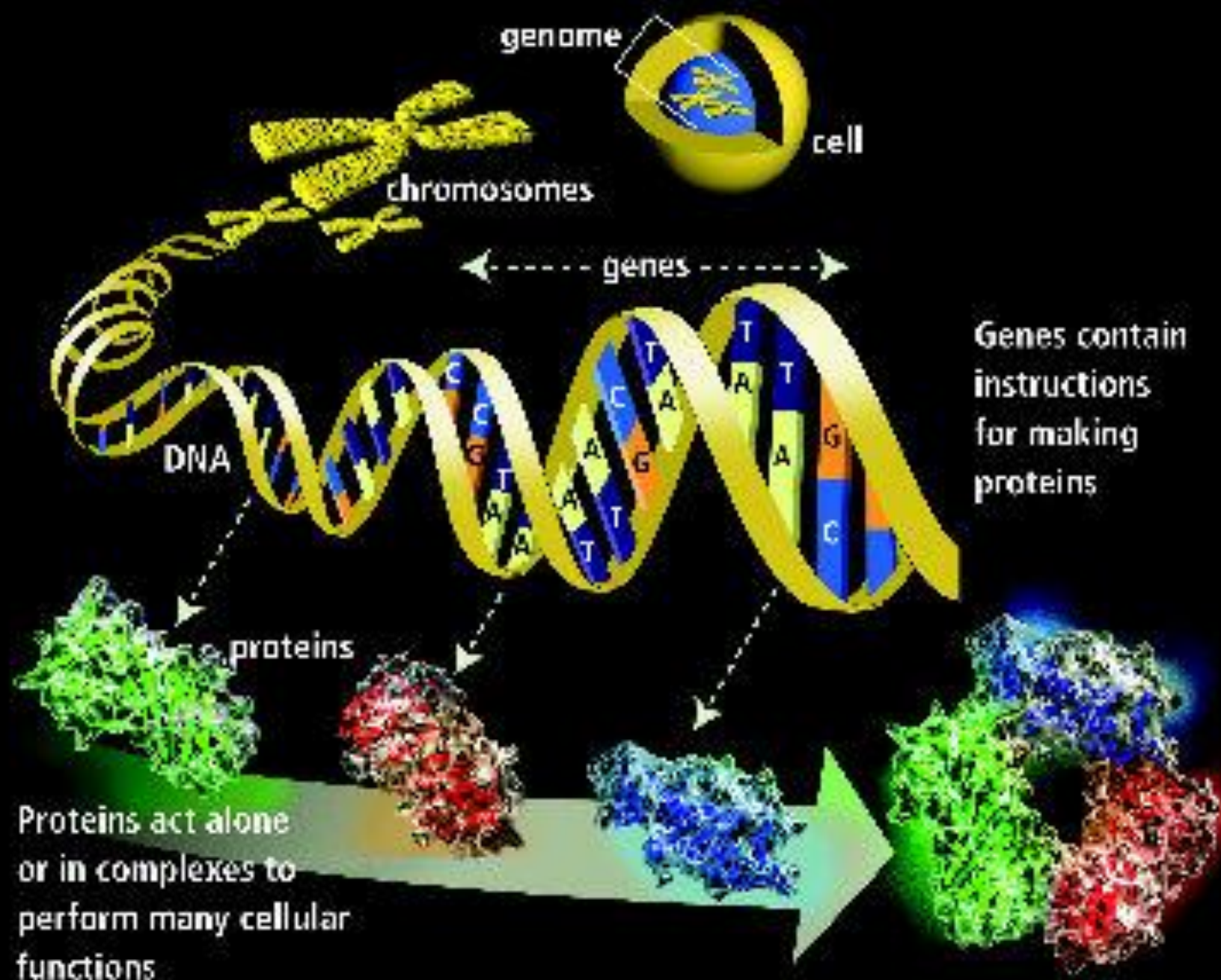


Direct and indirect mutation analysis I: PCR

Dr. Rasime Kalkan, Ph.D.
Faculty of Medicine



- The ability to prepare and isolate genomic DNA from a variety of sources is a crucial step in many molecular biology protocols.
- The quality and purity of genomic DNA are some of the most critical factors for downstream applications such as **polymerase chain reaction (PCR) and sequencing**.
- In order to obtain **high-quality** (i.e., intact) and **high-purity** (i.e., free from contaminants or inhibitors) genomic DNA, suitable isolation methods should be applied.

Extraction and Purification of DNA from Biological Samples

- Various sources of genomic DNA include:
- -Clinical samples (e.g., blood and stool) -
-lymphocytes-
- -Cultured cells (e.g., bacteria and yeast)
- -Animal tissues (e.g., tail)
- -Plant tissues (e.g., leaves and needles)
- -Fungal materials (e.g., mycelium)

Extraction and Purification of DNA from Biological Samples

- DNA is a relatively stable molecule; however, the introduction of **nucleases** to working solutions should be avoided as these enzymes will degrade DNA.
- Genomic DNA consists of very long DNA molecules, which are fragile and subject to **double-strand breaks**. To ensure the integrity of genomic DNA, excessive and rough pipetting, mixing and vortexing should be avoided.
- DNA is subject to **acid hydrolysis** when stored in water, and should therefore be stored in **Tris-EDTA (TE)** buffer.

Basic Protocol

- Most DNA extraction protocols consist of two parts
 1. A technique to lyse the cells gently and solubilize the DNA
 2. Enzymatic or chemical methods to remove contaminating proteins, RNA, or macromolecules

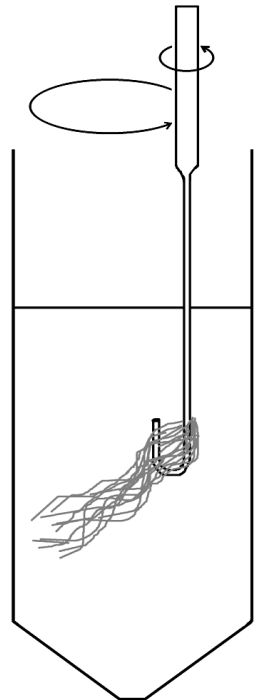
- The successful isolation of genomic DNA has three basic steps:
- -Cell disruption (i.e., disruption of the cellular organisation to solubilise nucleic acids)
chemical, mechanical/physical or enzymatic means
- -Nucleic acid extraction (i.e., separation of nucleic acids from cell debris and insoluble proteins)
- -DNA purification (i.e., separation of DNA from soluble proteins, RNA and inhibitors)

- Chemical disruption generally involves the use of a lysis buffer containing a **detergent**, such as **sodium dodecyl sulphate (SDS)**, to break apart the cell and organelle membranes.
- Because of the similar structure of both the lipid molecules and the detergent molecules, the detergent component of the lysis buffer has the function of capturing the lipids constituting the plasma membrane and the nuclear, mitochondrial and chloroplast membranes.
- This allows for the release of genomic DNA.

- Following cell lysis and nuclease inactivation, cell debris may easily be removed from the lysate by **filtration or centrifugation**.
- Next is the removal of proteins and RNA.
- It is usual to remove most of the protein by digesting with proteolytic enzymes such as **proteinase K**, which is active against a **broad spectrum of native proteins**.
- Much of the RNA is cut by **RNases** that are released when the cells are **broken open**.
- The presence of the RNA in DNA extracts is not a major problem as this does not interfere with PCR or restriction digestion.
- In some cases, however, it is important to isolate DNA free from RNA in order to be able to quantify the DNA yield accurately using a spectrophotometer.

- The most widely used method for concentrating DNA is **precipitation with ethanol**.

- Precipitate DNA with an alcohol
— usually [ethanol](#) or [isopropanol](#).
Since DNA is insoluble in these alcohols
, it will aggregate together, giving a *pellet* upon centrifugation.
This step also removes alcohol-soluble salt



- It is essential to inactivate DNases, which will otherwise digest DNA.
- Ethylene diamine tetraacetate (EDTA) is a chelating agent that acts to remove the divalent cations from the solution, thus inactivating DNase I and preventing it from degrading the genomic DNA.

What are the essential components of a DNA extraction Procedure?

