Polymerase Chain Reaction (PCR)

A Brief History of PCR

--Newer than you might think--

1869 DNA first isolated

1985

Invitro amplification achieved
--with *E.coli* DNA polymerase--

Saiki R, K.; Scharf S; Faloona F; Mullis K. B; Horn G. T; Erlich H. A.; Arnheim N., Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science, 1985 Dec 20, 230(4732):1350-4.

Mullis K. B; Faloona F. A; Scharf S; Saiki R. K; Horn G; Erlich H. A., Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symposia on Quantitative Biology, 1986

Scharf S. J; Horn G. T; Erlich H. A. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science, 1986 Sep 5, 233(4768):1076-8.

1988

Invitro amplification achieved
--with *Taq* DNA polymerase--

Saiki R. K; Gelfand D. H; Stoffel S; Scharf S. J; Higuchi R; Horn G. T; Mullis K. B; Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 1988 Jan 29, 239(4839):487-91.

1989

Taq DNA polymerase cloned and expressed in *E.coli*

Lawyer F. C; Stoffel S; Saiki R. K; Myambo K; Drummond R; Gelfand D. H. Isolation, characterization, and expression in Escherichia coli of the DNA polymerase gene from Thermus aquaticus. Journal of Biological Chemistry, 1989 Apr 15, 264(11):6427-37.

What is PCR?

PCR is an <u>exponentially</u> progressing synthesis of the defined target DNA sequences *in vitro*.

It was invented in 1983 by Dr. Kary Mullis, for which he received the Nobel Prize in Chemistry in 1993.

Why “Polymerase”?

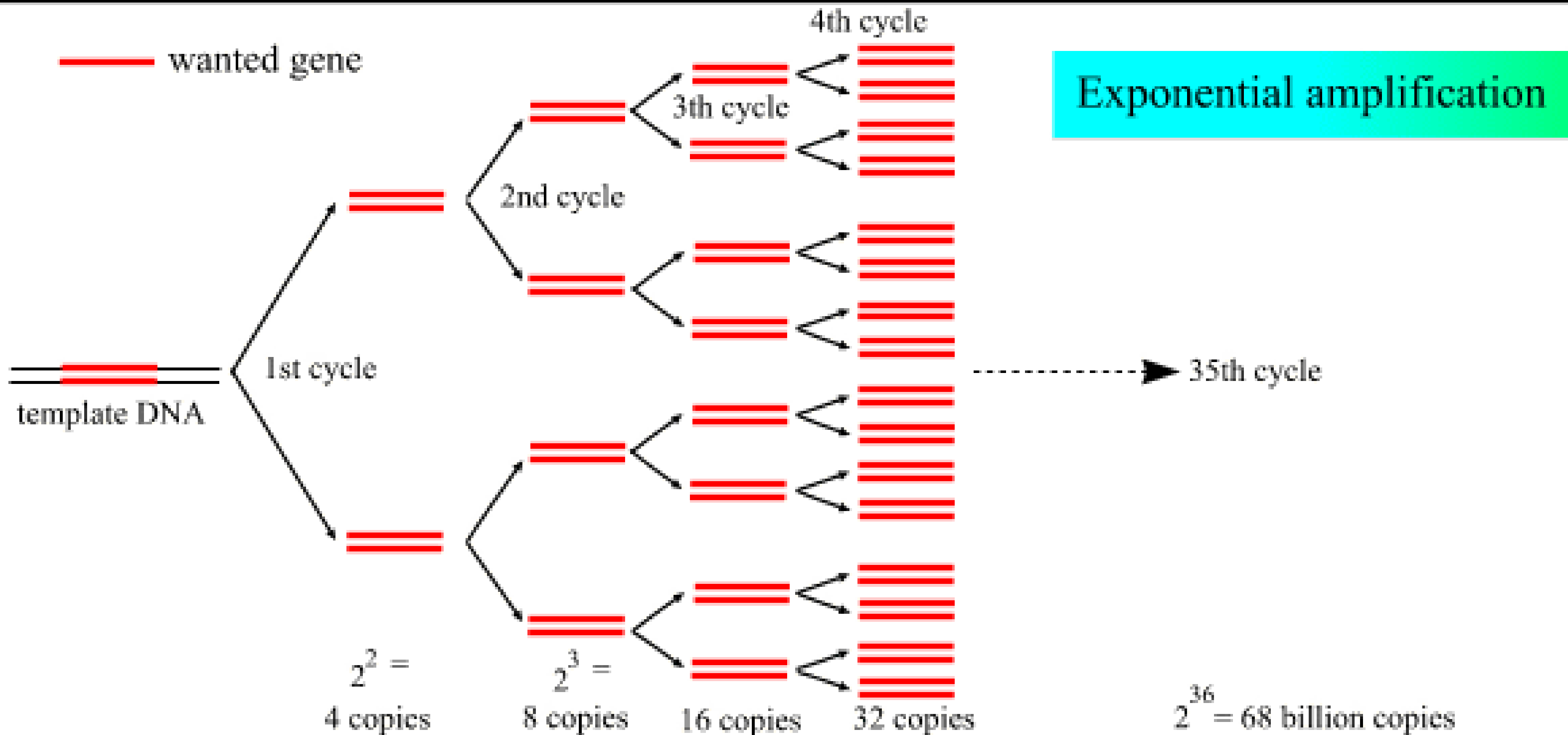
It is called “polymerase” because the only enzyme used in this reaction is DNA polymerase.

Why “Chain”?

It is called “chain” because the products of the first reaction become substrates of the following one, and so on.

PCR Amplification

Exponential Amplification of template DNA



The “Reaction” Components

1) **Target DNA** - **contains the sequence to be amplified.**

2) **Pair of Primers** - **oligonucleotides that define the sequence to be amplified.**

3) **dNTPs** -**deoxynucleotidetriphosphates: DNA building blocks**

4) **Thermostable DNA Polymerase** - **enzyme that catalyzes the reaction**

5) **Mg^{++} ions** - **cofactor of the enzyme**

6) **Buffer solution** – **maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme**

Polymerase Chain Reaction (PCR)

- PCR is a technique which is used to amplify the number of copies of a **specific region of DNA**, in order to produce enough DNA to be adequately tested.

- The purpose of a PCR is to make a **huge number of copies of a gene**. As a result, it now becomes possible to analyze and characterize DNA fragments found in minute quantities in places like a drop of blood at a crime scene or a pathogen within a cell or plasma

What PCR Can Do ?

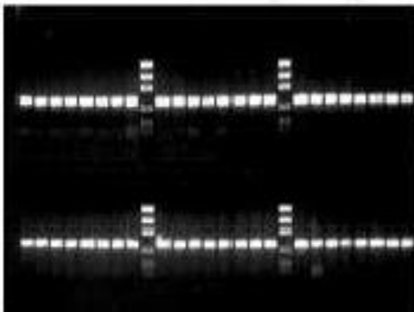
- Starting with one original copy an almost infinite number of copies can be made using PCR
- “Amplified” fragments of DNA can be sequenced, cloned, probed or sized using electrophoresis
- Defective genes can be amplified to diagnose any number of illnesses
- Genes from pathogens can be amplified to identify them (*i.e.*, HIV, Vibrio sp., Salmonella sp. etc.)
- Amplified fragments can act as genetic fingerprints

PROCEDURE …..

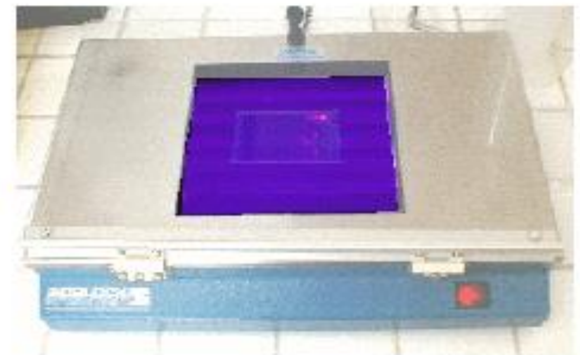
PCR

Agarose gel electrophoresis

3-4 hours



The final product



UV visualisation

Polymerase Chain Reaction (PCR)

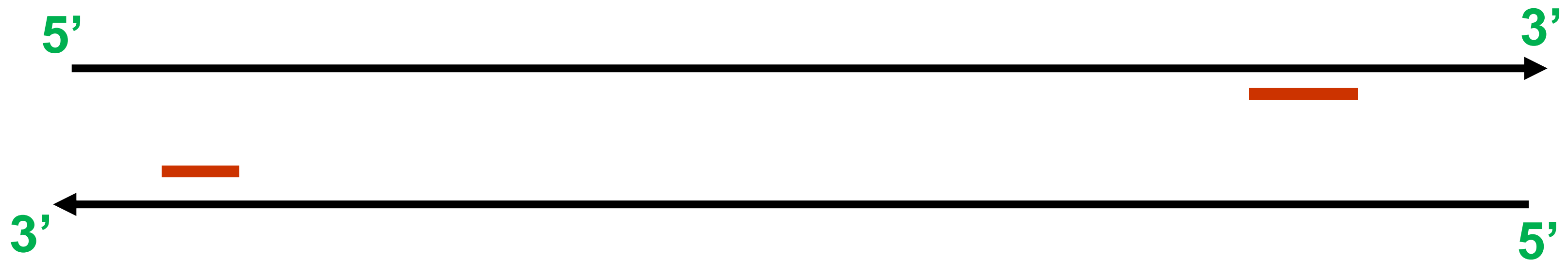
- PCR performs the chemistry of DNA duplication *in vitro*
- Numerous PCR applications make this process routine in most biology laboratories
- Understanding properties of DNA polymerases helps understanding PCR

