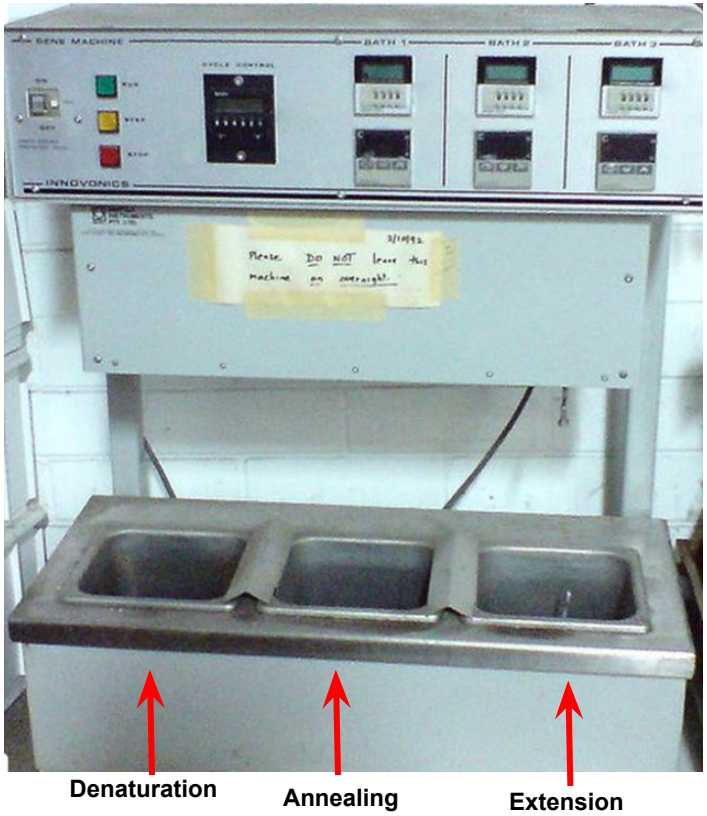
RNA Polymerase types

a. RNA dependent RNA Polymerase e.g. RNA polymerase found in RNA viruses for their replication
b. DNA dependent RNA Polymerase e.g. RNA polymerase found in most cells for transcription.

Thermus aquaticus “hot water”

A secret of PCR’s success is the **continuing evolution of the DNA polymerase** used to build new DNA strands using a template strand. The story of modern PCR begins in 1976 with the isolation of thermostable yet error prone enzyme, **Taq DNA polymerase** from the **thermophilic bacterium *Thermus aquaticus*** capable of repeat PCR cycling without the need to add fresh DNA polymerase after each cycle.

Taq DNA polymerase catalyzes the primer-dependent incorporation of nucleotides into duplex DNA in the 5′→3′ direction in the presence of Mg2+. **Taq does not possess 3′→5′ exonuclease activity** but has 5′→3′ exonuclease activity and therefore has less fidelity.



An early PCR machine which, rather than cycling through different temperatures, uses three different water baths at constant temperatures between which samples are moved with a robotic arm.

In the **initial phase of PCR** discovery, the PCR reaction was a very time-consuming process. All of the necessary PCR steps has to be performed manually.