1. Maximize DNA recovery
2. Remove inhibitors
3. Remove or inhibit nucleases
4. Maximize the quality of DNA
5. Double strand vs. Single strand (RFLP or PCR)

Isolation of Nucleic Acids

Goals:

- removal of proteins
- DNA vs RNA
- isolation of a specific type of DNA (or RNA)

Types of Methods:

- differential solubility
- 'adsorption' methods
- density gradient centrifugation

Types of DNA:

- genomic (chromosomal)
- organellar (satellite)
- plasmid (extra-chromosomal)
- phage/viral (ds or ss)
- complementary (mRNA)
- Mitochondrial DNA

General Features:

- denaturing cell lysis (SDS, alkali, boiling, chaotropic)
- \pm enzyme treatments
 - protease
 - RNase (DNase-free)
 - DNase (RNase-free)

UV–Visible Absorbance Spectrophotometric Measurement of DNA Concentration and Purity

DNA	A_{260}	$1.0 \approx 50 \mu\text{g/ml}$
	A_{260}/A_{280}	$1.6 - 1.8$
RNA	A_{260}	$1.0 \approx 40 \mu\text{g/ml}$
	A_{260}/A_{280}	~ 2.0

DNA Replication in Cells

- DNA replication is copying 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

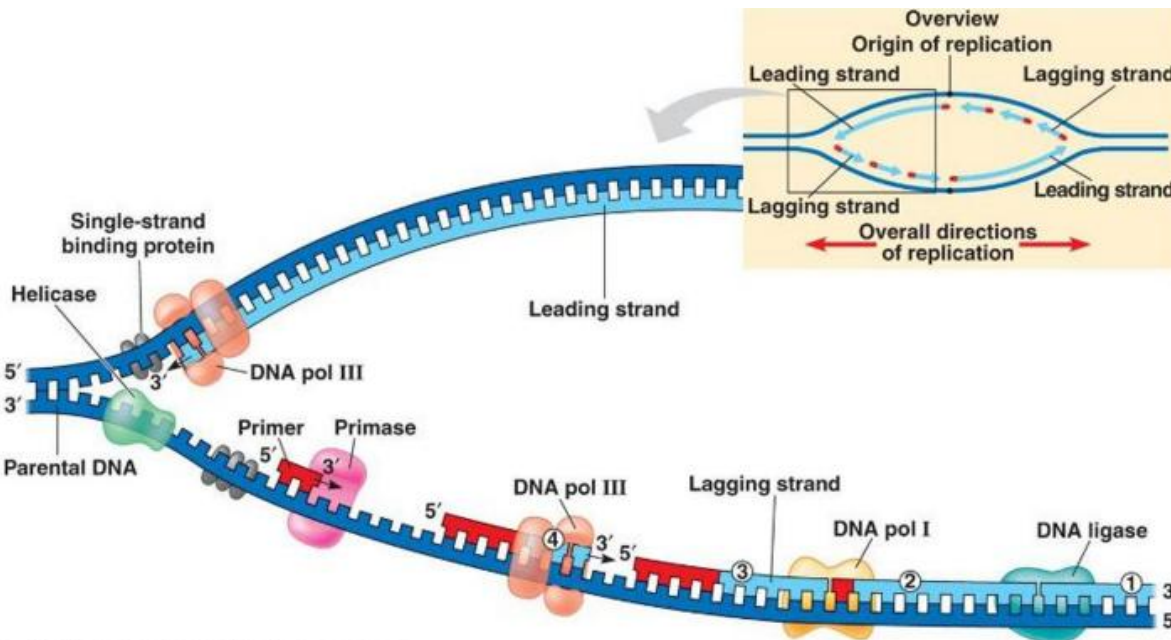
DNA Replication enzymes:

DNA Ligase

- 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*

• **DNA polymerases** can only add nucleotides to the 3' end, thus:

–one strand (referred to as the **leading strand**) of DNA is synthesized continuously
–the other strand (referred to as the **lagging strand**) is synthesized in fragments (called **Okazaki fragments**) that are joined together by **DNA ligase**.



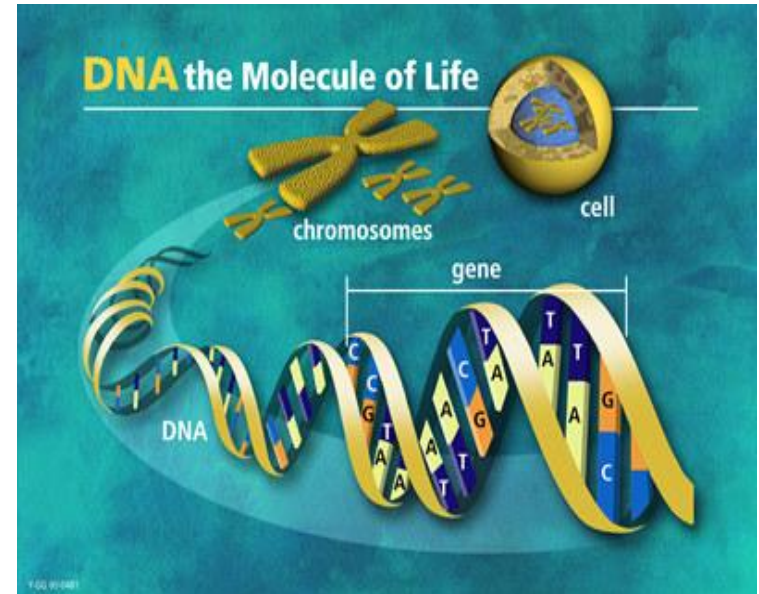
DNA Replication enzymes:

Helicase, Topoisomerase and Single-strand binding protein

- **Helicase** separates two parallel DNA strands
- **Topoisomerase** regulate the overwinding or underwinding of DNA and relieves the stress of this twisting
- **Single-strand binding protein** binds to and stabilizes the unpaired DNA strands

Polymerase chain reaction (PCR)

- *in vitro* version of DNA Replication
- Multiple copies of specific DNA sequence
 - 'Molecular photocopying'



Polymerase chain reaction

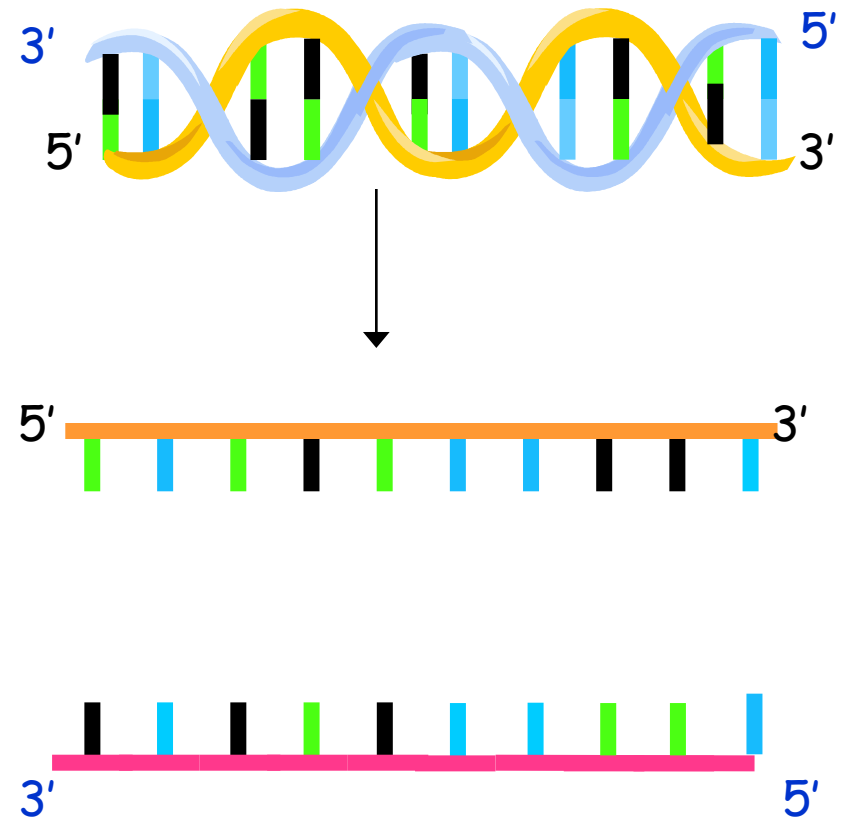
- “discovered” in 1983 by Kary Mullis
- 1993: Nobel Prize for Chemistry



Stages in PCR

- **Denaturation**

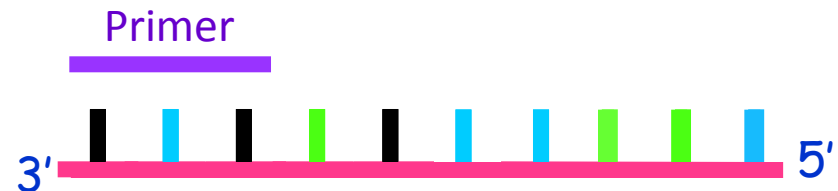
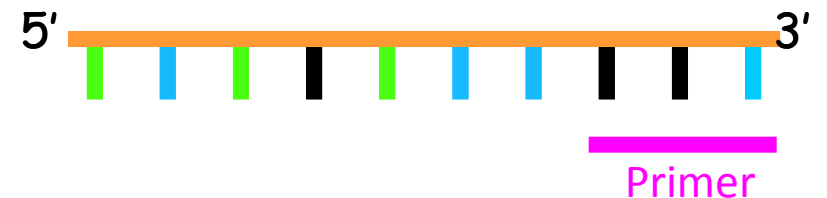
- Heat to separate double strands
- This occurs at 95 °C
- Mimics the function of **helicase** in the cell.