DNA polymerase

- Duplicates DNA
- Necessary for reproduction of new cells
- More than one DNA polymerases exist in different organisms

Properties of DNA polymerase

- Needs a pre-existing DNA to duplicate
  - Cannot assemble a new strand from components
  - Called template DNA
- Can only extend an existing piece of DNA
  - Called primers

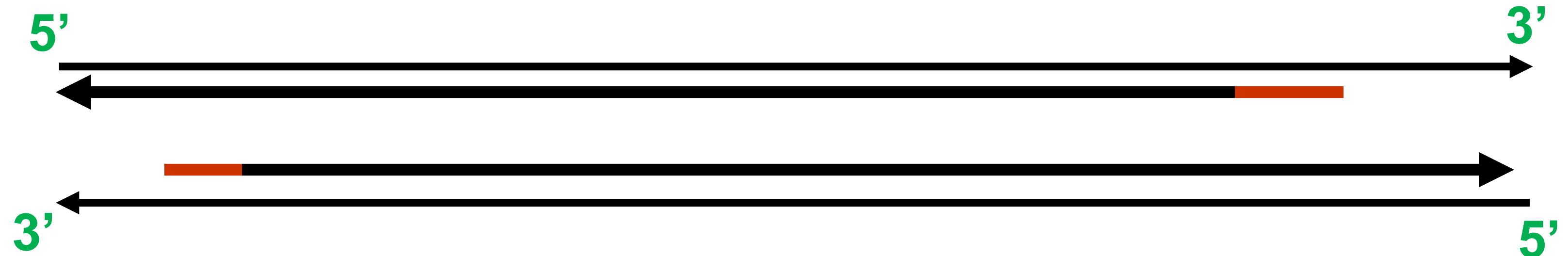


Properties of DNA polymerase

- DNA polymerase needs Mg^{++} as cofactor
- Each DNA polymerase works best under optimal temperature, pH and salt concentration
- PCR buffer provides optimal pH and salt condition

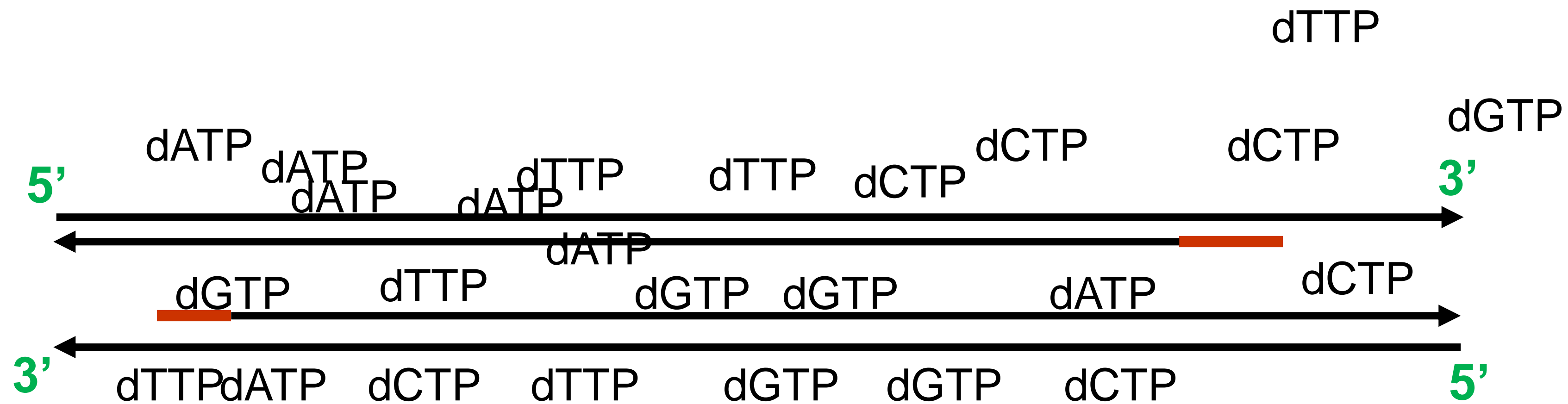
Properties of DNA polymerase

- DNA strands are anti-parallel
  - One strand goes in 5’ → 3’
  - The complementary strand is opposite
- DNA polymerase always moves in one direction (from 5’ → 3’)



Properties of DNA polymerase

- DNA polymerase incorporates the four nucleotides (A, T, G, C) to the growing chain
- dNTP follow standard base pairing rule



Properties of DNA polymerase

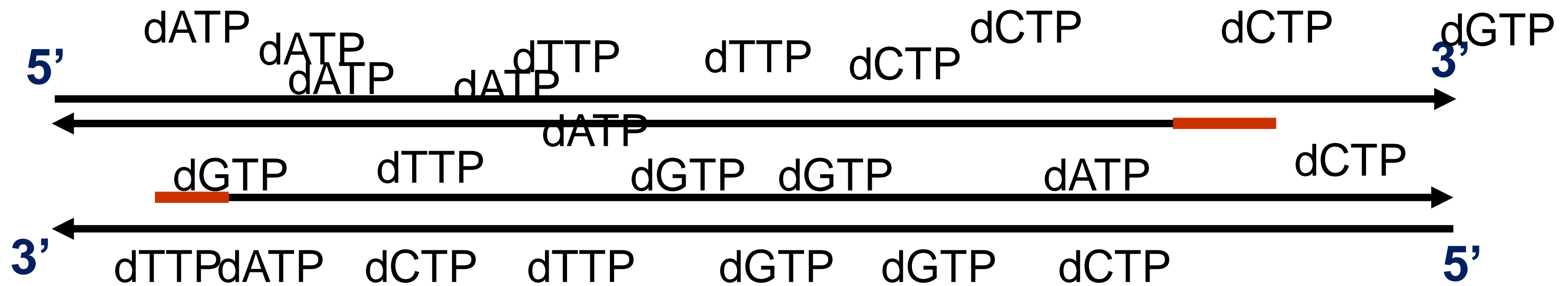
- The newly generated DNA strands serve as template DNA for the next cycle
- PCR is very sensitive
- Widely used

Taq DNA polymerase

1. Derived from *Thermus aquaticus*
2. Heat stable DNA polymerase
3. Ideal temperature 72C

Setting up a PCR Reaction

- **Add template DNA** and **primers**
- **Add dNTPs**
- **Add DNA polymerase**



Thermal Cycling

- A PCR machine controls temperature
- Typical PCR go through **three steps**

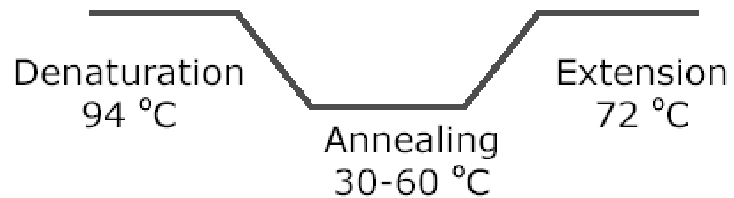
1. Denaturation
2. Annealing
3. Extension

PCR reaction contains

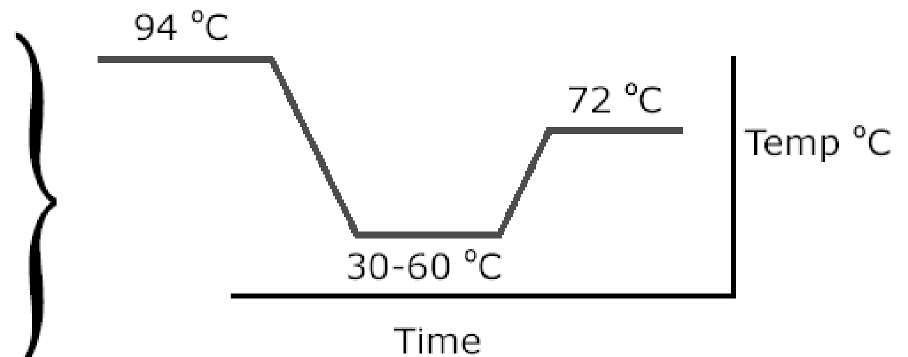
- Target DNA (example: environmental DNA)
- 2 primers (20-30 nts long)
- Thermostable DNA polymerase
- Nucleotides (dNTPs)
- Mg^{2+} (cofactor for DNA polymerase)

