

POLYMERASE CHAIN REACTION (PCR)



Polymerase Chain Reaction (PCR)

- PCR is a means to amplify a particular piece of DNA
 - *Amplify= making numerous copies of a segment of DNA*
- PCR can make **billions** of copies of a target sequence of DNA in a few hours
- PCR was invented in the 1984 as a way to make numerous copies of DNA fragments in the laboratory
- Its applications are vast and PCR is now an integral part of Molecular Biology

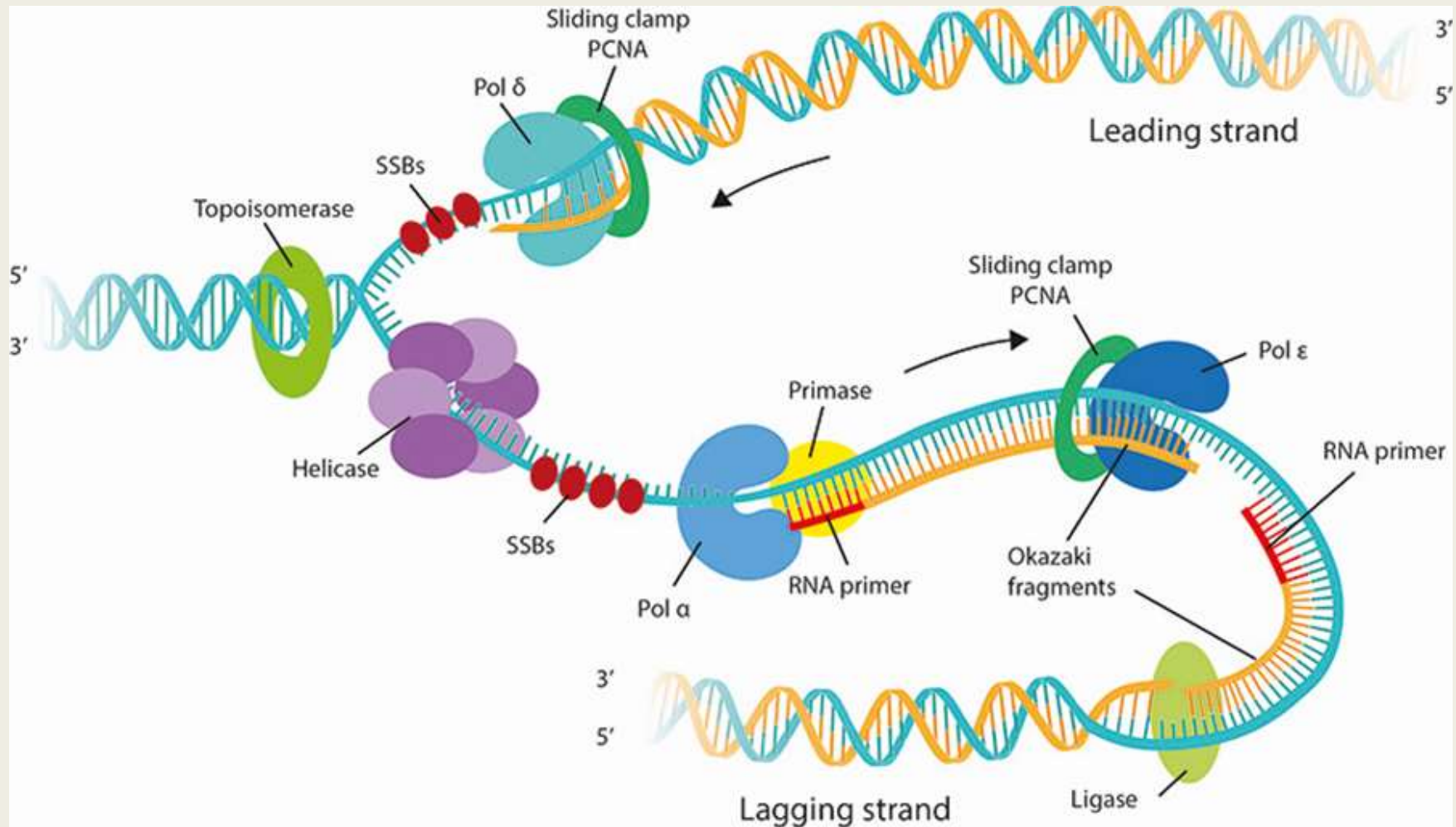
DNA Replication vs. PCR

- PCR is a laboratory version of DNA Replication in cells
 - The laboratory version is commonly called “*in vitro*” since it occurs in a test tube while “*in vivo*” signifies occurring in a living cell.

DNA Replication in Cells (*in vivo*)

- DNA replication is the copying of DNA
- It typically takes a cell just a few hours to copy all of its DNA
- DNA replication is semi-conservative (i.e. one strand of the DNA is used as the template for the growth of a new DNA strand)
- This process occurs with very few errors (on average there is one error per 1 billion nucleotides copied)
- More than a dozen enzymes and proteins participate in DNA replication
- **Key enzymes involved in DNA Replication**
 - DNA Polymerase
 - DNA Ligase
 - Primase
 - Helicase
 - Topoisomerase
 - Single strand binding protein

DNA Replication in Cells (*in vivo*)



DNA Replication enzymes:

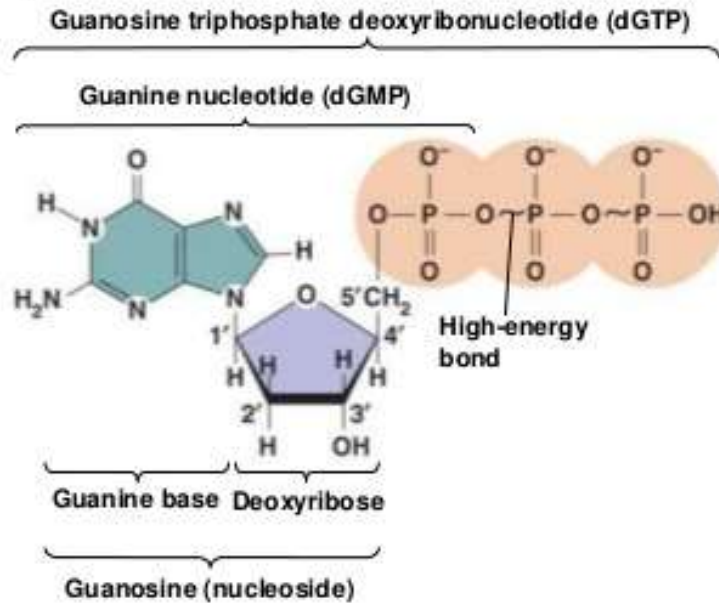
DNA Polymerase

- catalyzes the elongation of DNA by adding deoxynucleoside triphosphates to the 3' end of the growing strand
 - A deoxynucleotide triphosphate is a 1 sugar + 1 base + 3 phosphates
 - When a deoxynucleoside triphosphate joins the DNA strand, two phosphates are removed.
- DNA polymerase can *only* add nucleotides to 3' end of growing strand

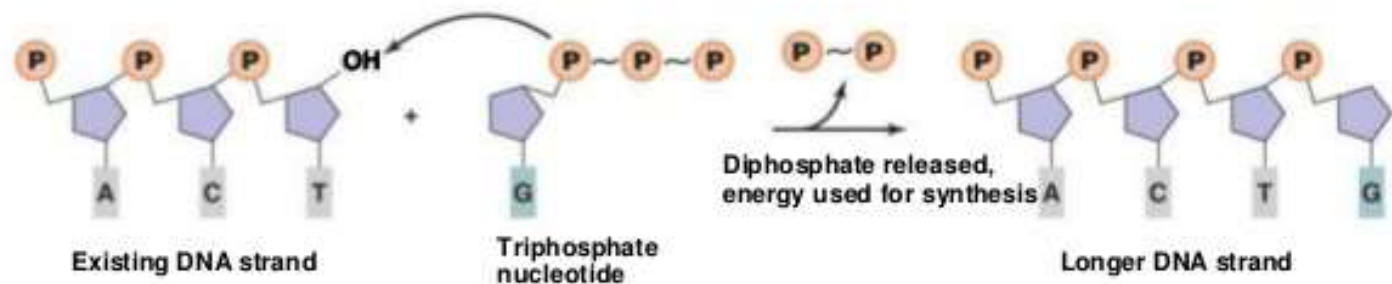
DNA replication

DNA Polymerase

Figure 7.5 The dual role of triphosphate deoxyribonucleotides as building blocks and energy sources in DNA synthesis.



(a)



(b)

Complementary Base-Pairing in DNA

- DNA is a double helix, made up of nucleotides, with a sugar-phosphate backbone on the outside of the helix.
- The two strands of DNA are held together by pairs of nitrogenous bases that are attached to each other via hydrogen bonds.
 - Adenine will only pair with thymine (2 H-bonds)
 - Guanine will only pair with cytosine (3 H-bonds)
- During replication, once the DNA strands are separated, DNA polymerase uses each strand as a template to synthesize new strands of DNA with the precise, complementary order of nucleotides.

DNA Replication enzymes:

DNA Ligase

- The two strands of DNA in a double helix are **antiparallel** (i.e. they are oriented in opposite directions with one strand oriented from 5' to 3' and the other strand oriented from 3' to 5')
 - *5' and 3' refer to the numbers assigned to the carbons in the 5 carbon sugar*
- Given the antiparallel nature of DNA and the fact that DNA polymerases can only add nucleotides to the 3' end, one strand (referred to as the **leading strand**) of DNA is synthesized continuously and the other strand (referred to as the **lagging strand**) is synthesized in fragments (called **Okazaki fragments**) that are joined together by **DNA ligase**.

DNA Replication enzymes:

Primase

- DNA Polymerase *cannot* initiate the synthesis of DNA
 - Remember that DNA polymerase can *only* add nucleotides to 3' end of an already existing strand of DNA
- In humans, **primase** is the enzyme that can start an RNA chain from scratch and it creates a **primer (a short stretch RNA with an available 3' end)** that DNA polymerase can add nucleotides to the strand during replication
- *RNA primer is subsequently replaced with DNA*

DNA Replication enzymes:

Helicase, Topoisomerase and Single-strand binding protein

- **Helicase** untwists the two parallel DNA strands
- **Topoisomerase** relieves the stress of this twisting
- **Single-strand binding protein** binds to and stabilizes the unpaired DNA strands

PCR: the *in vitro* version of DNA Replication

The following components are needed to perform PCR in the laboratory:

- 1) DNA of interest that contains the target sequence we want to copy
- 2) A heat-stable DNA Polymerase (like Taq Polymerase)
- 3) All four deoxynucleotide triphosphates (dNTP)
- 4) Buffers
- 5) Two short, single-stranded DNA molecules that serve as primers
- 6) Thin walled tubes
- 7) Thermal cycler (a device that can change temperatures dramatically in a very short period of time)

Old versus New method

Standard tube, ↑volume,
↑cost
evaporation & heat
transfer concerns



Thin walled tube, ↓ volume, ↓
cost
↓ evaporation & heat transfer
concerns



PCR

The DNA, DNA polymerase, buffer, deoxynucleoside triphosphates, and primers are placed in a thin-walled tube and then these tubes are placed in the PCR thermal cycler



PCR Thermocycler

The three main steps of PCR

- The basis of PCR is temperature changes and the effect that these temperature changes have on the DNA.
- In a PCR reaction, the following series of steps is repeated 20-40 times
(note: 25 cycles usually takes about 2 hours and amplifies the DNA fragment of interest 100,000 fold)

Step 1: **Denature** DNA

At 95°C, the DNA is denatured (i.e. the two strands are separated)

Step 2: Primers **Anneal**

At 40°C- 65°C, the primers anneal (or bind to) their complementary sequences on the single strands of DNA

Step 3: DNA polymerase **Extends** the DNA chain

At 72°C, DNA Polymerase extends the DNA chain by adding deoxynucleotides to the 3' ends of the primers.