Stages in PCR

- **Annealing**

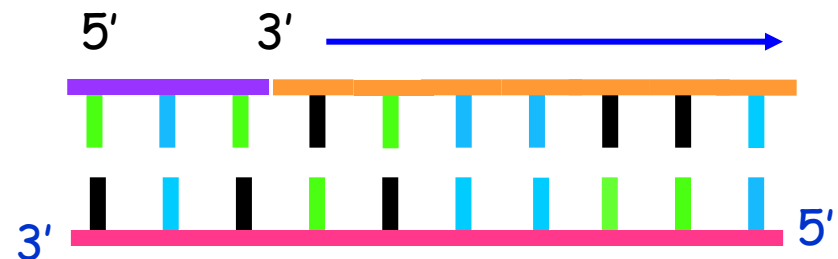
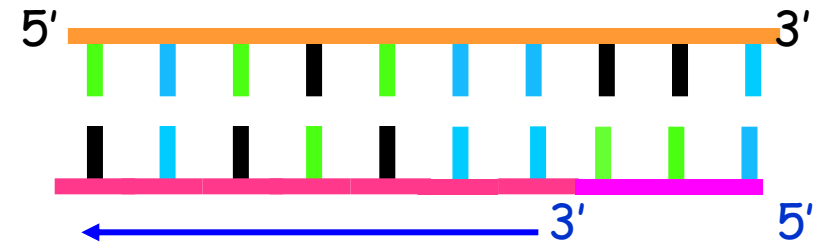
- Primer binds to template sequence
- Primers are chosen such that one is complimentary to the one strand at one end of the target sequence and that the *other* is complimentary to the *other* strand at the other end of the target sequence.



Stages in PCR

- **Elongation**

- Primer is extended with addition of dNTPs with Taq polymerase
- the extension of the strand in the 5-3 direction starting at the primers attaching the appropriate nucleotide (A-T, C-G)

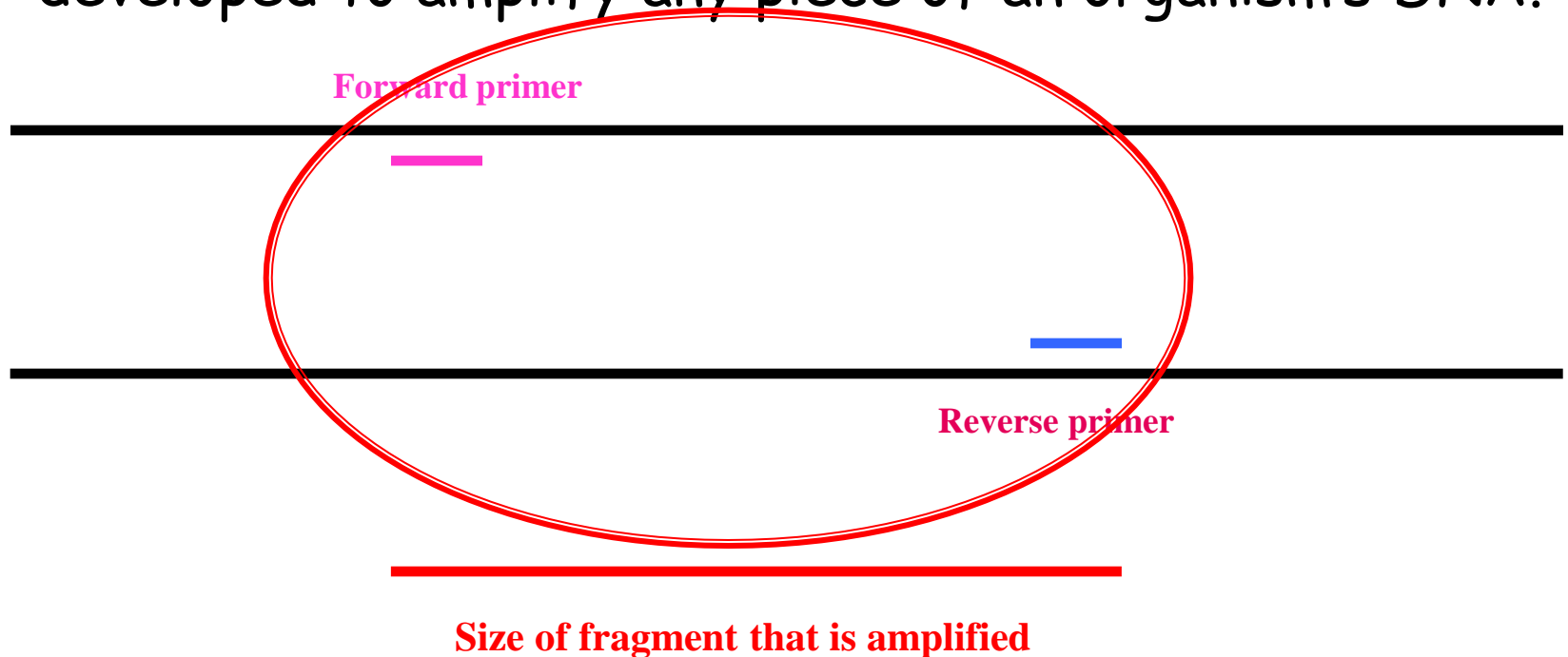


Steps of a single PCR cycle involve:

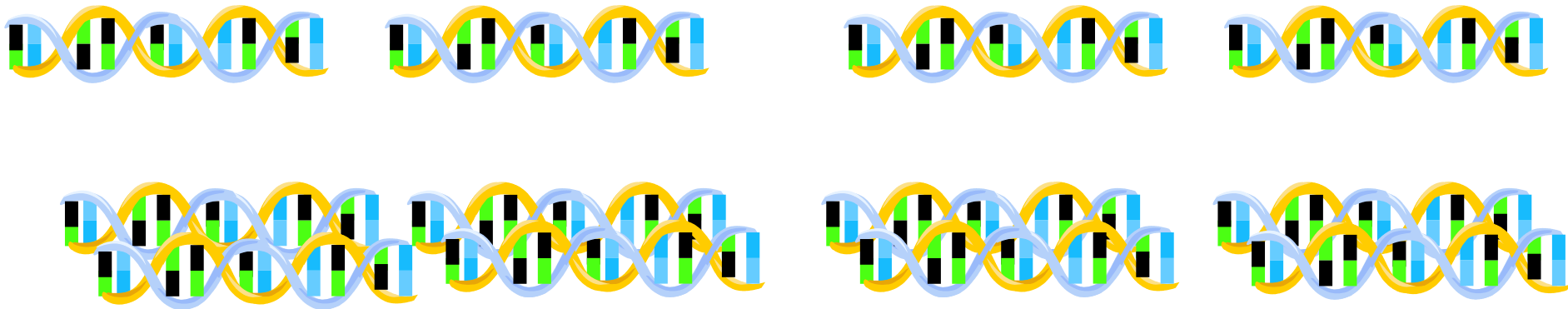
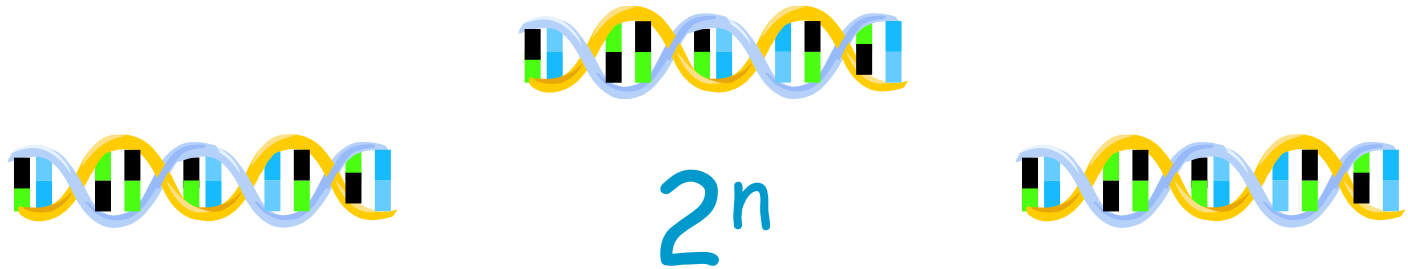
- **-DNA denaturation:** expose the DNA template to high temperatures to separate the two DNA strands and allow access by DNA polymerase and PCR primers.
 - **-Primer annealing:** lower the temperature to allow primers to anneal to their complementary sequence.
 - **-Primer extension:** adjust the temperature for optimal thermostable DNA polymerase activity to extend primers.
- PCR uses a thermostable DNA polymerase so that the DNA polymerase is not heat-inactivated during the DNA denaturation step. *Taq DNA polymerase is the most commonly used DNA polymerase for PCR.*