Mix is subjected to temperature cycling

Each cycle

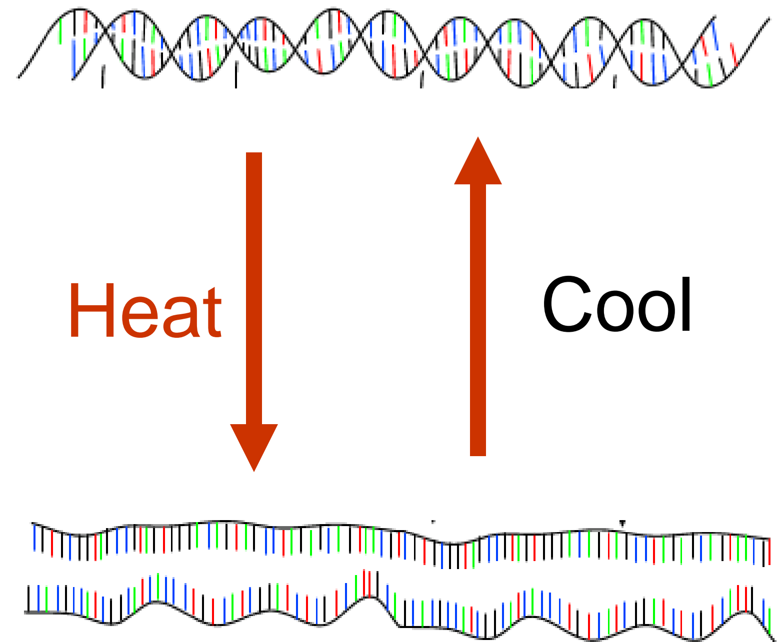


(adjust temperature to balance between specificity and amplification)

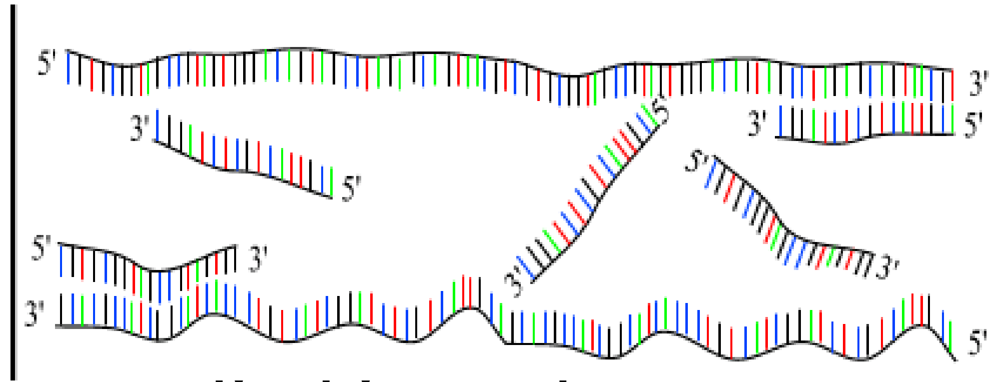


Denaturation

- Heating separates the double stranded DNA
  - Denaturation
- Slow cooling anneals the two strands
  - Renaturation



Annealing

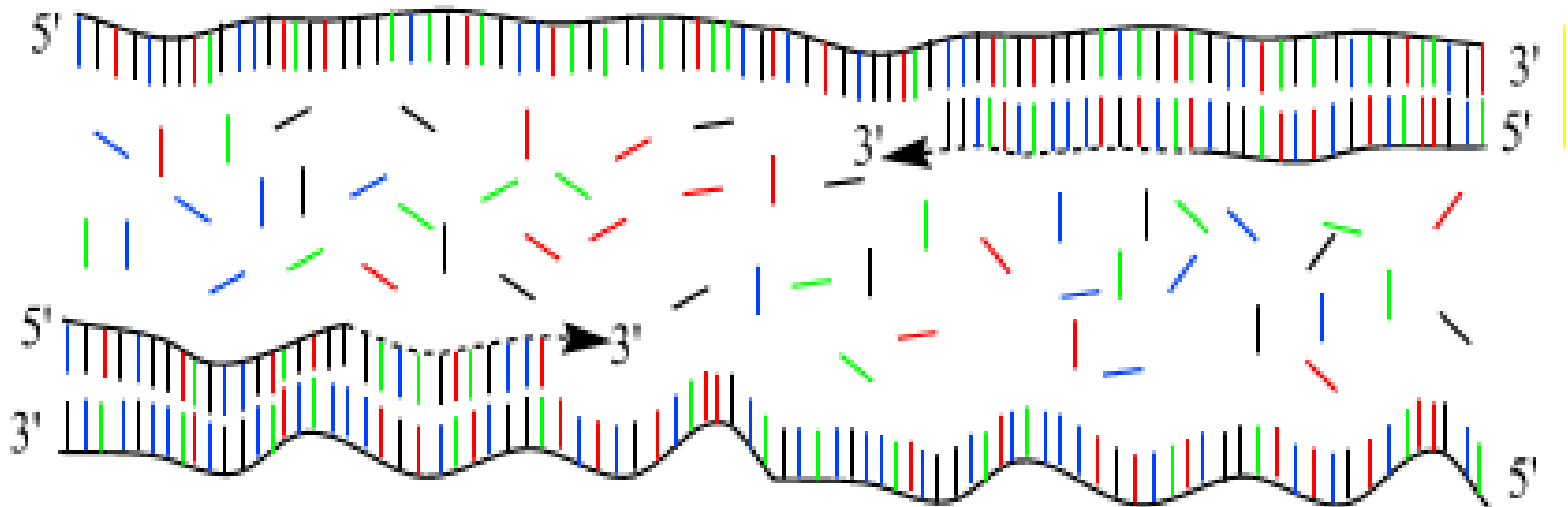


- Two primers are supplied in molar excess
- They bind to the complementary region
- As the DNA cools, they wedge between two template strands
- Optimal temperature varies based on primer length etc.

Typical temperature from 40 to 60 C

Extension

- DNA polymerase duplicates DNA
- Optimal temperature 72°C



Typical PCR mix

In a thin wall Eppendorf tube assemble the following

PCR components	Amount
Template DNA (5-200 ng)	variable
1 mM dNTPs (200 uM final)	10 uL
10 X PCR buffer	5 uL
25 mM MgCl2 (1.5 mM final)	3 uL
20 uM forward primer (20 pmoles final)	1 uL
20 uM reverse primer (20 pmoles final)	1 uL
5 units/uL Taq DNA polymerase (1.5 units)	0.3 uL
Water	Variable
Final Volume	50 uL

Primer selection

- **Primer is an oligonucleotide sequence –targets a specific sequence of opposite base pairing (A-T, G-C only) of single-stranded nucleic acids**

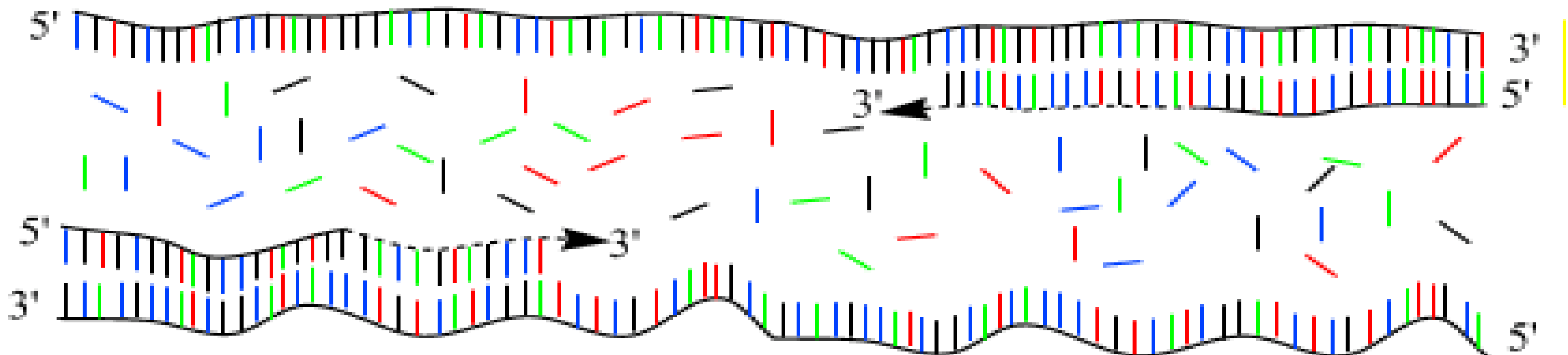
- For example, there is a
  - ¼ chance (4-1) of finding an A, G, C or T in any given DNA sequence; there is a
  - 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a
  - 1/256 chance of finding a given 4-base sequence.
- Thus, a **sixteen base sequence will statistically be present only once in every 416 bases (=4 294 967 296, or 4 billion)**: this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*.