Heat-stable DNA Polymerase

- Given that PCR involves very high temperatures, it is imperative that a heat-stable DNA polymerase be used in the reaction.
 - Most DNA polymerases would denature (and thus not function properly) at the high temperatures of PCR.
 - Initially PCR used the Klenow fragment of *E. coli* DNA polymerase - inactivated by high temperatures
- Taq DNA polymerase was purified from the hot springs bacterium *Thermus aquaticus* in 1976

DNA polymerase

- Taq has maximal enzymatic activity at 75 °C to 80 °C, and substantially reduced activities at lower temperatures.
- $K_{\text{cat}} = 150$ nucleotides/sec/enzyme (at T_{opt})

$Taq_{1/2} =$

92.5 °C  130 min

95.0 °C  40 min

97.5 °C  5 min

Pfu DNA polymerase

- ***Pfu* DNA polymerase** is an enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*
- Characterized by having superior thermostability and proofreading properties compared to *Taq* DNA polymerase.
- Unlike *Taq* DNA polymerase, *Pfu* DNA polymerase possesses 3' to 5' exonuclease proofreading activity, meaning that as the DNA is assembled from the 5' end to 3' end, the exonuclease activity immediately removes nucleotides misincorporated at the 3' end of the growing DNA strand.
- Consequently, *Pfu* DNA polymerase-generated PCR fragments will have fewer errors than *Taq*-generated PCR inserts

Magnesium Chloride

(MgCl_2 - usually 0.5-5.0mM)

Magnesium ions have a variety of effects

Mg^{2+} acts as cofactor for *Taq* polymerase

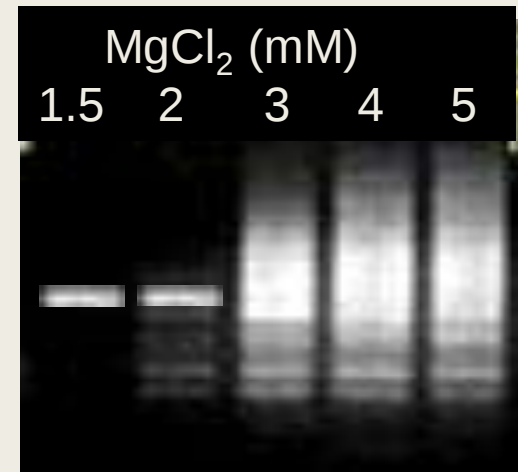
Required for *Taq* to function

Mg^{2+} binds DNA - affects primer/template interactions

Mg^{2+} influences the ability of *Taq* pol to interact with primer/template sequences

More magnesium leads to less stringency in binding

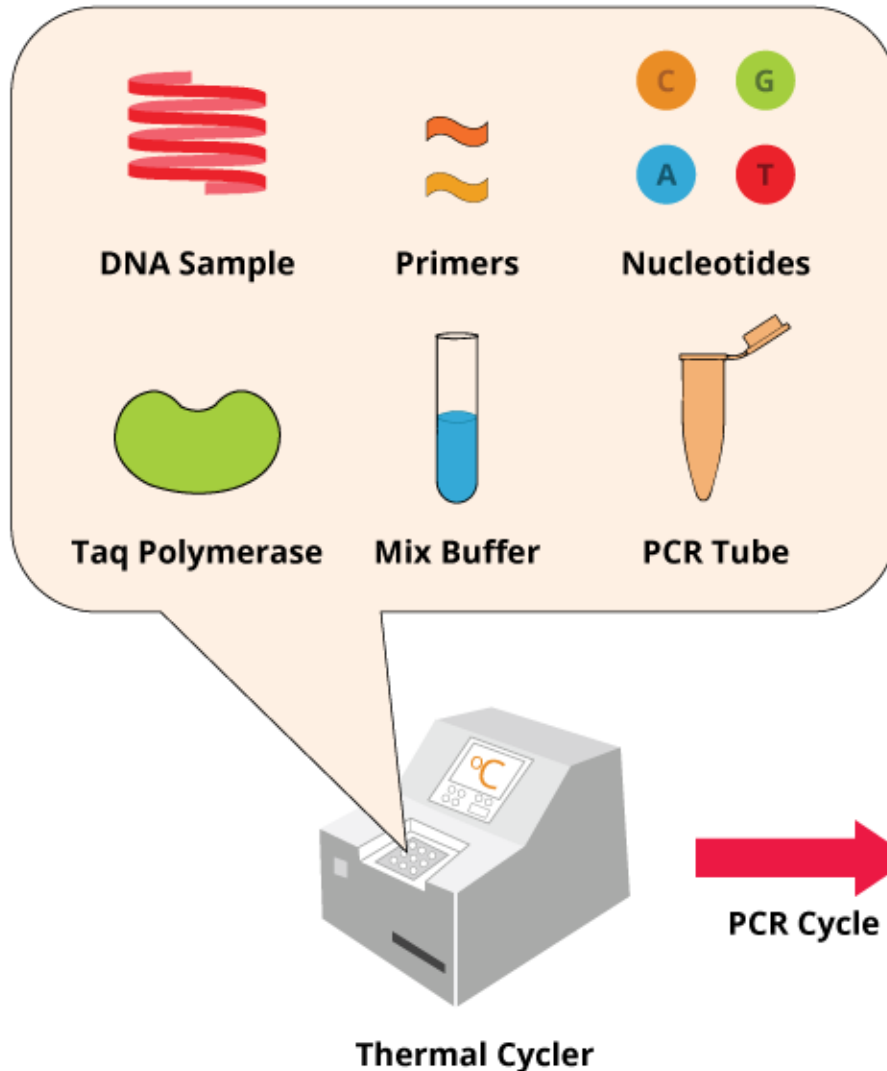
Manganese (Mn^{2+}) can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis



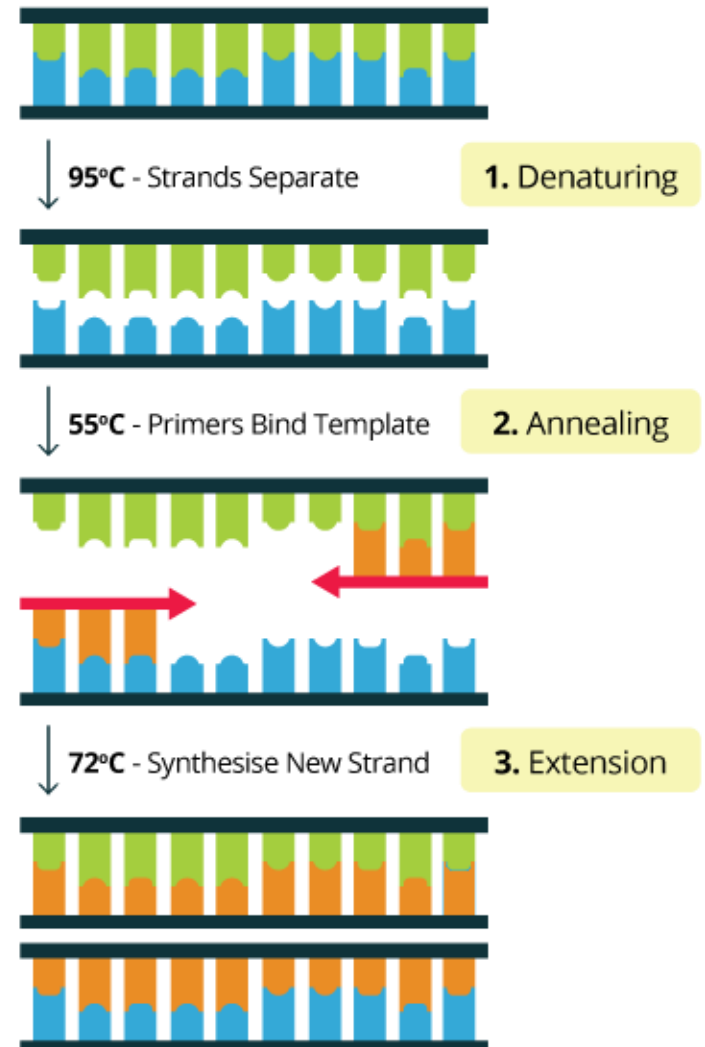
Primers

- PCR primers are short, single stranded DNA molecules (15-40 bp)
- They are manufactured commercially and can be ordered to match any DNA sequence
- Primers are sequence specific, they will bind to a particular sequence in a genome
- As you design primers with a longer length (15 → 40 bp), the primers become more selective.
- DNA polymerase requires primers to initiate replication

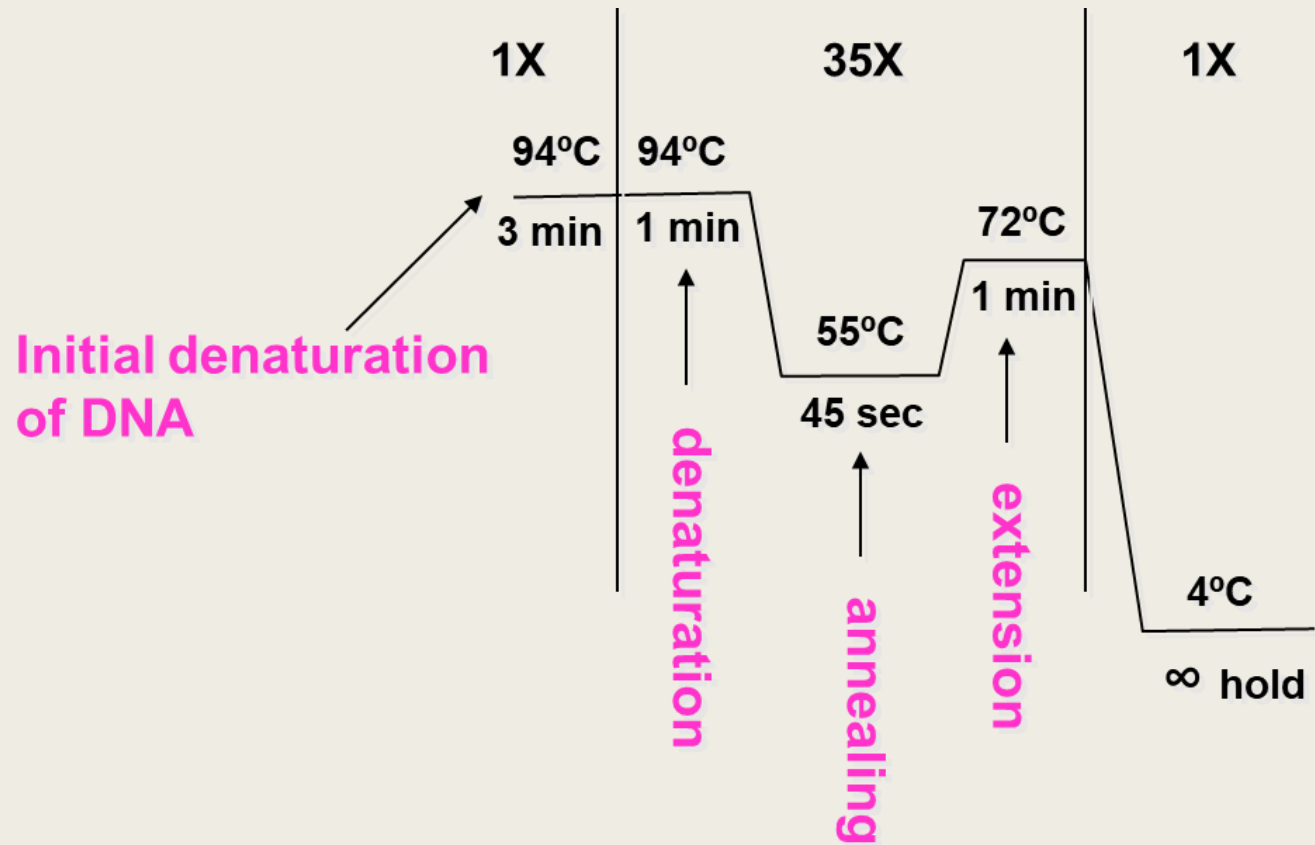
PCR Components



PCR Process (One Cycle)