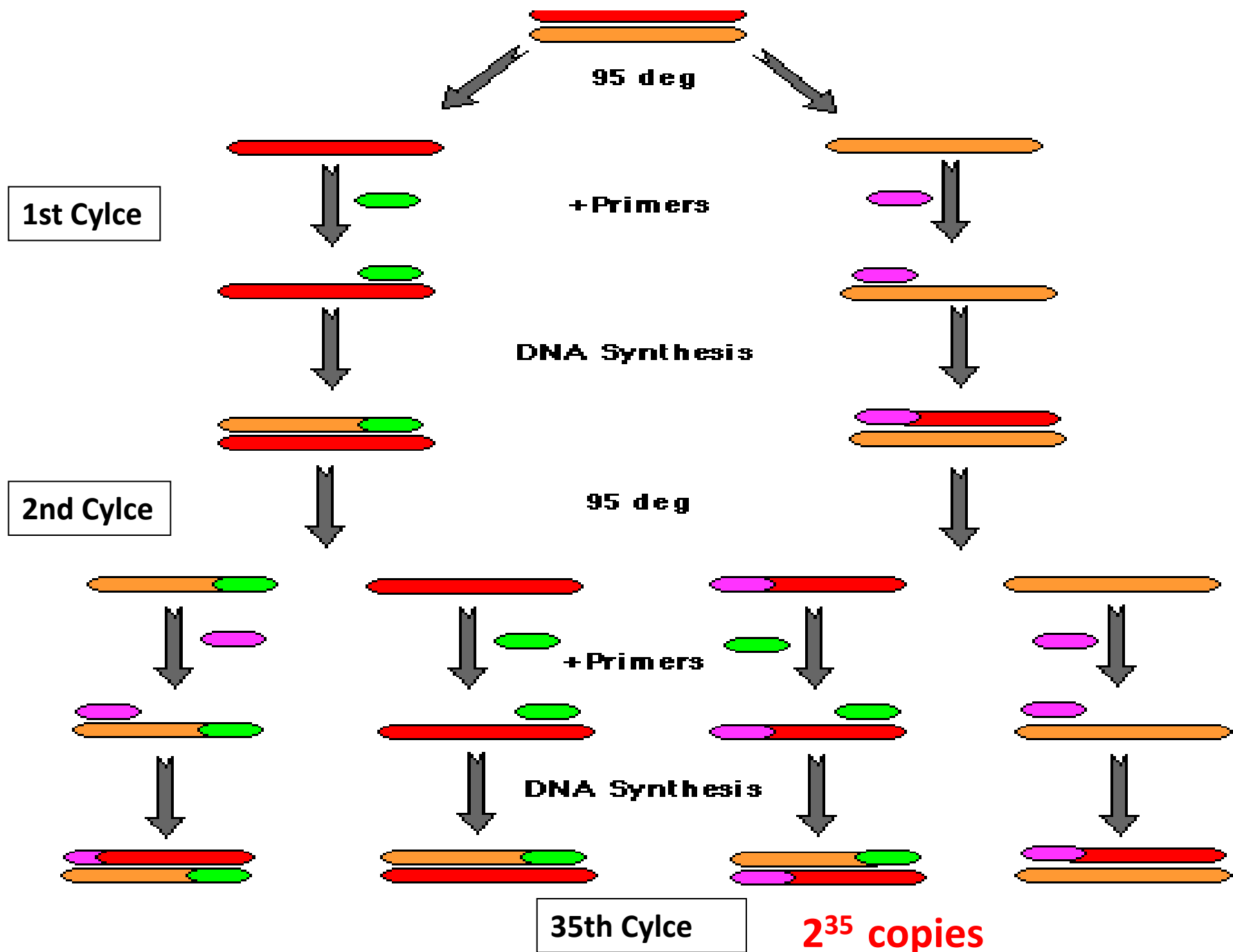
The Size of the DNA Fragment Produced in PCR is Dependent on the Primers

- The PCR reaction will amplify the DNA section between the two primers.
- If the DNA sequence is known, primers can be developed to amplify any piece of an organism's DNA.



PCR amplification





Genomic DNA

PCR inhibitor	Inhibiting concentration
SDS	> 0.005%
Phenol	> 0.2%
Ethanol	> 1%
Isopropanol	> 1%
Sodium acetate	> 5 mM
Sodium chloride	> 25 mM
EDTA	> 0.5 mM
Hemoglobin	> 1 mg ml ⁻¹
Heparin	> 0.15 IU ml ⁻¹
Urea	> 20 mM

Reagents for PCR

What we need in the laboratory:

- DNA template
- Primers
- DNA polymerase
- Buffer
- dNTPS (bases)
- $MgCl_2$

Reagents for PCR

- Typical components of a PCR mixture include:
 - **-DNA:** the template used to synthesise new DNA strands.
 - **-DNA polymerase:** an enzyme that synthesises new DNA strands.
 - **-Two PCR primers:** short DNA molecules (oligonucleotides) that define the DNA sequence to be amplified.
 - **-Deoxynucleoside triphosphates (dNTPs):** the building blocks for the newly synthesized DNA strands.
 - **-Reaction buffer:** a chemical solution that provides the optimal environmental conditions.
 - **-Magnesium:** a necessary cofactor for DNA polymerase activity.

Reagents for PCR

- **Primers**

- ssDNA sequences flanking region of interest
- Present in excess - up to $0.5\mu\text{M}$
 - (too high - primer dimers)

- **Primer Design**

- Homology of primers
- Length (18-28bp)
- GC content (45-60%) $T_m = 2X(A+T) + 4X(C+G)$

Reagents for PCR

- **DNA polymerase**
- Enzyme responsible for copying the sequence starting at the primer from the single DNA strand by adding nucleoside triphosphates to the 3' end of the growing strand
- DNA polymerase uses each strand as a template to synthesize new strands of DNA, complementary order of nucleotides.
- This enzyme is heat-tolerant → thermally tolerant (survives the melting temperature of DNA denaturation) which also means the process is more specific, higher temperatures result in less mismatch - more specific replication
- Many types available
 - Some modified to allow hot start
 - Some have long half life / stable at high temperature
 - Some have high rate of processivity

Reagents for PCR

- MgCl_2
- required for enzyme activation and amplification
- It stabilizes dsDNA and raises the T_m
- Mg^{2+} concentration controls the specificity of the reaction.

Reagents for PCR

- **Buffer** - providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
- **dNTPs (bases)**

PCR - before the thermocycler



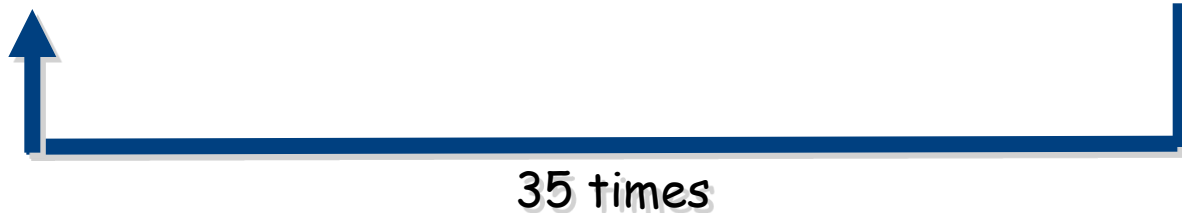
95° C
5 min



55° C
3 min



72° C
5 min



8 **BORING** hours per PCR!

PCR



PCR tube with all the reagents

THERMOCYCLER

- The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step.
- Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.
- Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube.

PCR is not usually 100% efficient and the reaction conditions may need to be optimized to increase yield, sensitivity of detection or amplification specificity.