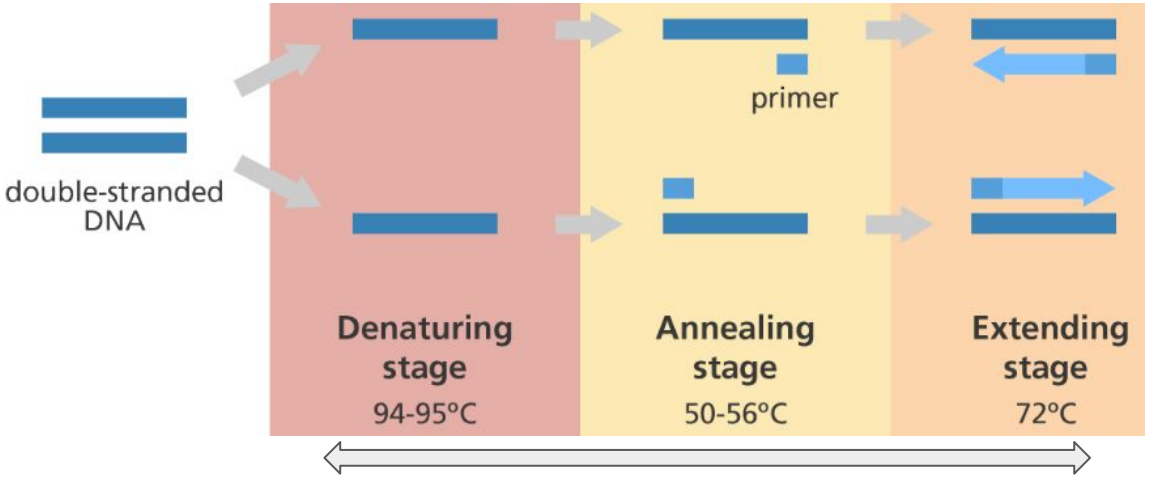
Many of these steps required moving the DNA sample back and forth between **three large water baths** of different temperatures for **Denaturation, Annealing and Extension.**

When PCR was first conducted in the laboratory, PCR enzymes (DNA polymerase i.e. the Klenow fragment) **were not heat-stable**; their highest activity is at 37°C, however, as temperature increase the activity decreases.

Therefore, **polymerase had to be added during each cycle manually** and the transfer of DNA samples between the different temperatures.

There are three main stages in polymerase chain reaction:

- **Denaturation**– when the double-stranded template DNA is heated (melted) to separate it into two single strands.
- **Annealing** – when the temperature is lowered to enable the DNA primers to attach to the template DNA.
- **Extension**– when the temperature is raised and the new strand of DNA is made by the DNA polymerase enzyme.



Cycle Repeated 30-35 times

The earliest thermal cyclers were designed for use with the Klenow fragment of DNA polymerase I.

Since this enzyme is destroyed during each heating step of the amplification process, new enzyme had to be added every cycle. This led to a cumbersome machine based on an automated pipettor, with open reaction tubes.

Later, the PCR process was adapted to the use of thermostable DNA polymerase from *Thermus aquaticus*, which greatly simplified the design of the thermal cycler.

While in some old machines the block is submerged in an oil bath to control temperature, in modern PCR machines a Peltier element is commonly used.

Quality thermal cyclers often contain silver blocks to achieve fast temperature changes and uniform temperature throughout the block.

Other cyclers have multiple blocks with high heat capacity, each of which is kept at a constant temperature, and the reaction tubes are moved between them by means of an automated process.

Miniaturized thermal cyclers have been created in which the reaction mixture moves via channel through hot and cold zones on a microfluidic chip. Thermal cyclers designed for quantitative PCR have optical systems which enable fluorescence to be monitored during reaction cycling.

The key players for modern PCR

Two significant advances have enabled PCR to become the technology it is today are:

Taq polymerase and the thermal cycler.

In 1986, Cetus (US based biotech company) scientists isolated the Taq polymerase from *Thermus aquaticus,* a bacterium found in hot springs. Because **Taq could withstand high temperatures, it removed the need for human intervention during the reaction,streamlining and shortening the process.** Without a heat-resistant enzyme like Taq polymerase, PCR could not be used on a large scale as the process would have been too cumbersome.

Prior to Taq DNA polymerase, Klenow fragment of DNA polymerase from E. coli was used and manually replaced in the second step of PCR as it became denatured from the heat .

In 1987, PerkinElmer, another US-based biotech company, launched a thermal cycler, an instrument that is programmed to regulate the temperature of a reaction, heating or cooling the samples as needed.

DNA polymerase in given suitable conditions, can perform DNA synthesis or polymerisation *in vitro* (outside living cell) with the help of dNTPs and chemically synthesized oligonucleotide usually of DNA called a primer.