PCR Protocol



PCR Problems

Taq is active at low temperatures

At low temperatures mis-priming is likely

SO The design of the primer is important

Temp	Extension Rate
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55° C	24 nt/sec
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37° C	1.5 nt/sec
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22° C	0.25 nt/sec		150 nucleotides in 10 min
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Primer Design

1. Typically 20 to 30 bases in length (sense and antisense)
2. Annealing temperature dependent upon primer sequence (~ 50% GC content)
3. Avoid secondary structure, particularly 3'
4. Avoid primer complementarity (primer dimer)
5. The last 3 nucleotides at the 3' end is the substrate for DNA polymerase - G or C
6. Many good freeware programs available

Selectivity of Primers

- Primers bind to their complementary sequence on the target DNA
 - *A primer composed of only 3 letter, ACC, for example, would be very likely to encounter its complement in a genome.*
 - *As the size of the primer is increased, the likelihood of, for example, a primer sequence of 35 base letters repeatedly encountering a perfect complementary section on the target DNA become remote.*

A Review of Probability

A COIN THROW

The probability of a heads (H) or a tails (T) is always 0.5 for every throw. What is the probability of getting this combination of tails in a row?

Event	Probability	
Tails	0.5	= 0.5
T,T	0.5 x 0.5	= 0.25
T,T,T	0.5 x 0.5 x 0.5	= 0.125
T,T,T,T,T	$(0.5)^5$	= 0.03125
T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{11}$	= 0.0004883
T,T,T,T,T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{16}$	= 0.00001526

So it become increasing unlikely that one will get 16 tails in a row (1 chance in 65536 throws). In this same way, as the primer increases in size the chances of a match other than the one intended for is highly unlikely.

Probability in Genetics

- There are 4 bases in the DNA molecule A,C,G,T
- The probability of encountering any of these bases in the code is 0.25 (1/4)
- So let us look at the probability of encountering a particular sequence of bases

Event	Probability
A	$0.25 = 0.25$
A,T	$0.25 \times 0.25 = 0.0625$
A,T,A	$0.25 \times 0.25 \times 0.25 = 0.015625$
A,T,A,G,G	$(0.25)^5 = 0.0009765$
A,T,A,G,G,T,T,T,A,A,C	$(0.25)^{11} = 0.000002384$
A,T,A,G,G,T,T,T,A,A,C,C,T,G,G,T	$(0.25)^{16} = 0.0000000002384$

So it become increasing unlikely that one will get 16 bases in this particular sequence (1 chance in 4.3 billion). In this same way, one can see that as the primer increases in size, the chances of a match other than the one intended for is highly unlikely.

Primer Design Software

Many free programs available online

OLIGO

PRIMER

PrimerQuest

DNA Star

Primer Dimers

- Pair of Primers

5'-ACGGATACGTTACGCTGAT-3'

5'-TCCAGATGTACCTTATCAG-3'

- Complementarity of primer 3' ends

5'-ACGGATACGTTACGCTGAT-3'

3'-GACTATTCCATGTAGACCT-5'

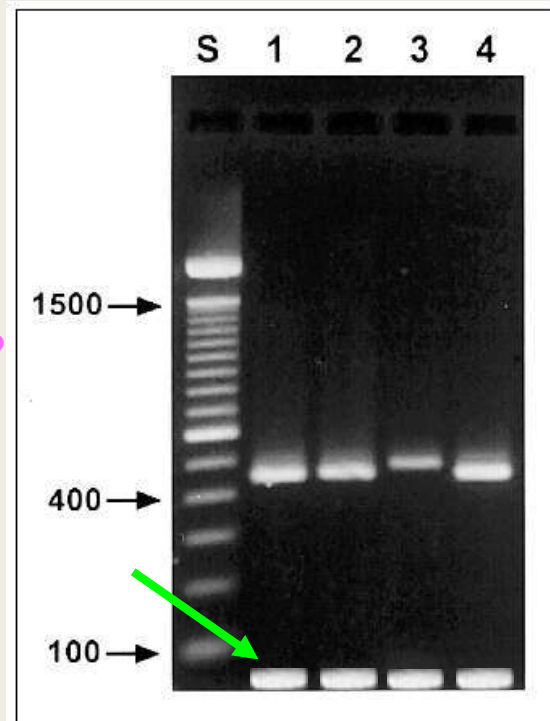
- Results in PCR product

Primer 1

5'-ACGGATACGTTACGCTGATAAGGTACATCTGGA-3'

3'-TGCCTATGCAATGCGACTATTCCATGTAGACCT-5'

Primer 2



Rules of thumb for PCR conditions

- Add an extra 3-5 minute (longer for Hot-start *Taq*) to your cycle profile to ensure everything is denatured prior to starting the PCR reaction
- Approximate melting temperature (T_m) = $[(2 \times (A+T)) + (4 \times (G+C))]$ °C
 - If GC content is < 50% start 5°C beneath T_m for annealing temperature
 - If GC content $\geq$ 50% start at T_m for annealing temperature
- Extension at 72°C: rule of thumb is ~500 nucleotide per minute. Use 3 minutes as an upper limit without special enzymes

Common PCR additives

BSA (usually at 0.1 to 0.8 $\mu\text{g}/\mu\text{L}$ final concentration)

Stabilize *Taq* polymerase & overcome PCR inhibitors

DMSO (usually at 2-5% v/v, inhibitory at $\leq 10\%$ v/v)

Denaturant - good at keeping GC rich template/primer strands from forming secondary structures.

Glycerol (usually at 5-10% v/v)

Increases apparent concentration of primer/template mix, and often increases PCR efficiency at high temperatures.