- The usual parameters to vary include:

- Magnesium concentration
- Primer annealing temperature
- PCR primer design
- DNA quality
- DNA quantity

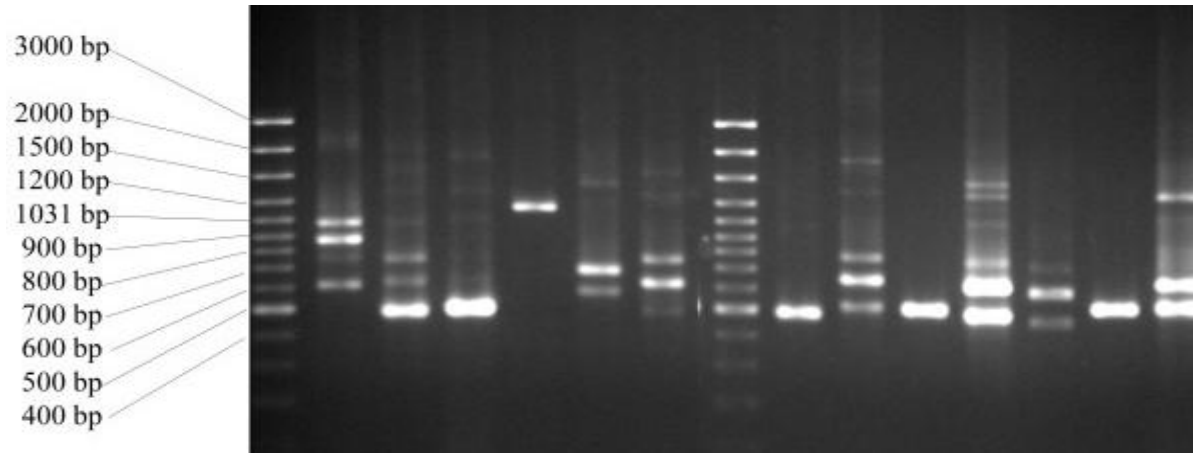
- This is very important when trying to amplify a particular target from a population of other sequences (*e.g., one gene from genomic DNA*).

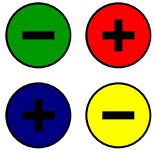
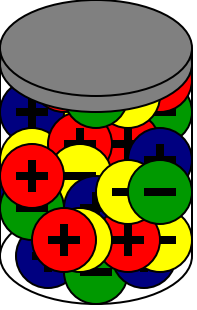
How do we analyse the PCR products?

- Gel electrophoresis
- Fluorescent PCR
- Sequencing
- Blotting
- Short tandem repeat (STR) analysis

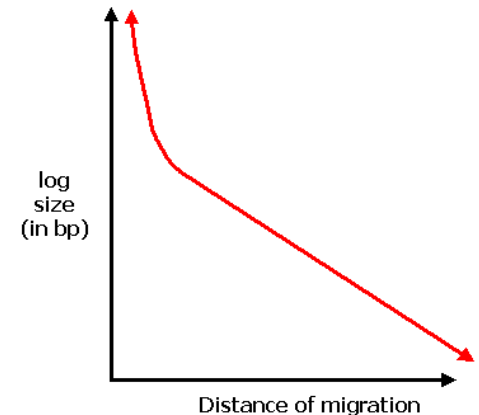
Gel Electrophoresis

- Fragmentation products of differing length are separated
- Separation of DNA fragments
- Separation based (mostly) on length
 - longer molecules move slower.
- Size and shape



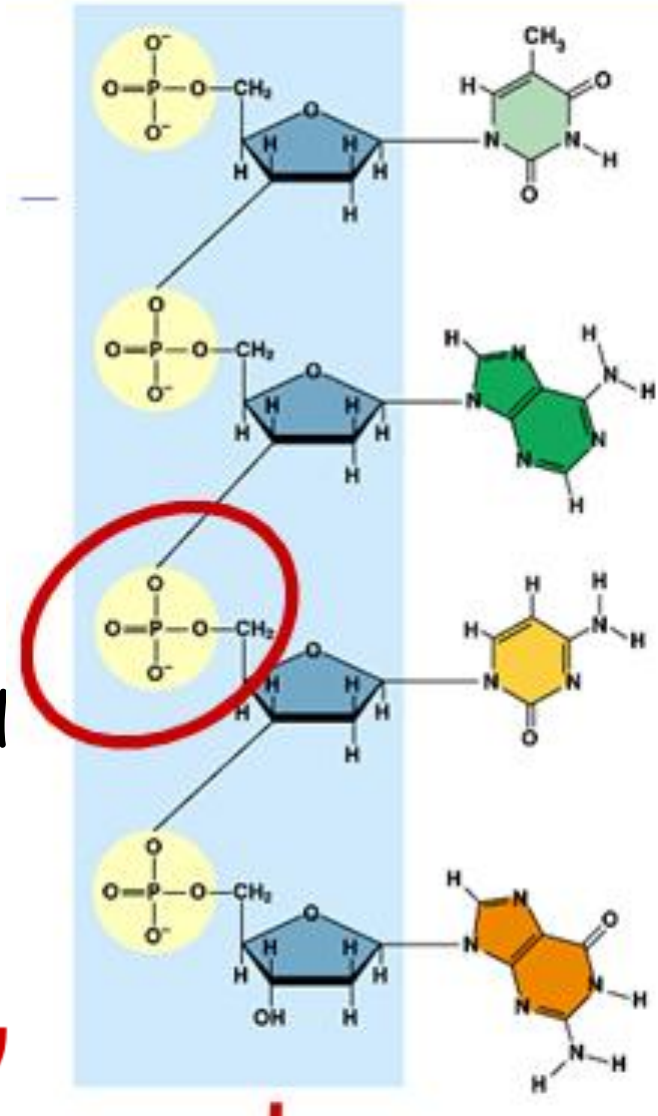
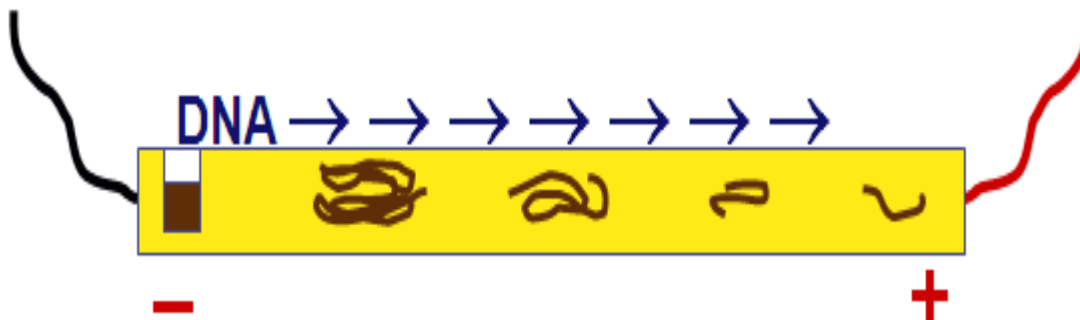


- Gel electrophoresis separates molecules on the basis of their **charge** and **size**.
- The charged macromolecules migrate across a span of gel because they are placed in an electrical field. The gel acts as a sieve to retard the passage of molecules according to their size and shape.



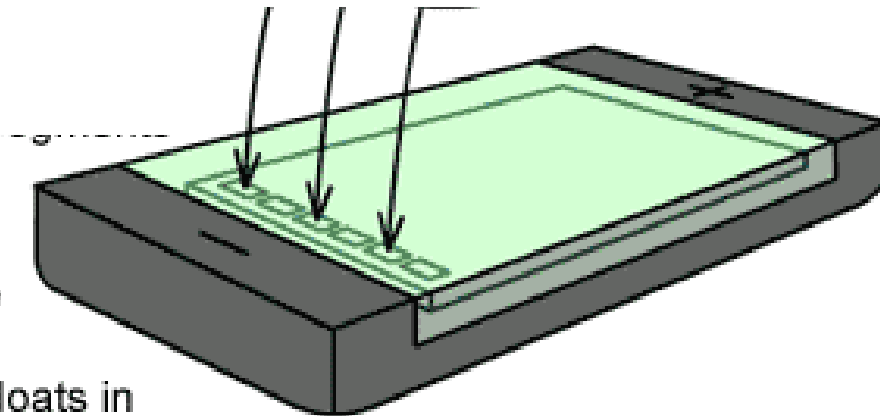
Gel electrophoresis

- A method of separating DNA in a gelatin-like material using an electrical field
- DNA is negatively charged
- When it's in an electrical field it moves toward the positive side.



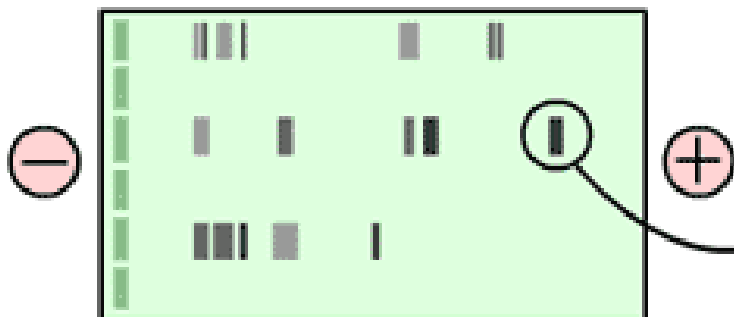
Gel Electrophoresis

DNA (or RNA) samples loaded into wells



2. DNA segments are loaded into wells in a porous gel. The gel floats in a buffer solution within a chamber between two electrodes.