Kary Mullis

American Biochemist

In 1980s , Kary Mullis (who shared the 1993 Nobel Prize for chemistry for inventing PCR) and his group at Cetus Corporation (first recombinant DNA startup company) found a way to start and stop the polymerase action at specific points along a single strand of DNA.

Kary Mullis conceived the chain reaction that looked possible by adding polymerase to a DNA molecule in the presence of short oligonucleotide primers and nucleotide triphosphates. The chain reaction (a series of events, each caused by the previous one) leads the target DNA exponentially amplified.

The starting material for PCR is the "target sequence," or segment of DNA or any gene.

The complementary strands of a double-stranded molecule of DNA are separated by heating also known as melting.

Two small pieces of synthetic DNA, each complimenting a specific sequence at one end of the target sequence, serve as primers.

Each primer binds to its complementary sequence. Polymerases start at each primer and copy the sequence of that strand.

Within a short time, exact replicas of the target sequence have been produced.

In subsequent cycles, double-stranded molecules of both the original DNA and the copies are separated; primers bind again to complementary sequences and the polymerase replicates them.

At the end of many cycles, the pool is greatly enriched in the small pieces of DNA that have the target sequences, and this amplified genetic information is then available for further analysis.

PRIMER SELECTION

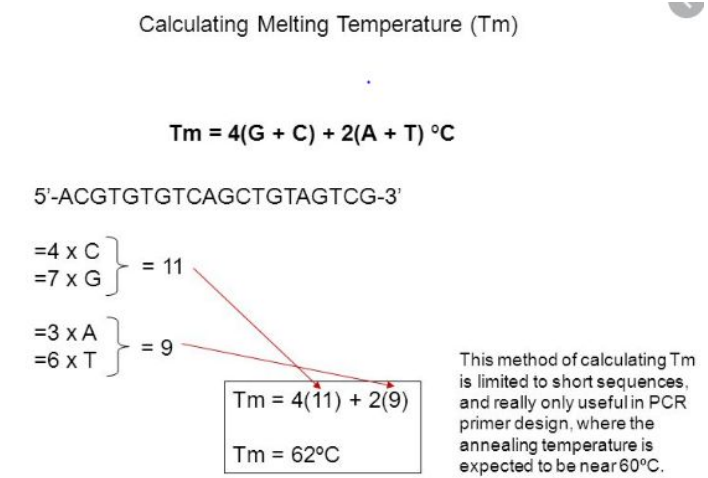
There is no set of rules that will ensure the synthesis of an effective primer pair. Yet it is the primers more than anything else that determine the success or failure of an amplification reaction. Fortunately, the majority of primers can be made to work and the following guidelines will help in their design.

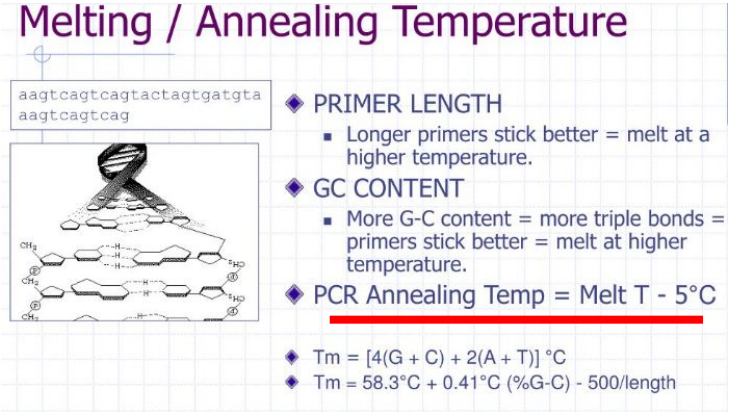
1. Where possible, *select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Try to avoid primers with stretches of polypurines, polypyrimidines, or other unusual sequences.*

2. Avoid sequences with *significant secondary structure, particularly at the 3'-end of the primer*.

3. *Check the primers against each other for complementarity*. In particular, avoiding primers with 3' overlaps will reduce the incidence of "primer dimer".