Primer Specificity

1. **Universal** – amplifies ALL bacterial DNA for instance (gram positive)
2. **Group Specific** – amplify all denitrifies for instance (genus level)
3. **Specific** – amplify just a given sequence (species level)

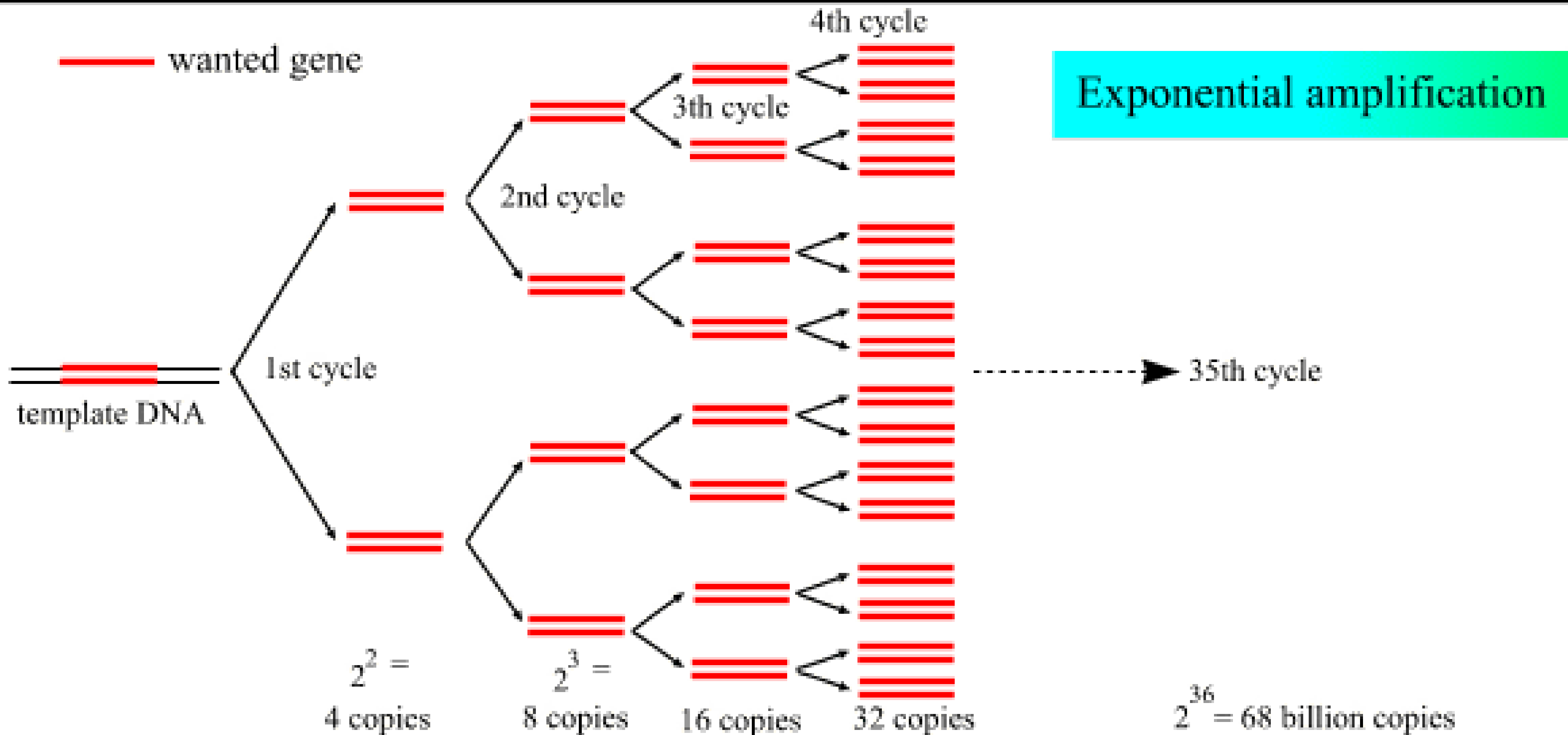
Forward and reverse primers

- If you know the sequence targeted for amplification, you know the size which the primers should be annealing across.
- If you don't know the sequence… What do you get?



PCR Amplification

Exponential Amplification of template DNA



Applications

- Ubiquitous applications
- Revolutionized how we study biology
  1. Research
  2. Diagnostics
  3. Forensics

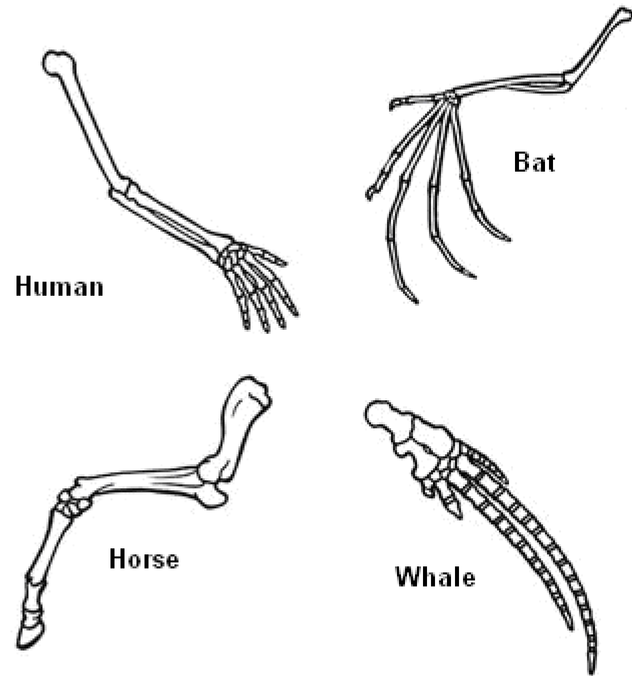
Specific PCR Applications

- Classification of organisms
- Genotyping
- Molecular archaeology
- Mutagenesis
- Mutation detection
- Sequencing
- Cancer research
- Detection of pathogens
- DNA fingerprinting
- Drug discovery
- Genetic matching
- Genetic engineering
- Pre-natal diagnosis

Traditional classification

Classification of organisms into closely related species, then more distant genuses, phyla and kingdoms was originally done on the basis of how similar organisms were in form.

The limbs in this picture show the ways that the same basic structure is adapted in four mammals.

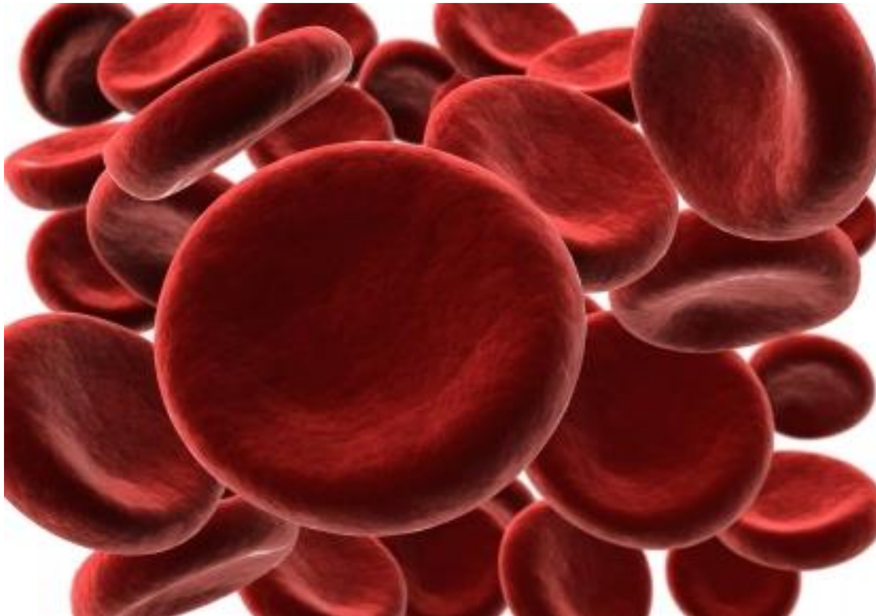


Traditional classification

Embryology is also used to identify similar structures in early development of animals, signifying common ancestry.

Traditional classification

Biochemical similarities can be used to identify closely related organisms. Proteins such as enzymes and haemoglobin (found in red blood cells) have more differences in their amino acid sequences as organisms become more distantly related.