3. When an electric current is passed through the chamber, DNA fragments move toward the positively-charged cathode.



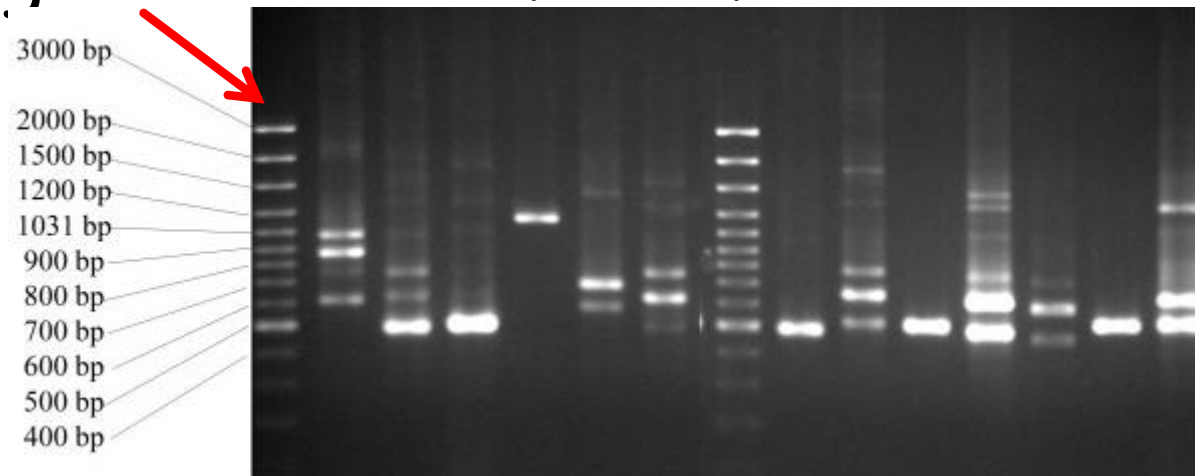
4. Smaller DNA segments move faster and farther than larger DNA segments.

How does gel electrophoresis work?

- DNA is forced by an **electrical current** through a firm gel
 - Phosphate group in DNA is **negatively charged** so it is moved towards a **positive electrode** by the current
 - **Longer fragments have more nucleotides**
 - So have a larger molecular weight
 - So are bigger in size
 - So aren't able to pass through the small holes in the gel and get hung up at the beginning of the gel
 - **Shorter fragments are able to pass through and move farther along the gel**
 - Fragments of intermediate length travel to about the middle of the gel

How does gel electrophoresis work?

- DNA fragments are then visualized in the gel with a special dye
- The number of nucleotides are then estimated by comparing it to a known sample of DNA fragments which is run through the gel at the same time → ladder



Reagents Needed for gel electrophoresis

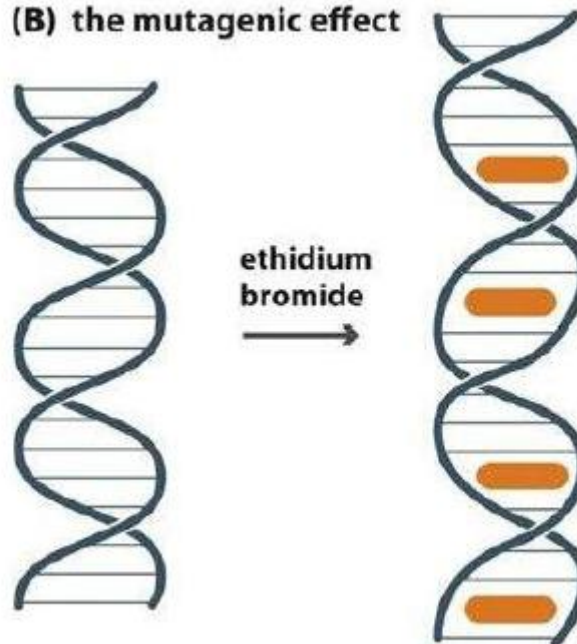
- Sample of DNA fragments
- Known sample of DNA fragments
 - DNA ladder
- Gel
 - Agarose
- Dye to visualize the movement of the sample as it is traveling through the gel
 - Loading dye
- Dye to visualize DNA after it has traveled to its final spot in the gel
 - Ethidium bromide
- Buffer

- The migrated DNA is visualised under UV light with the help of an intercalating dye, **ethidium bromide**, which fluoresces when irradiated with UV

(A) ethidium bromide

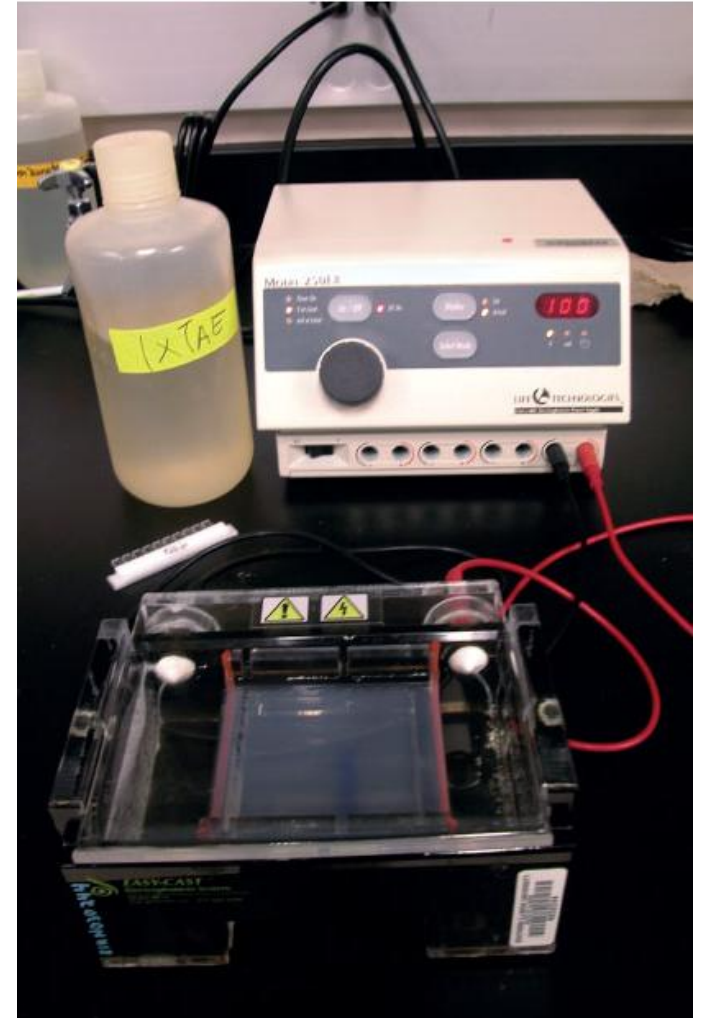


(B) the mutagenic effect



Equipment Needed for gel electrophoresis work

- Box to hold the gel
- Comb to create small wells in the agarose gel to put the DNA sample in
- Positive and negative electrodes to create the electrical current
- Power supply
- Gel photo imaging system



What are the different types of
PCRs?

MOLECULAR AMPLIFICATION TECHNIQUES

- Nucleic acid (NA) amplification methods fall into 3 categories
 - Target amplification systems
 - Probe amplification systems
 - Signal amplification

Target Amplification Methods

- PCR –
 - PCR using specific probes
 - RT PCR
 - Nested PCR-increases sensitivity, uses two sets of amplification primers, one internal to the other
 - Multiplex PCR-two or more sets of primers specific for different targets
 - Arbitrarily Primed PCR/Random Primer PCR
- NASBA - Nucleic Acid Sequence-Based Amplification
- TMA - Transcription Mediated Amplification
- SDA - Strand Displacement Amplification

Signal and Probe Amplification Methods

- Signal Amplification
 - bDNA - Branched DNA probes
 - Hybrid Capture - Anti-DNA-RNA hybrid antibody
- Probe Amplification
 - LCR - Ligase Chain Reaction
 - Cleavase Invader - FEN-1 DNA polymerase (cleavase)

PCR Modifications

- Nested PCR
- Multiplex PCR
- Tailed primers
- Sequence-specific PCR
- Reverse-transcriptase PCR
- Long-range PCR
- Whole-genome amplification
- RAPD PCR (AP-PCR)
- Quantitative real-time PCR

RT-PCR

- PCR of cDNA is used to detect specific transcripts in RNA sample.
- In this procedure, known as RT-PCR, reverse transcriptase is used to copy all of the mRNAs in an RNA sample into cDNA.
- Usually, oligo dT molecules, that anneal to the poly A tails of the mRNA, are used as primers.
- This single stranded cDNA can then be amplified by PCR using primers that anneal to a specific transcript sequence.