4. *Most primers will be between 20 and 30 bases in length* and the optimal amount to use in an amplification will vary. Longer primers may be synthesized but are seldom necessary.

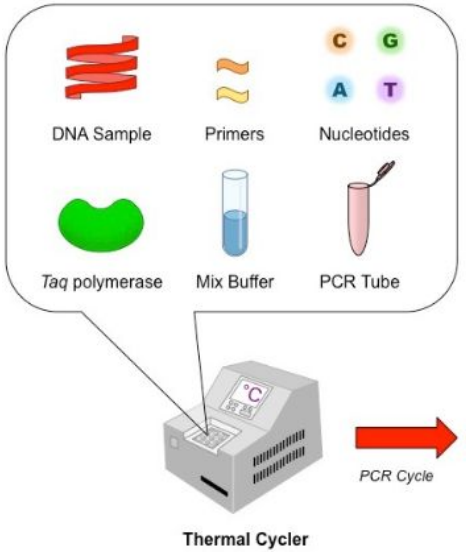




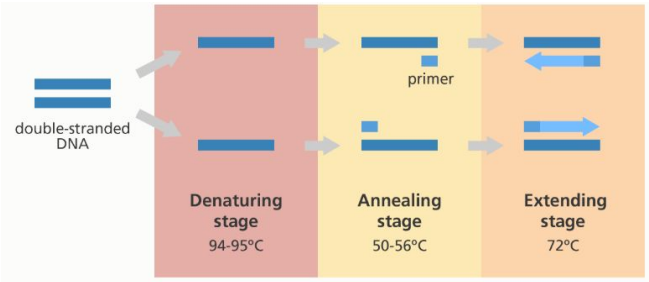
The Design and Optimization of the PCR

THE "STANDARD" REACTION

The standard PCR is typically done in a 25 or50 µl volume and in addition to the sample DNA, Buffer { 50 mM KCl, 10 mM Tris.HCl (pH 8.4 at room temp.), 1.5 mM MgCl2, 100 µg/ml gelatin} 0.25 µM of each primer, 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 units of Taq polymerase.



Reagent	Volume (µl)	Final concentration
Nuclease-free water	16.175	-
10X PCR buffer + $MgCl_2$	2.5	1X
10mM DNTP mix	0.4	200 µM each
10µM forward primer	0.4	0.2µM
10µM reverse primer	0.4	0.2µM
5U/µl Taq polymerase	0.125	0.5U
Template DNA	5	(≤1 µg/reaction)
TOTAL volume	**25**	



Thus, we can now understand that “**PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA**.”

A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase **results in the exponential accumulation** of a specific fragment whose termini are defined by the 5' ends of the primers.

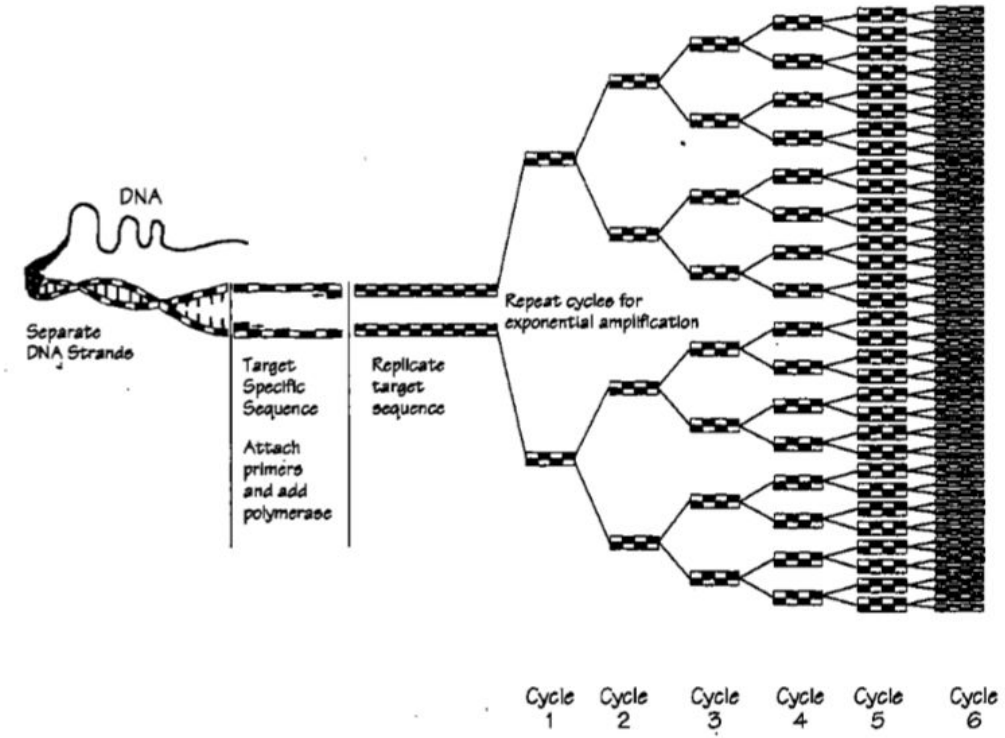
Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR yields 2 ^n i.e. 2^20 amplification.