Stringency enhancers (Formamide, Betaine, TMAC)

Concentrations used vary by type

Enhances yield and reduces non-specific priming

Non-ionic detergents (Triton X, Tween 20 or Nonidet P-40) (0.1–1%)

NOT SDS (0.01% SDS cuts *Taq* activity to $\sim 10\%$ of normal)

Stabilize *Taq* polymerase & suppress formation of 2° structure

Typical PCR Temps/Times

Initial denaturation	90° – 95° C	1 – 3 min	
Denature	90° – 95° C	0.5 – 1 min	 25 – 40 cycles
Primer annealing	45° – 65° C	0.5 – 1 min	
Primer extension	70° – 75° C	0.5 – 2 min	
Final extension	70° – 75° C	5 – 10 min	
Stop reaction	4° C or 10 mM EDTA	hold	

Solution for mis-priming

- **Cheap fixes**
 - Physical separation – “DNA-in-the-cap”
 - Set up reactions on ice
- **Hot-start PCR – holding one or more of the PCR components until the first heat denaturation**
 - Manually - delay adding polymerase
 - Wax beads
 - Polymerase antibodies
- **Touch-down PCR** – set stringency of initial annealing temperature high, incrementally lower with continued cycling
- **PCR additives**
 - 0.5% Tween 20
 - 5% polyethylene glycol 400
 - betaine
 - DMSO

Troubleshooting PCR

Non-specific bands on your gel

Reagents, set-up

Template concentration inappropriate

Annealing temp too low

Extension time too short

Cycle number too high

Primer design not appropriate

Primer concentration too high

Non-specific priming

MgCl₂ concentration too high

GC-rich template, ↑ 2° structure

Contaminating DNA

Run negative control

Review guidelines

Optimize by gradient PCR

↑ time for longer products

Review guidelines

↑ specificity

Optimize by titration

↑ specificity, Hot Start

Optimize by titration

PCR additives

Decontaminate work area:

*use ARTs, wear gloves,
pipettor, reagents,
UV treat plastics*

Troubleshooting PCR

Diffuse smearing on your gel

Template concentration inappropriate

Review guidelines

Taq concentration too high

Optimize by titration

Extension time inappropriate

Review guidelines

Cycle number too high

Reduce, review guidelines

Primer design not appropriate

↑ specificity

Primer concentration too high

Optimize by titration

Non-specific priming

use Hot Start

MgCl₂ concentration too high

Optimize by titration

GC-rich template, ↑ 2° structure

PCR additives

Contaminating DNA

Decontaminate work area:

use ARTs, wear gloves,
pipettor, reagents,
UV treat plastics

Troubleshooting PCR

Poor or no amplification of bands

Problem with thermocycler, set-up, reagents

Enzyme concentration low

Annealing temp too low

Extension time too short
products

Cycle number too low

Primer design not appropriate

Primer concentration too high

Non-specific priming

MgCl₂ concentration too low

GC-rich template, ↑ 2° structure

Run positive control

↑ Concentration

Optimize by gradient PCR

↑ Time for longer

Review guidelines

↑ Specificity

Optimize by titration

↑ Specificity, Hot Start

Optimize by titration

PCR additives

Klenow fragment

- It is a large protein fragment produced when DNA polymerase I from *E. coli* is enzymatically cleaved by the protease **subtilisin**.
- It retains the 5' → 3' polymerase activity and the 3' → 5' exonuclease activity for removal of precoding nucleotides and proofreading, but loses its 5' → 3' exonuclease activity.
- The other smaller fragment formed when DNA polymerase I from *E. coli* is cleaved by subtilisin retains the 5' → 3' exonuclease activity but does not have the other two activities exhibited by the Klenow fragment (i.e. 5' → 3' polymerase activity, and 3' → 5' exonuclease activity)

Klenow fragment

- The Klenow fragment, which lacks 5' to 3' activity, can be very useful in research. The Klenow fragment is extremely useful for research-based tasks such as:
 - *Synthesis of double-stranded DNA from single-stranded templates*
 - *Filling in recessed 3' ends of DNA fragments to make 5' overhang blunt*
 - *Digesting away protruding 3' overhangs*
 - *Preparation of radioactive DNA probes*
- The Klenow fragment was also the original enzyme used for greatly amplifying segments of DNA in the polymerase chain reaction (PCR) process, before being replaced by thermostable enzymes such as Taq and Pfu polymerases