RT-PCR

- The amplified DNA fragments that are produced can be analysed by agarose gel electrophoresis or fluorescent PCR or real time PCR.
- The amount of amplified fragment produced is proportional to the amount of target mRNA in the original RNA sample.
- RT-PCR is extremely sensitive and can be used to detect very rare mRNA species.

Synthesize cDNA Using RT

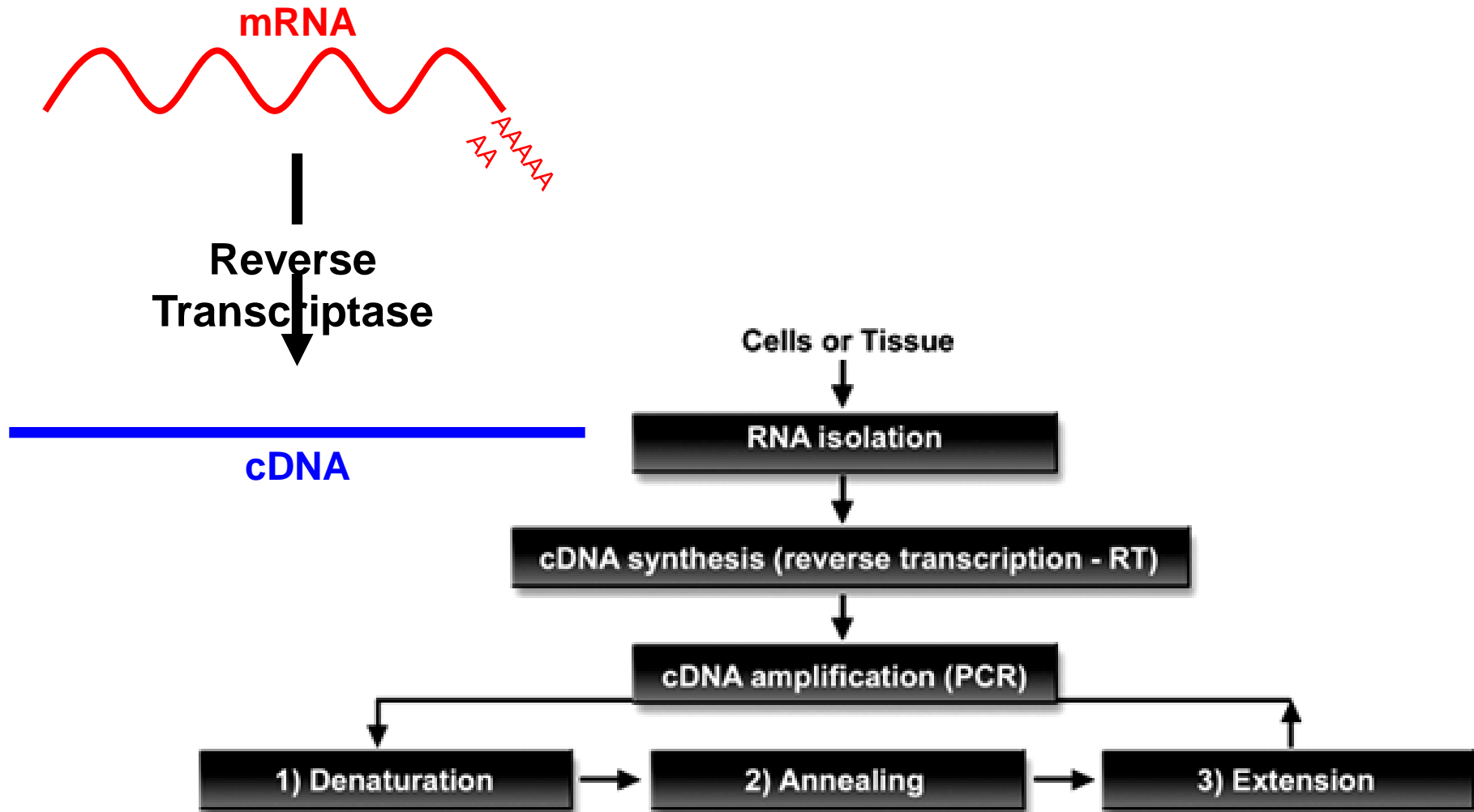
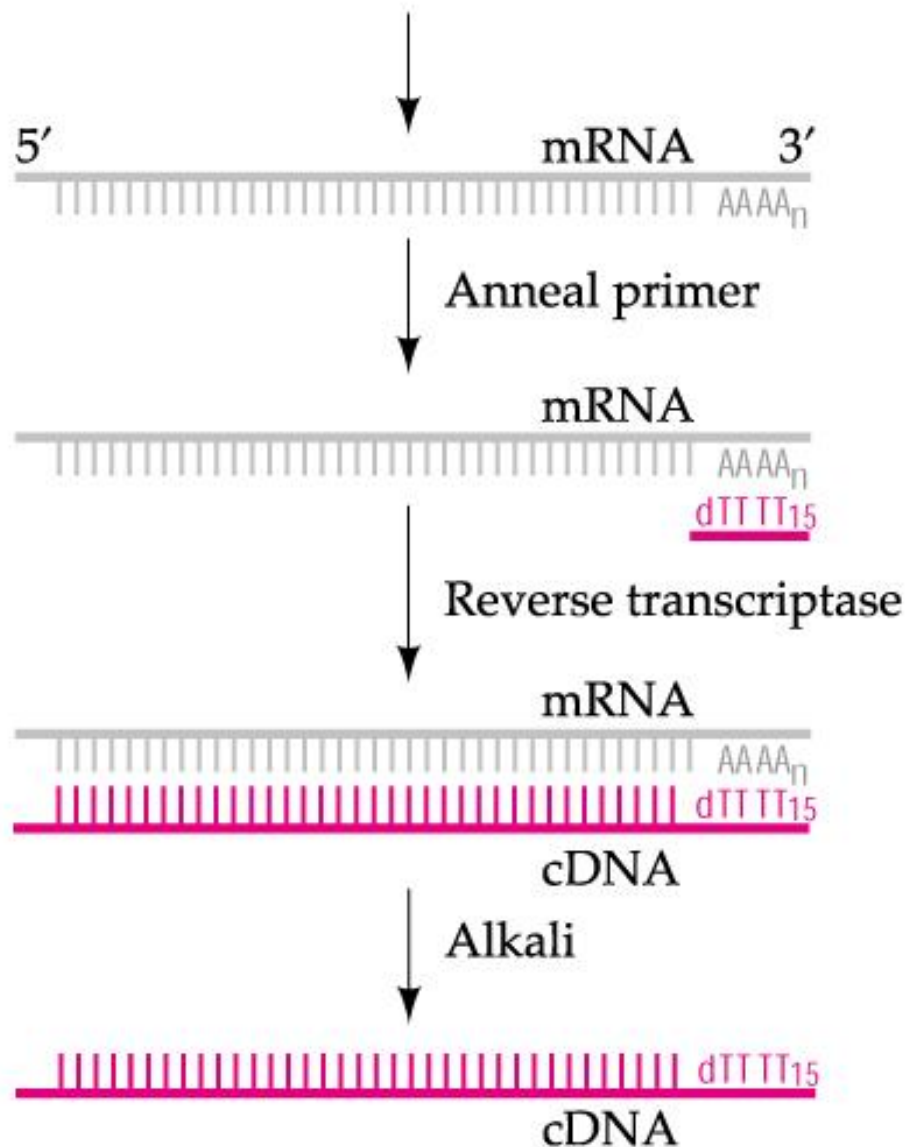


FIGURE 3 - Schematic diagram of RT-PCR showing that RNA isolated from cells or tissue is used as substrate in reverse transcription for synthesis of cDNA that will serve as template for amplification by PCR.

Reverse Transcription



- mRNA can be copied to complementary DNA sequence (cDNA) using reverse transcriptase—a DNA polymerase that uses ssRNA as template.
- Processed mRNA will match protein coding sequence while unprocessed (nuclear) mRNA will contain intron sequences.

The Polymerase Chain Reaction (PCR) is a process for the amplification of specific fragments of DNA.

What is Real- Time PCR?

Real-Time PCR a specialized technique that allows a PCR reaction to be visualized “in real time” as the reaction progresses.

As we will see, Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!

Overview of Real -Time PCR

- Real-Time PCR combines DNA amplification with real time amplified product detection in a single tube.
- More specific than gel or hybridization assays.
- Less time consuming
- Quantitative results as opposed to semi-quantitative or qualitative results.
- Human (higher primate) or Y chromosome specific
- Monitors for PCR inhibition

What is Real- Time PCR used for?