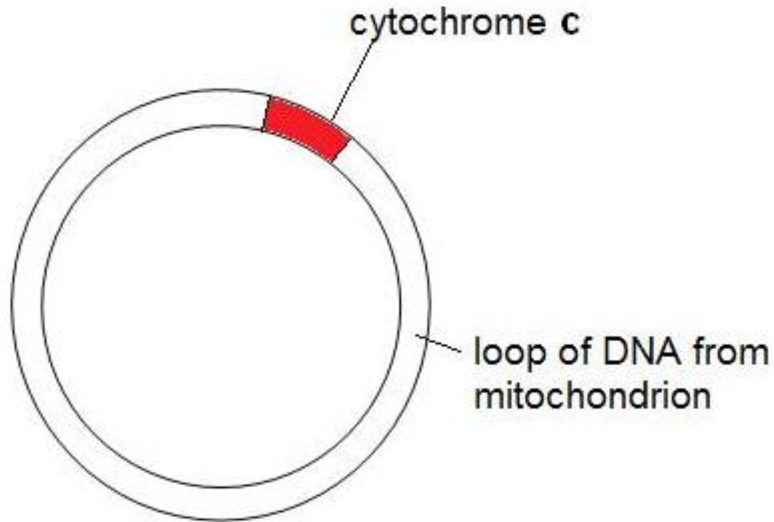
Process of sample/pathogen identification

The biological material to be identified is collected.

DNA extraction

DNA is extracted from the cells of the specimen.

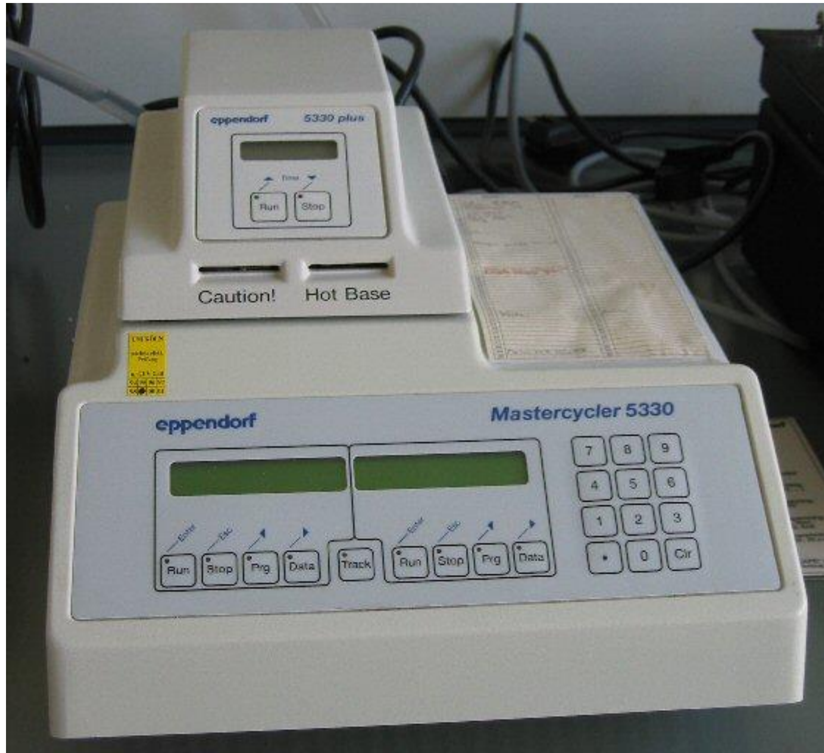
Finding a suitable gene



Mitochondria are ‘organelles’ within cells. They carry out respiration. The cytochrome c gene is needed in respiration, so is widely found in living organisms.

The DNA used to identify differences and similarities between organisms must be nearly identical within a species and show differences between species. A gene from mitochondrial DNA is commonly used.

Amplifying the DNA

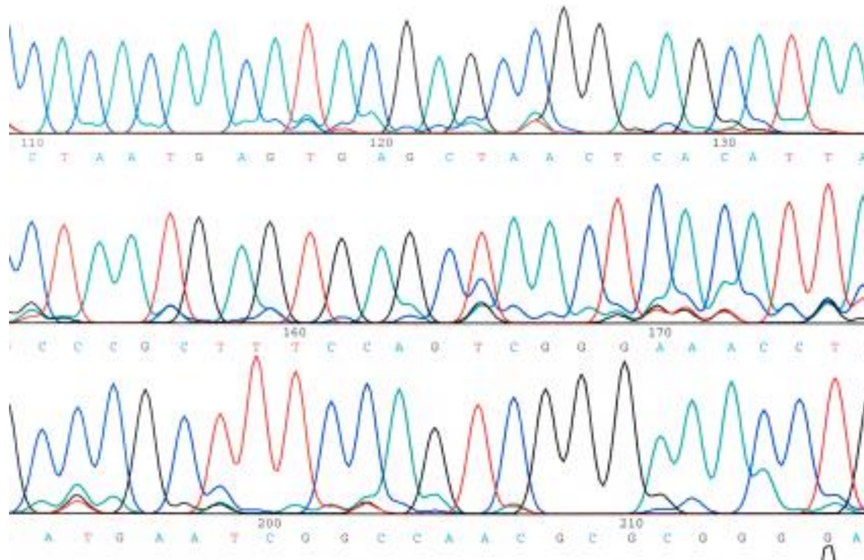


Many thousands of copies of the DNA sample are made in a process called PCR. This PCR machine heats and cools the DNA along with enzymes that rapidly make copies of the DNA.

Finding the individual’s code

The sequence of bases (GATC) in the gene is then found.

Identification of the species



The sequence of bases is then compared with a standard sequence to identify the species.

An international library of base sequences is developing to allow scientists across the world to compare their specimens.

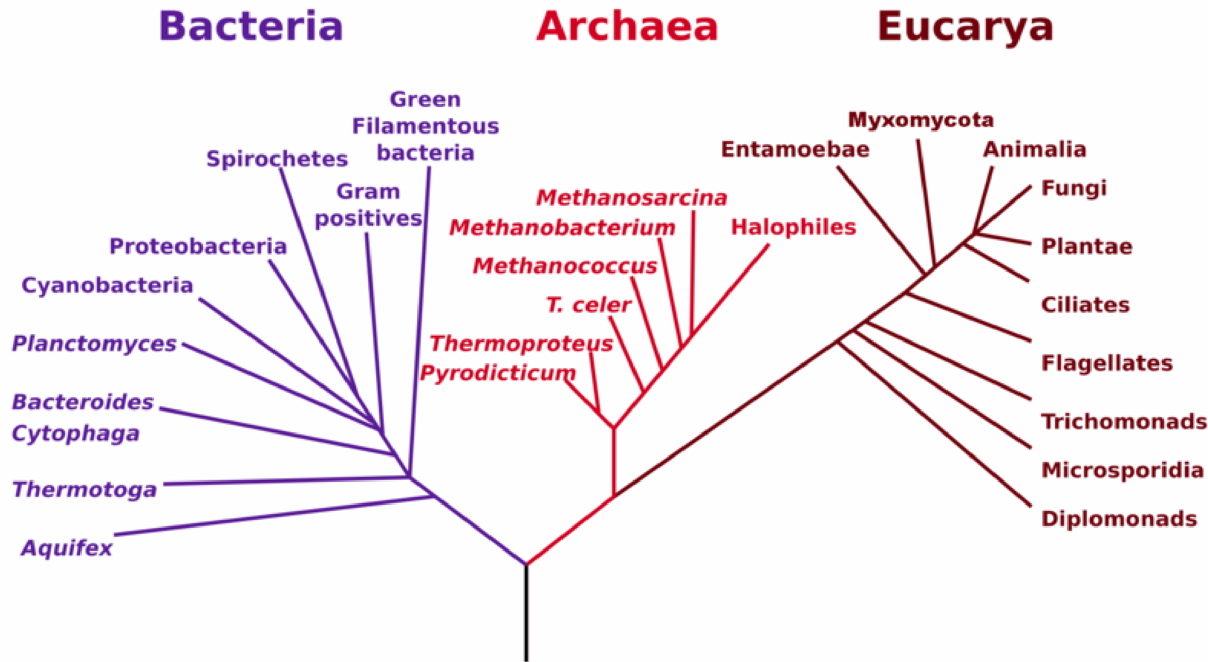
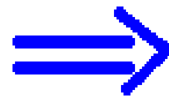


Image from http://commons.wikimedia.org/wiki/File:PhylogeneticTree.png

Most biologists recognise three domains of organisms, which can be shown in this tree of life connecting organisms according to the genes they have in common, and their common ancestry.

Molecular Identification:

DNA is unique for each single type of organism.



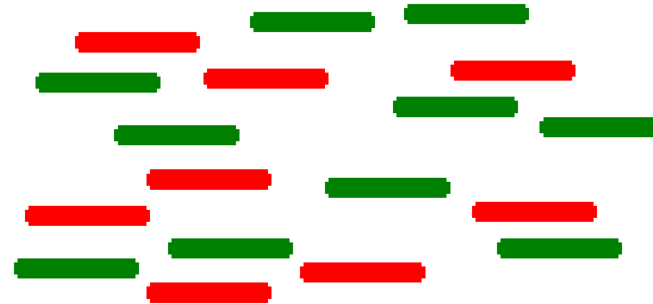
DNA can be used to identify an organism.

Organisms can be identified by using PCR.

PCR allows easy manipulation of DNA.