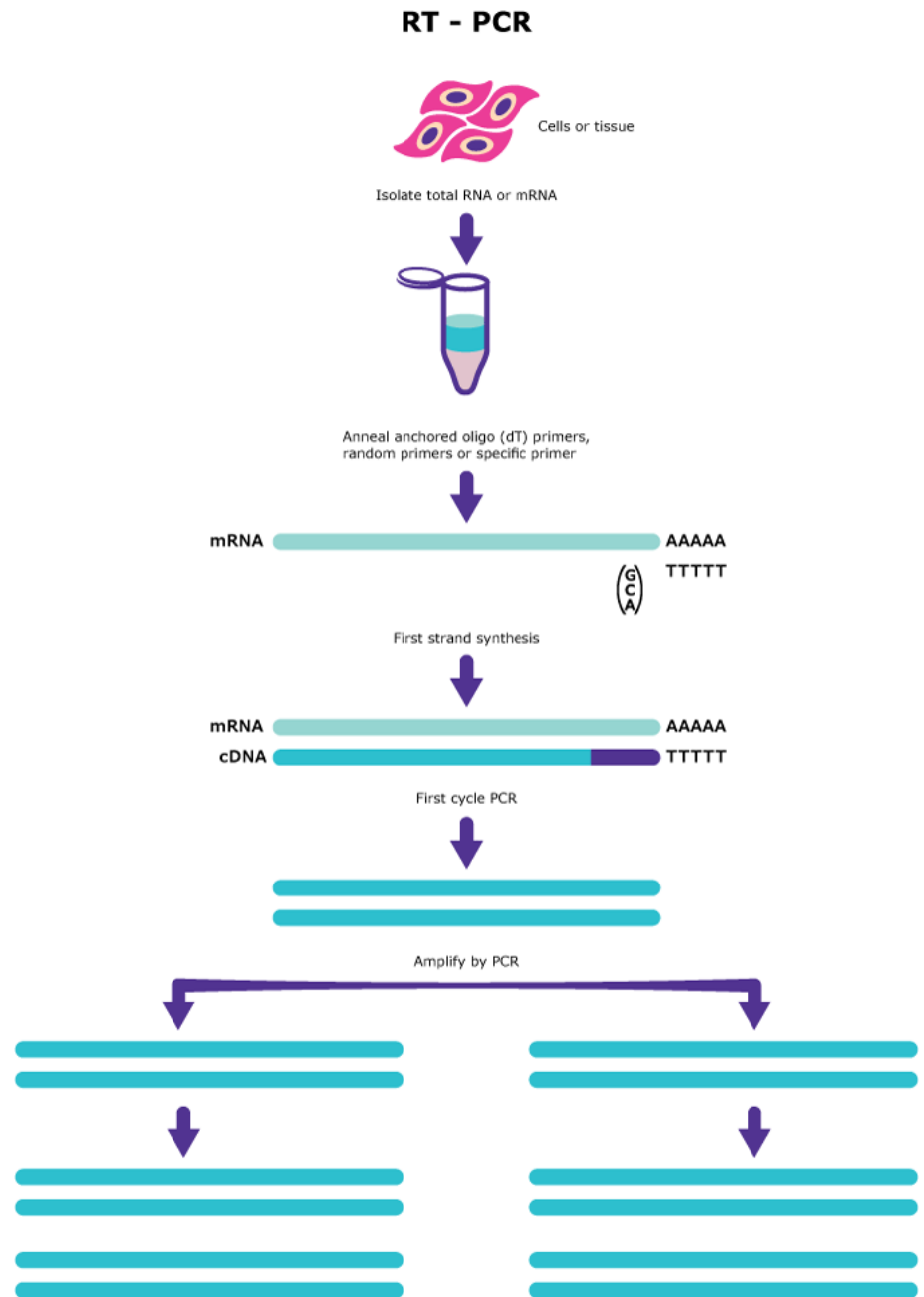
Klenow fragment

- Just as the 5' → 3' exonuclease activity of DNA polymerase I from *E.coli* can be undesirable, the 3' → 5' exonuclease activity of Klenow fragment can also be undesirable for certain applications. This problem can be overcome by introducing mutations in the gene that encodes Klenow. This results in forms of the enzyme being expressed that retain 5' → 3' polymerase activity, but lack any exonuclease activity (5' → 3' or 3' → 5'). This form of the enzyme is called the **exo- Klenow fragment**.
- The exo-Klenow fragment is used in some fluorescent labeling reactions for microarray, and also in dA and dT tailing, an important step in the process of ligating DNA adapters to DNA fragments, frequently used in preparing DNA libraries for Next-Gen sequencing

Reverse transcriptase- PCR

- RT-PCR is a PCR test that is designed to detect and measure RNA. Although initial PCR tests amplified DNA, many viruses and other biological components (for example, mitochondria) utilize RNA as their genetic material.
- RT-PCR differs from conventional PCR by first taking RNA and converting the RNA strand into a DNA strand.
- This is done by using an enzyme termed reverse transcriptase instead of the DNA polymerase which allows a single strand of RNA to be translated into a complementary strand of DNA.
- RT-PCR is used to detect and study many RNA viruses

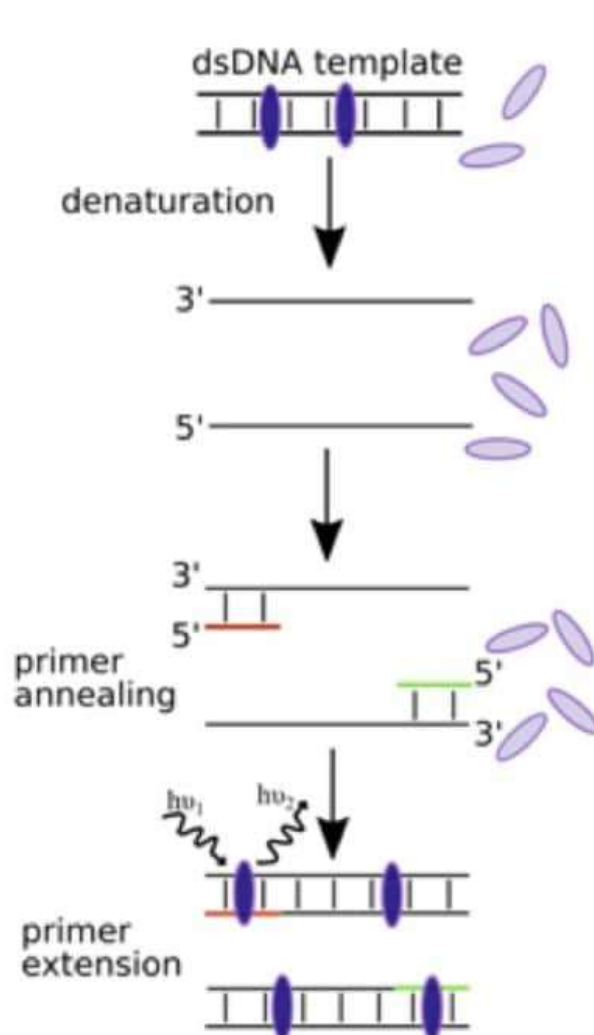
RT-PCR



Real time-PCR

- Real-Time PCR is a variation of PCR that allows analysis of the amplified DNA during the usual 40 cycles of the procedure. Although the procedure is similar to conventional PCR with cycling, Real-Time PCR uses fluorescent dyes attached to some of the building blocks or small nucleotide strands.
- Fluorescence occurs when the amplified DNA strands are formed. The amount of fluorescence can be measured throughout the 40 cycles, and allows the investigators to measure specific products and their amounts during the amplification cycles (no need for gel electrophoresis, producing more rapid results)