This method, which was invented by Kary Mullis, was originally applied by a group in the Human Genetics Department at Cetus to the amplification of human beta globin DNA and to the **prenatal diagnosis of sickle-cell anemia**

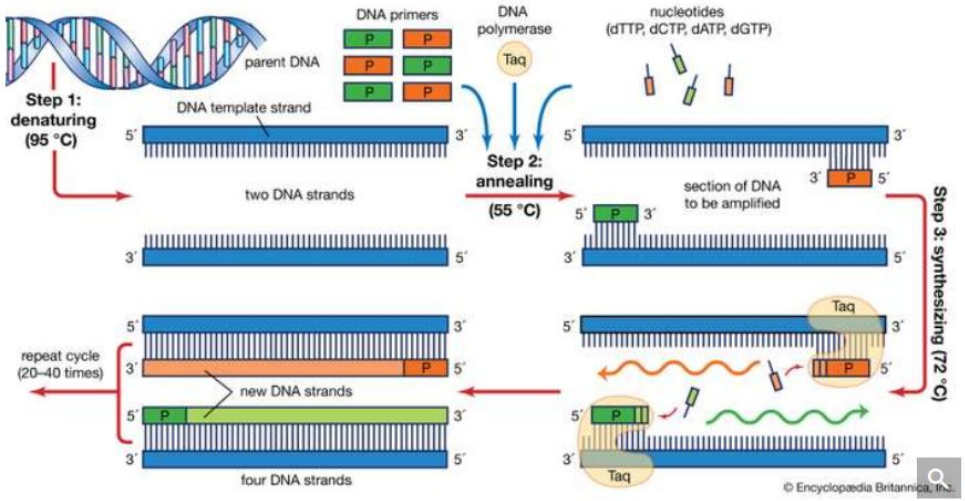
Although different template sequences may have a somewhat different "mutability" and different reaction conditions may influence the fidelity of the Taq polymerase, the original "high" error rate estimated for Taq polymerase PCR (-10^4) does not pose a problem for most applications.

CYCLES	COPIES
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
11	2048
12	4096
13	8192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824