Molecular Identification:

Detection of Unknown Mutations

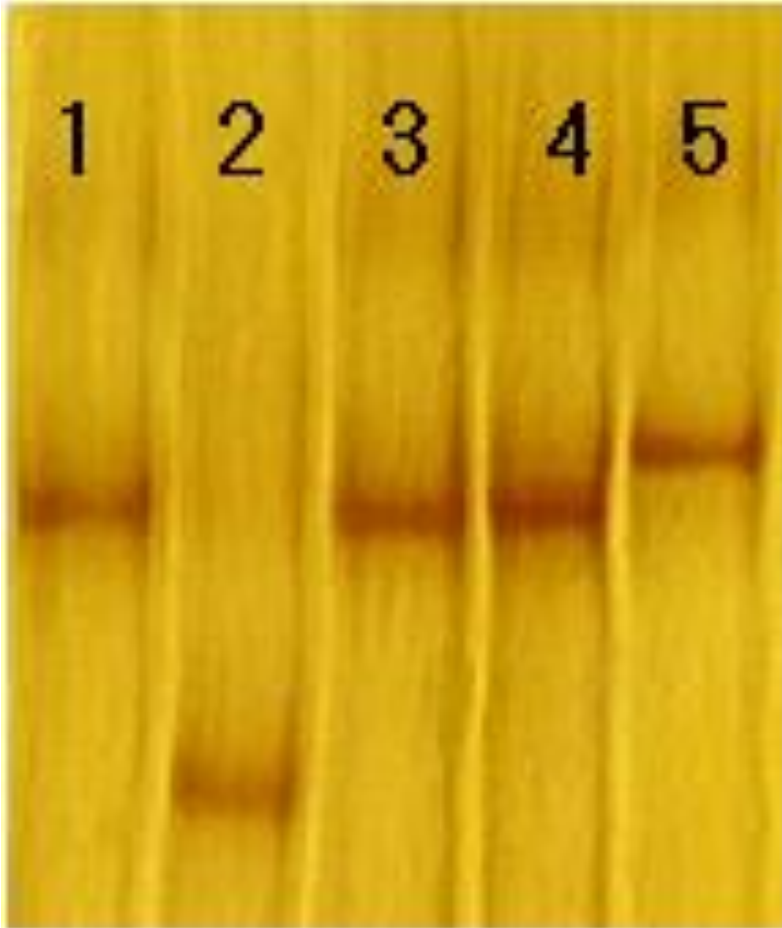


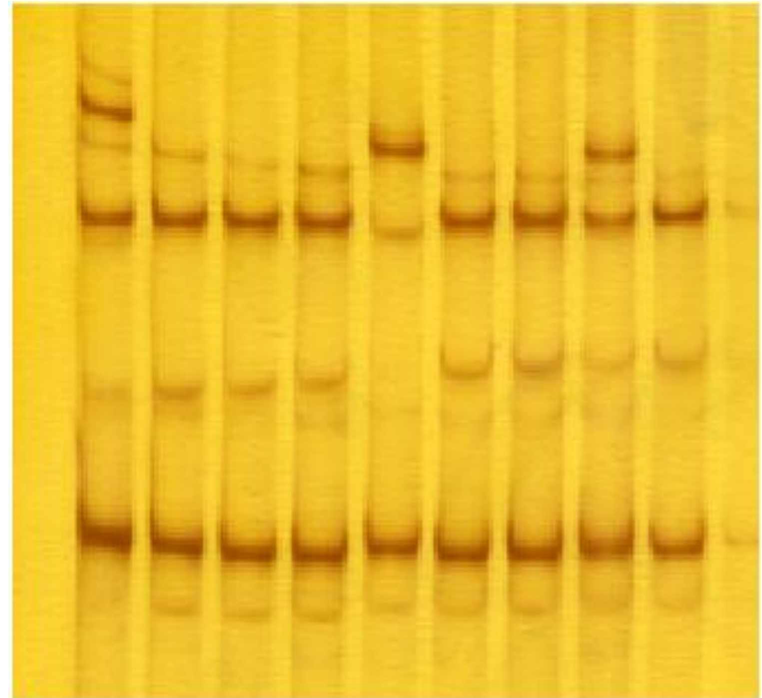
Whole Genomic DNA

PCR →

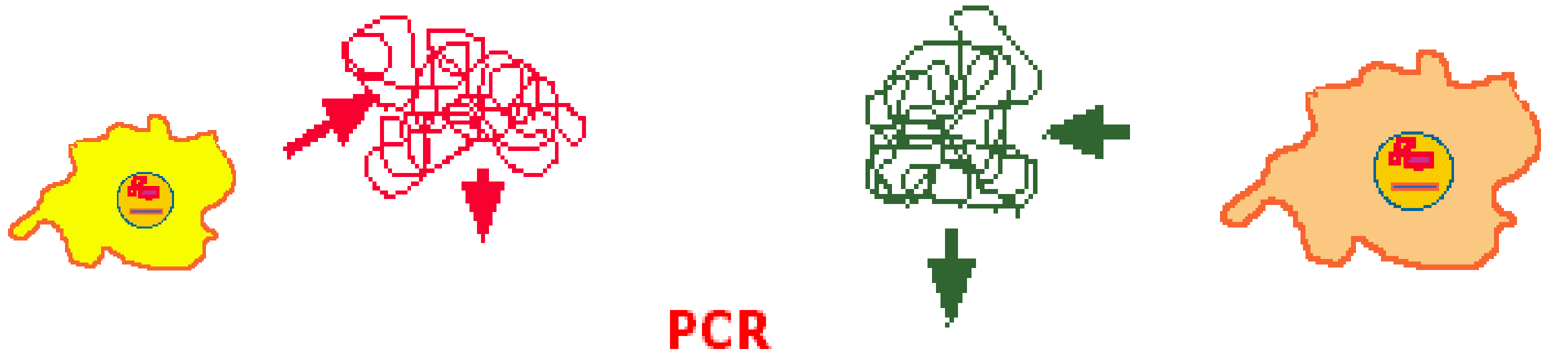
Desired DNA fragments that may contain a mutation in huge numbers.