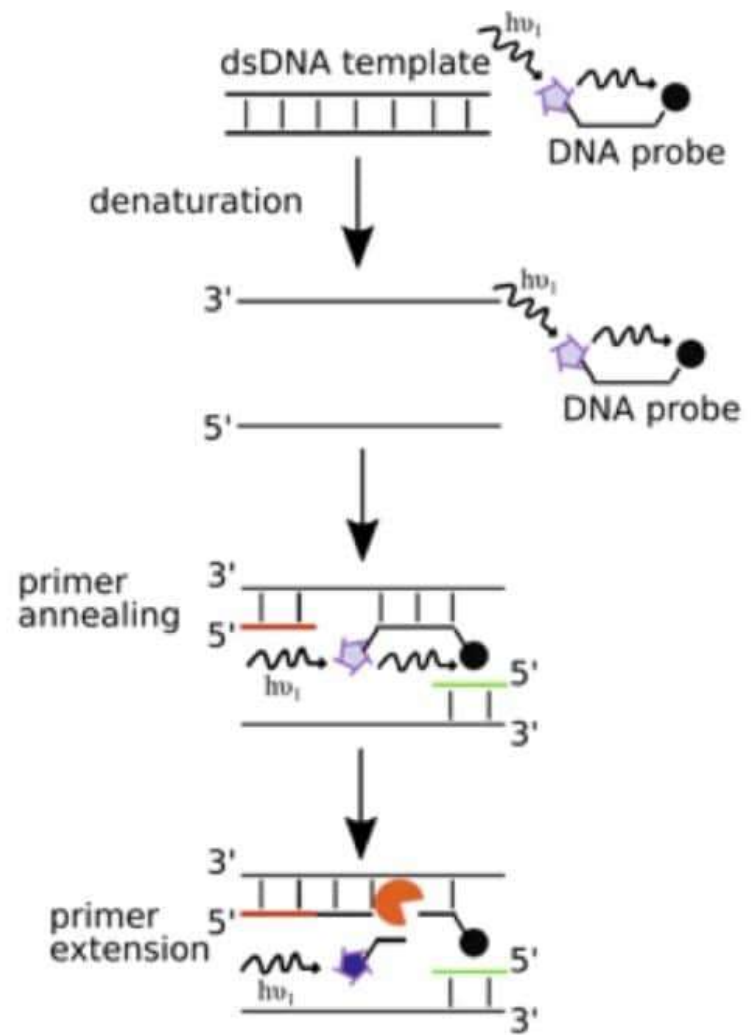
Fluorescent dye-based real-time PCR



Key:

- ground state fluorophore
- excited state fluorophore
- fluorescence quencher

DNA probe-based real-time PCR



- ☆ quenched fluorophore
- ★ excited state fluorophore
- Taq polymerase

Other PCR variations

- In addition to Real-Time PCR and RT-PCR, there are many more variations (at least 25) that exist and are used to solve specific problems. They all have different names such as Assembly PCR, Hot-start PCR, Multiplex PCR, Solid-phase PCR and many others

PCR has become a very powerful tool in molecular biology

- One can start with a single sperm cell or strand of hair and amplify the DNA sufficiently to allow for DNA analysis and a distinctive band on an agarose gel.
- One can amplify fragments of interest in an organism's DNA by choosing the right primers.
- One can use the selectivity of the primers to identify the likelihood of an individual carrying a particular allele of a gene.

Applications of PCR

- **Selective DNA isolation** (as in cloning or fingerprinting of organism)
- **Amplification and quantification of DNA** (forensic medicine when trace amounts remains)
- **Medical applications:**
 - *Genetic testing* looking for genetic disease mutations
 - *Tissue typing* important for organ transplantation
 - *In early detection of cancer* as leukemia and lymphoma
- **Infectious disease application** as in HIV virus, Tuberculosis and spread of disease organism in animals
- **Forensic applications:** as genetic fingerprinting of DNA in crime scene and DNA Paternity testing

PCR and Disease

- Primers can be created that will only bind and amplify certain alleles of genes or mutations of genes
 - This is the basis of genetic counseling and PCR is used as part of the diagnostic tests for genetic diseases.
- Some diseases that can be diagnosed with the help of PCR:
 - Huntington's disease
 - cystic fibrosis
 - Human immunodeficiency virus

Huntington's Disease (HD)

- HD is a genetic disorder characterized by abnormal body movements and reduced mental abilities
- HD is caused by a mutation in the Huntingtin (*HD*) gene
- In individuals with HD, the *HD* gene is “expanded”
 - *In non-HD individuals, the HD gene has a pattern called **trinucleotide repeats** with “CAG” occurring in repetition less than 30 times.*
 - *IN HD individuals, the “CAG” trinucleotide repeat occurs more than 36 times in the HD gene*
- PCR can be performed on an individual's DNA to determine whether the individual has HD.
 - *The DNA is amplified via PCR and **sequenced** (a technique by which the exact nucleotide sequence is determined) and the number of trinucleotide repeats is then counted.*

Cystic Fibrosis (CF)

- CF is a genetic disease characterized by severe breathing difficulties and a predisposition to infections.
- CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CTFR*) gene.
- In non-CF individuals, the *CTFR* gene codes for a protein that is a chloride ion channel and is involved in the production of sweat, digestive juices and mucus.
- In CF individuals, mutations in the *CTFR* gene lead to thick mucous secretions in the lungs and subsequent persistent bacterial infections.
- The presence of *CTFR* mutations in a individual can be detected by performing PCR and sequencing on that individual's DNA.

Human Immunodeficiency Virus (HIV)

- HIV is a retrovirus that attacks the immune system.
- HIV tests rely on PCR with primers that will only amplify a section of the viral DNA found in an infected individual's bodily fluids.

Therefore if there is a PCR product, the person is likely to be HIV positive. If there is no PCR product the person is likely to be HIV negative.

- Protein detection based tests are available as well but all US blood is tested by PCR.

PCR and Forensic Science

- Forensic science is the application of a broad spectrum of sciences to answer questions of interest to the legal system. This may be in relation to a crime or to a civil action.
- It is often of interest in forensic science to identify individuals genetically. In these cases, one is interested in looking at variable regions of the genome as opposed to highly-conserved genes.
- PCR can be used to amplify highly variable regions of the human genome. These regions contain runs of short, repeated sequences (known as variable number of tandem repeat (VNTR) sequences). The number of repeats can vary from 4-40 in different individuals.
- Primers are chosen that will amplify these repeated areas and the genomic fragments generated give us a unique “genetic fingerprint” that can be used to identify an individual.

PCR Applications to Forensic Science

- Paternity suits -Argentina's Mothers of the plaza and their search for abducted grandchildren
- Identifying badly decomposed bodies or when only body fragments are found - Bosnian , Iraq & Rwandan mass graves