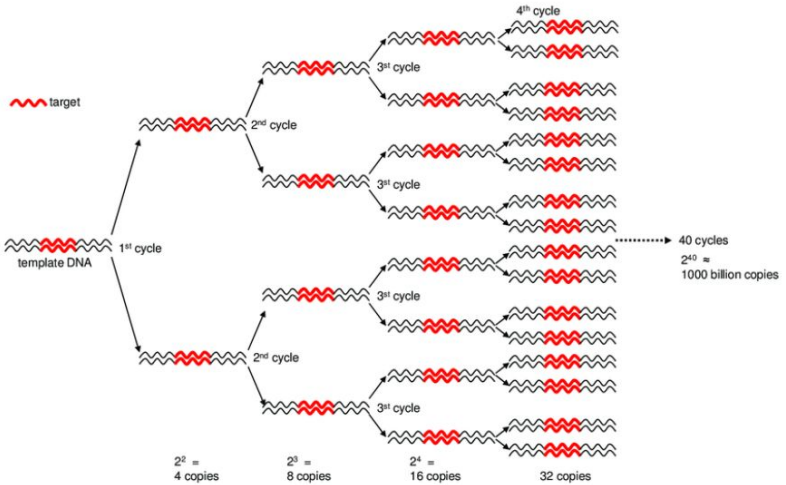
Exponential amplification



Polymerase Chain Reaction

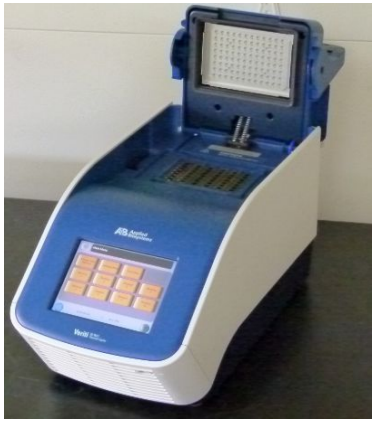


polymerase chain reaction



The exponential amplification of DNA in PCR.

WORKFLOW IN PCR EXPERIMENT



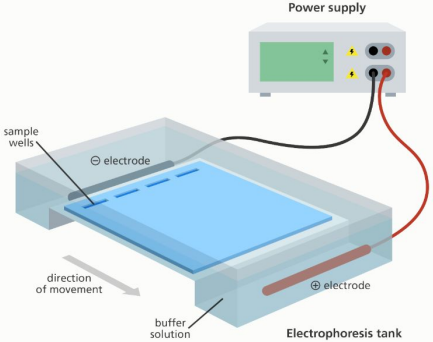


Illustration of DNA electrophoresis equipment used to separate DNA fragments by size. A gel sits within a tank of buffer. The DNA samples are placed in wells at one end of the gel and an electrical current passed across the gel. The negatively-charged DNA moves towards the postive electrode. Image credit: Genome Research Limited

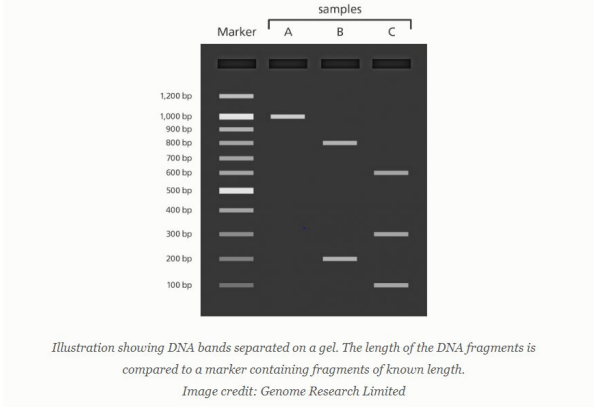
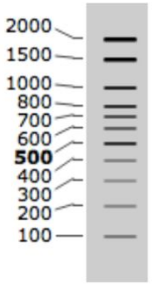
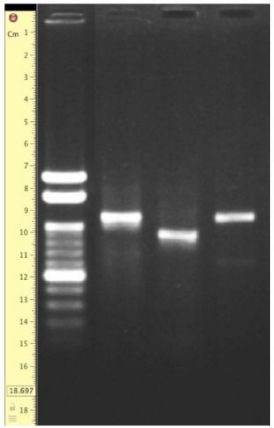


Illustration showing DNA bands separated on a gel. The length of the DNA fragments is compared to a marker containing fragments of known length.
Image credit: Genome Research Limited

Total Length DNA Marker 2 Kb
Mass = 0.3 µg/µL
Volume of Marker loaded = 15 µL
Volume of Sample loaded = 20 µL





THANKS

REFERENCE BOOK- Principles of Gene Manipulation and Genomics, 7th Edition
Sandy B. Primrose, Richard Twyman