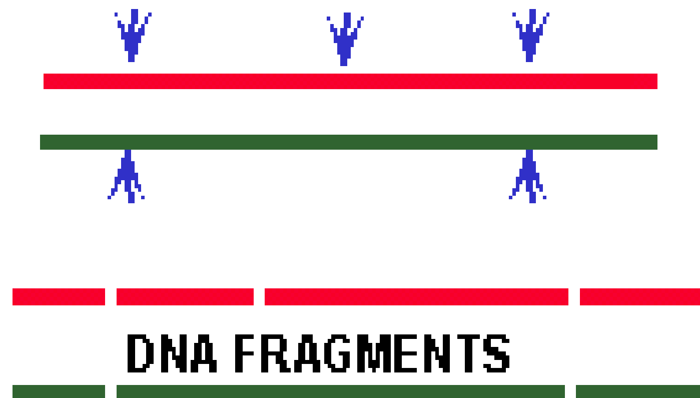
SSCP →



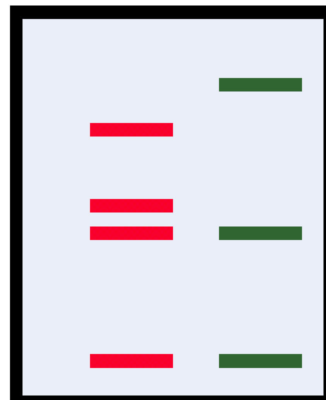


SSCP gels:
“shifts” representing a mutation in the amplified DNA fragment





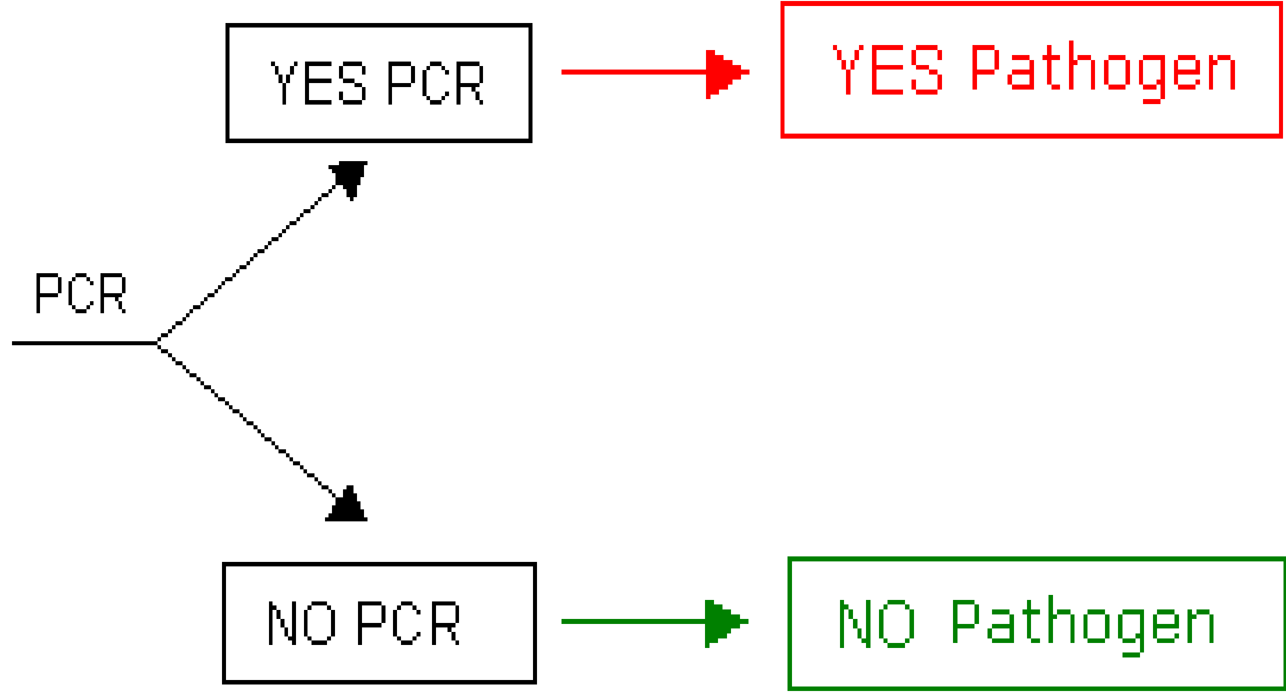
Specific PCR products are cut with restriction enzymes.



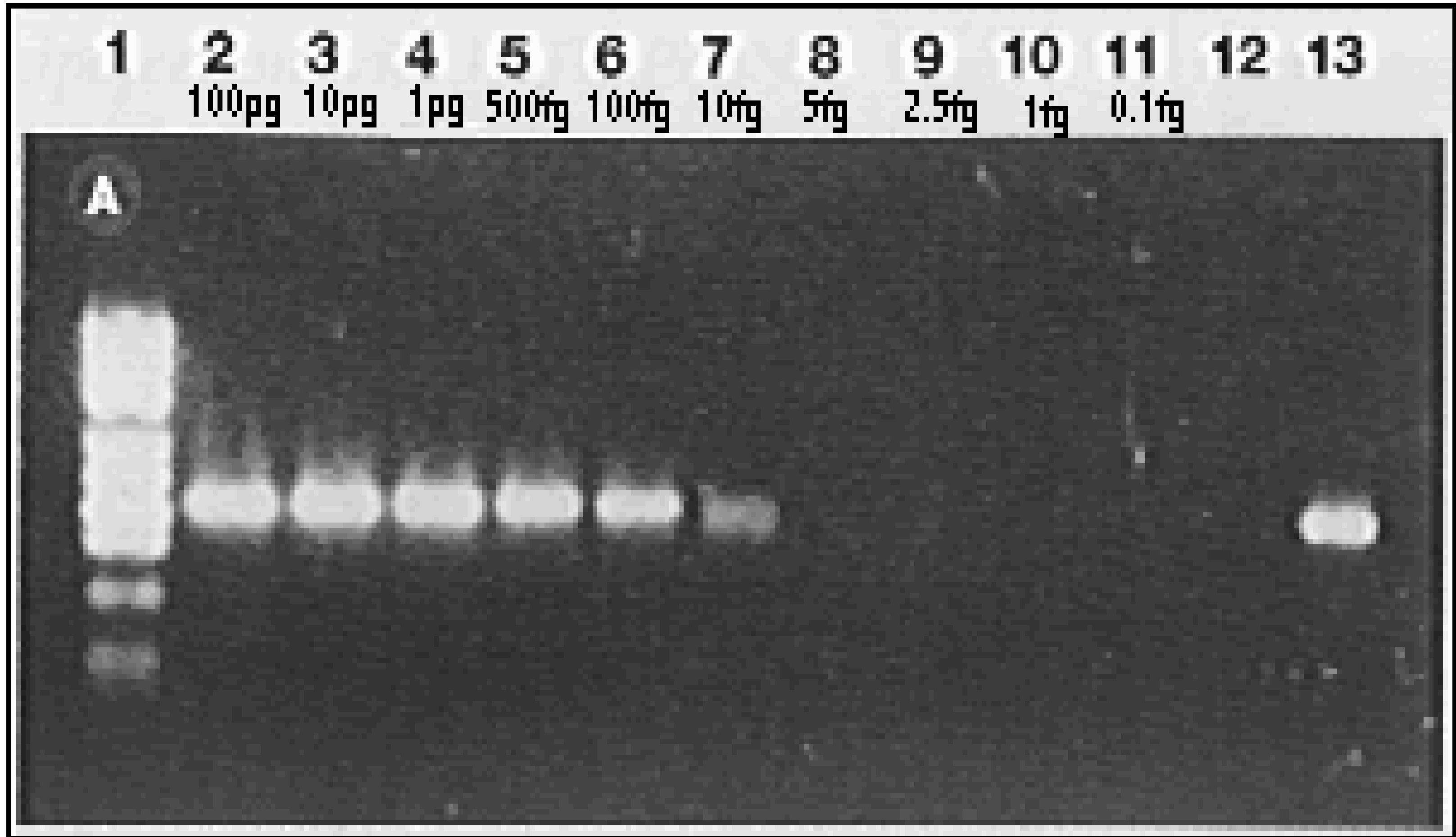
SEPARATE FRAGMENTS ON THE BASIS OF THEIR SIZE

Molecular Identification:

Detection Of Pathogens



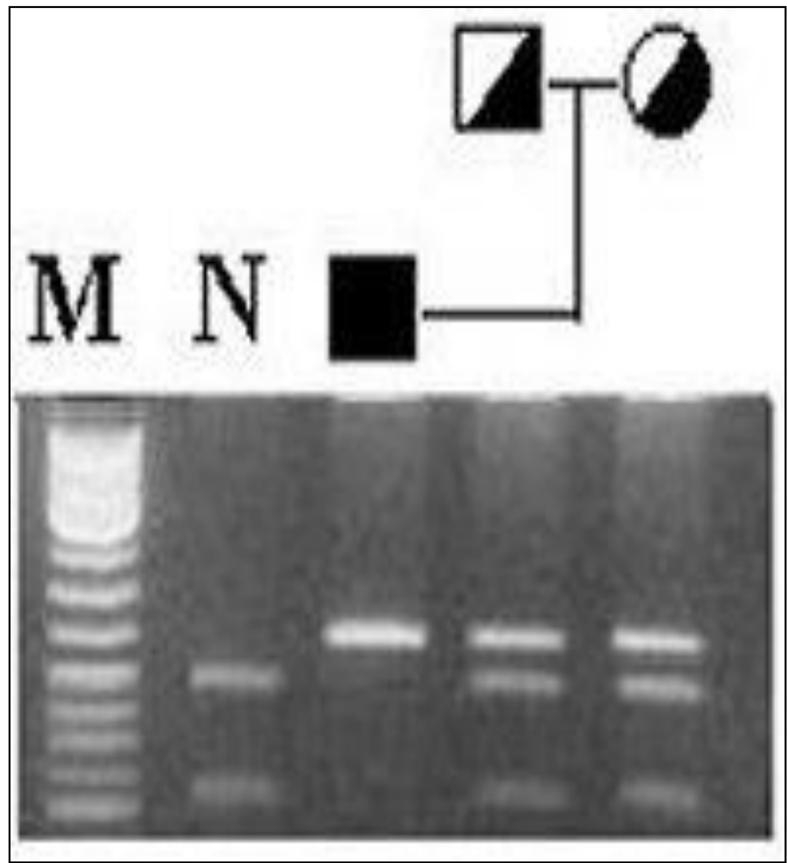
Detection Of Pathogens



Sensitivity of detection of PCR-amplified *M. tuberculosis* DNA. (Kaul *et al.*1994)

Molecular Identification:

Prenatal Diagnosis



- Chorionic Villus
- Amniotic Fluid

644 bp

440 bp

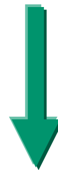
204 bp

Molecular analysis of a family with an autosomal recessive disease

SEQUENCING

Nucleotides (dNTP) are modified (dideoxynucleotides = ddNTP)

NO polymerisation after a dideoxynucleotide!

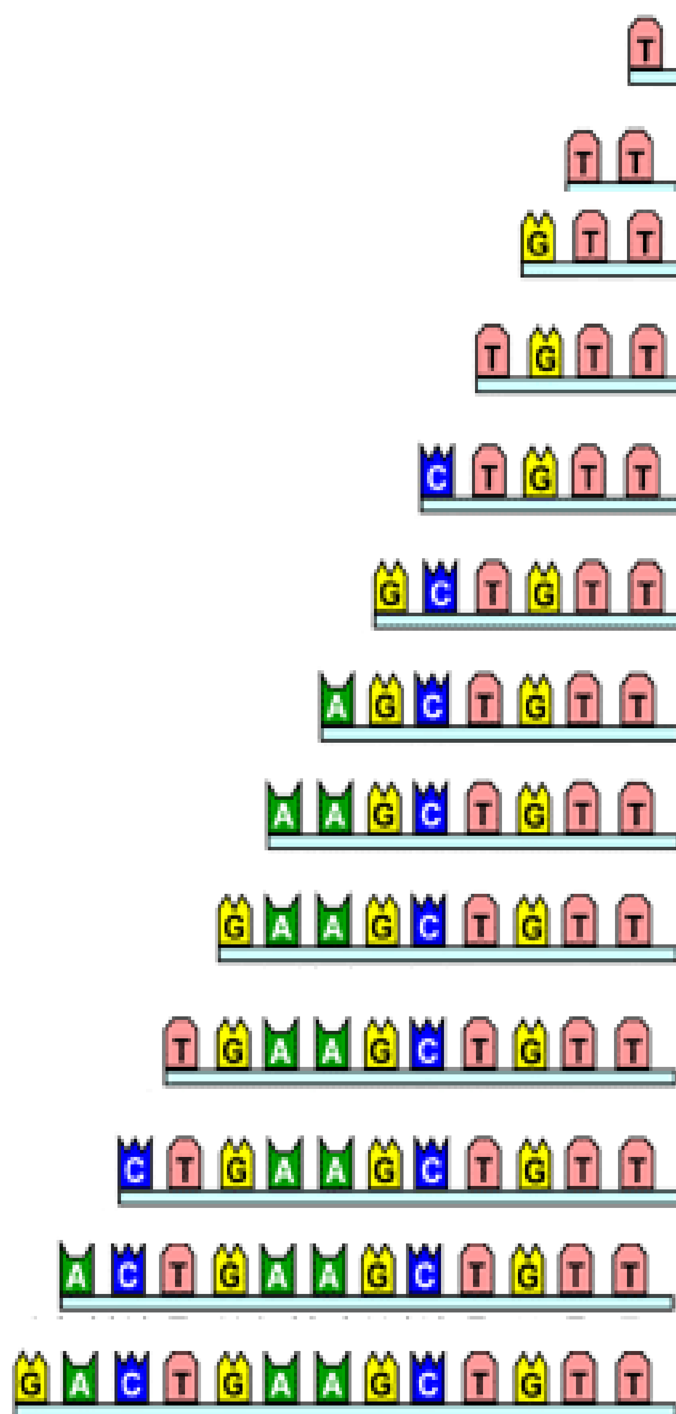


Fragments of DNA differing only by one nucleotide are generated

Nucleotides are either radioactive or fluorescent

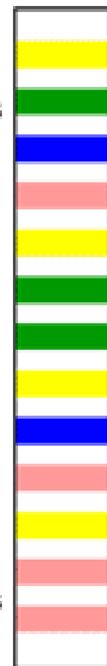
DNA Polymerase I
dATP
dGTP
dCTP
dTTP
plus limiting amounts of
fluorescently labelled

ddATP
ddGTP
ddCTP
ddTTP

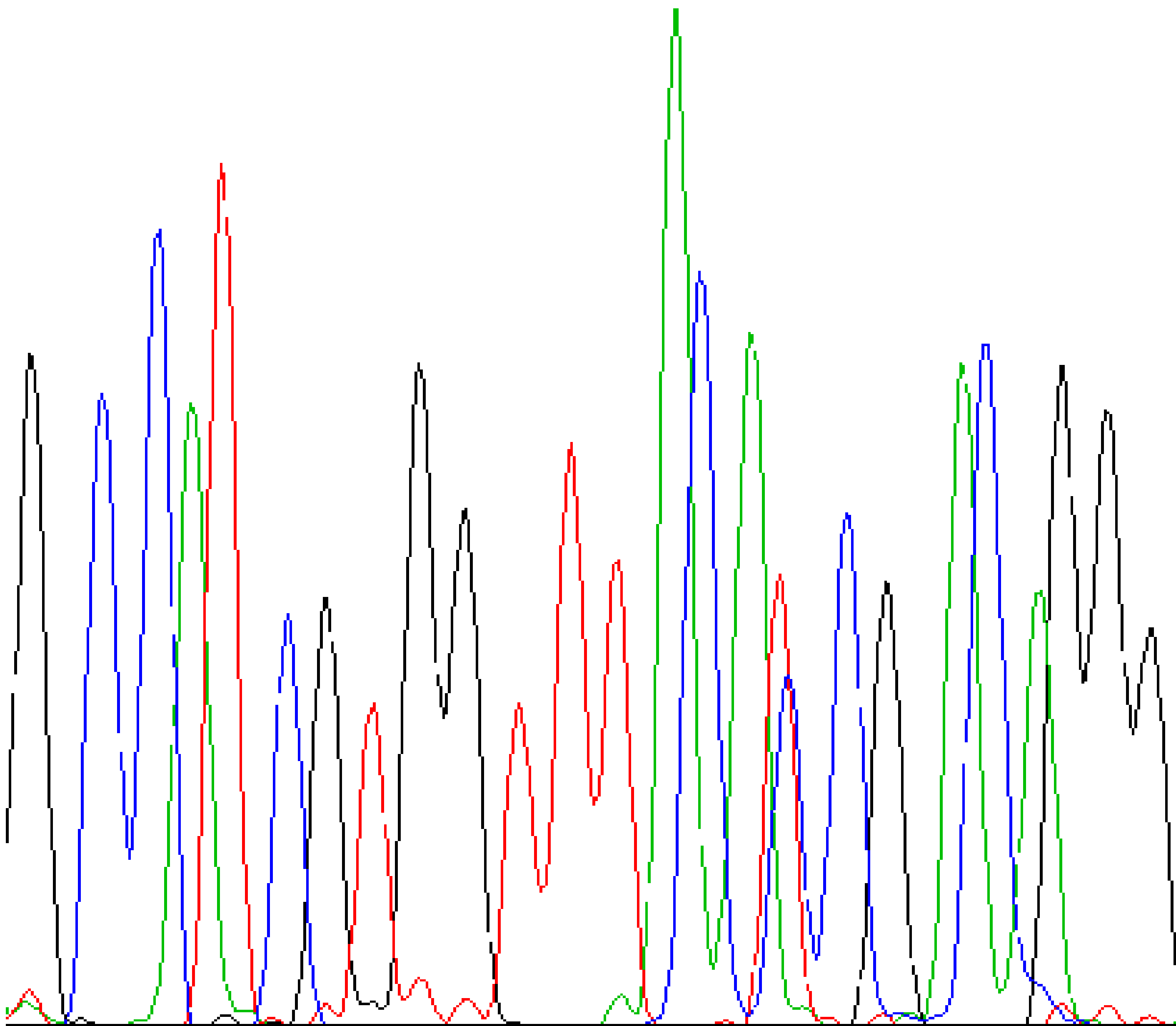


Larger fragments

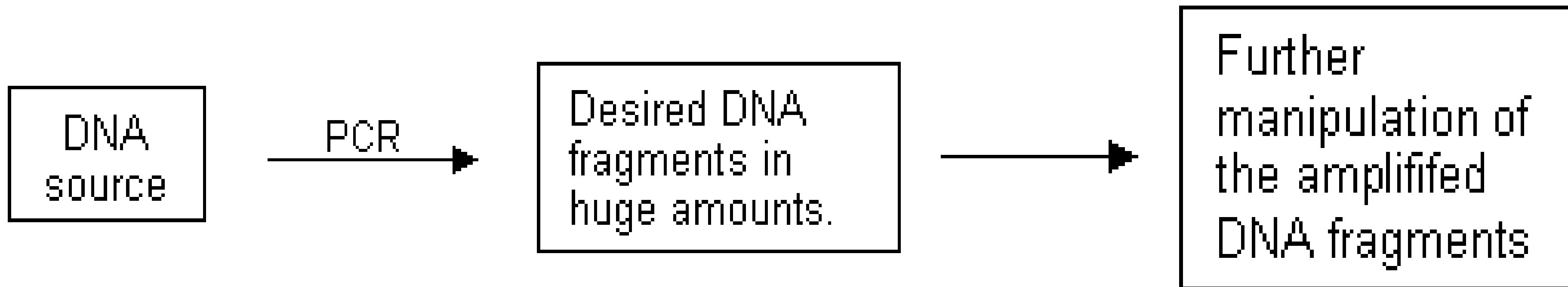
Smaller fragments



Sequencing:



Summary



blood, chorionic villus, amniotic fluid, semen, hair root, saliva

68,719,476,736 copies

Gel Analysis, Restriction Digestion, Sequencing

Conclusion

The <u>speed</u> and <u>ease</u> of use, <u>sensitivity</u>, <u>specificity</u> and <u>robustness</u> of PCR has revolutionised molecular biology and made PCR the most widely used and powerful technique with great spectrum of research and diagnostic applications.