Real-Time PCR has become a cornerstone of molecular biology:

- **Gene expression analysis**
 - Cancer research
 - Drug research
- **Disease diagnosis and management**
 - Viral quantification
- **Food testing**
 - Percent GMO food
- **Animal and plant breeding**
 - Gene copy number

Real-Time PCR in Gene Expression Analysis

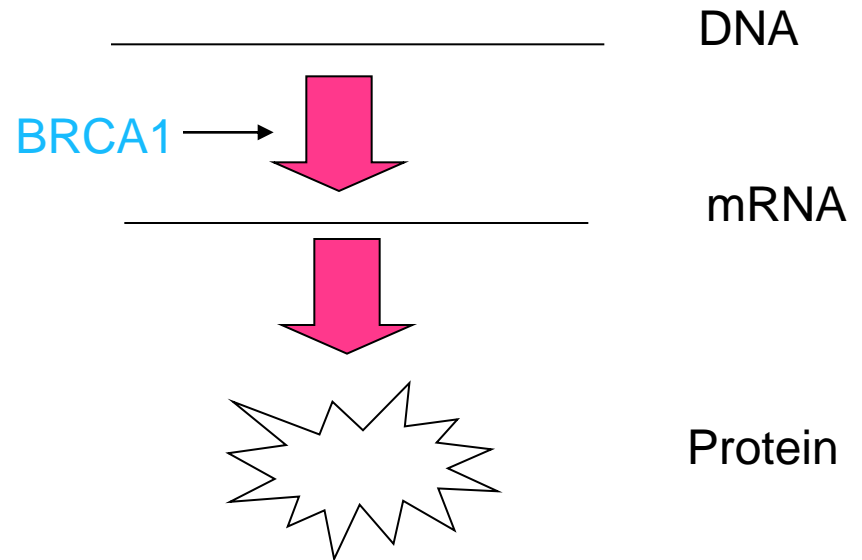
Example: BRCA1 Expression Profiling

BRCA1 is a gene involved in tumor suppression.

BRCA1 controls the expression of other genes.

In order to monitor level of expression of BRCA1, real-time PCR is used.

The screenshot shows a web browser window displaying a research article from *Breast Cancer Research*. The article title is "Real-time PCR-based expression profiling of BRCA1-induced genes in primary breast tumors". The authors listed are B Gur¹, B Bozkurt², O Konu¹, S Seckin³, and IG Yulug¹. The article was published on 17 June 2005. The background section begins with: "BRCA1 possesses a number of features common to transcriptional regulatory proteins, suggesting that it may regulate the expression of one or more downstream genes. It is important to determine which genes are transcriptionally influenced by BRCA1 in vivo to explain its role in tumor suppression and in cancer development. In our previous study, a BRCA1 overexpression system enabled us to define the genes whose expression levels were induced in MCF-7 breast cancer cells by using the PCR-dependent suppression subtractive hybridization technique [1]. Herein, we report the preliminary results obtained from our real-time expression profiling of normal-matched primary breast tumors for six genes, three of which were previously reported [1]. The

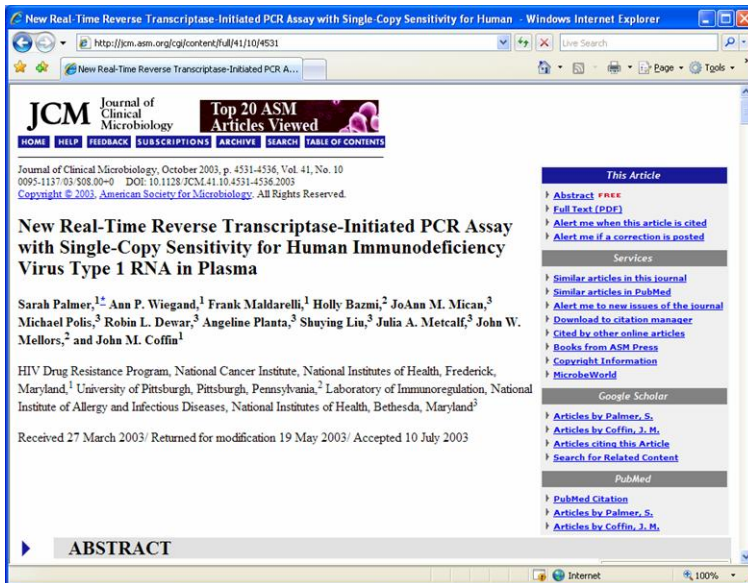


Real-Time PCR in Disease Management

Example: HIV Treatment

Drug treatment for HIV infection often depends on monitoring the “viral load”.

Real-Time PCR allows for direct measurement of the amount of the virus RNA in the patient.



The screenshot shows a Windows Internet Explorer browser window displaying a research article from the Journal of Clinical Microbiology. The article title is "New Real-Time Reverse Transcriptase-Initiated PCR Assay with Single-Copy Sensitivity for Human Immunodeficiency Virus Type 1 RNA in Plasma". The authors listed are Sarah Palmer, Ann P. Wiegand, Frank Maldarelli, Holly Bazmi, JoAnn M. Mican, Michael Polis, Robin L. Dewar, Angeline Plante, Shuying Liu, Julia A. Metcalf, John W. Mellors, and John M. Coffin. The article is from the October 2003 issue, volume 41, number 10. The browser window also shows a sidebar with links to the abstract, full text, and other services.

Journal of Clinical Microbiology
Top 20 ASM Articles Viewed

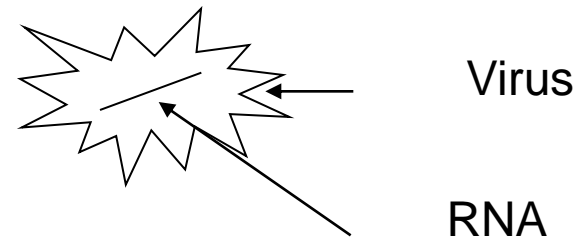
New Real-Time Reverse Transcriptase-Initiated PCR Assay with Single-Copy Sensitivity for Human Immunodeficiency Virus Type 1 RNA in Plasma

Sarah Palmer,^{1,2} Ann P. Wiegand,¹ Frank Maldarelli,¹ Holly Bazmi,² JoAnn M. Mican,³ Michael Polis,³ Robin L. Dewar,³ Angeline Plante,³ Shuying Liu,³ Julia A. Metcalf,³ John W. Mellors,² and John M. Coffin¹

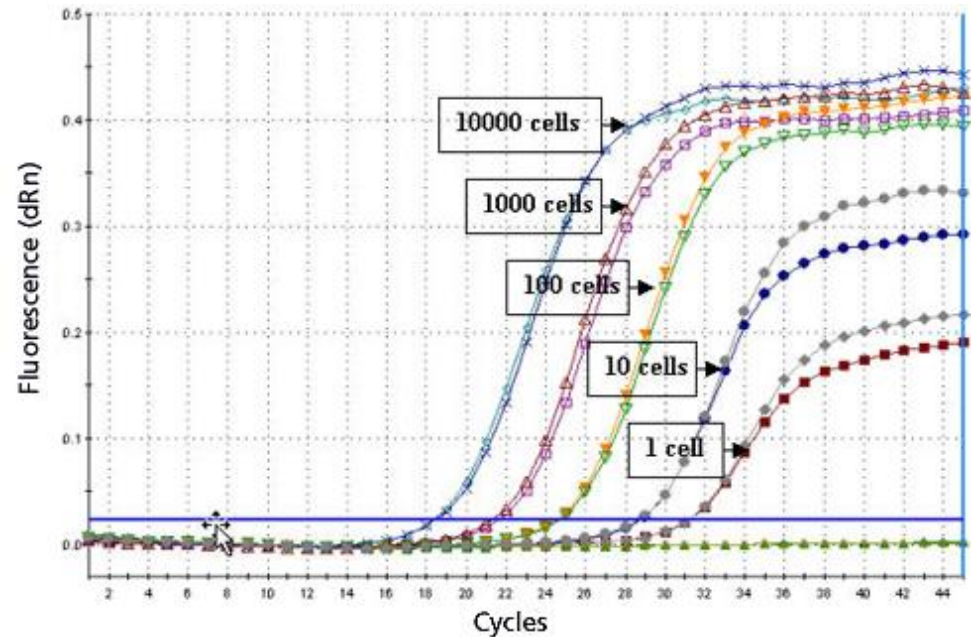
HIV Drug Resistance Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland,¹ University of Pittsburgh, Pittsburgh, Pennsylvania,² Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland³

Received 27 March 2003/ Returned for modification 19 May 2003/ Accepted 10 July 2003

ABSTRACT



Real-time quantitative PCR amplification plot:



How do We Measure DNA in a PCR Reaction?

We use reagents that fluoresce in the presence of amplified DNA!

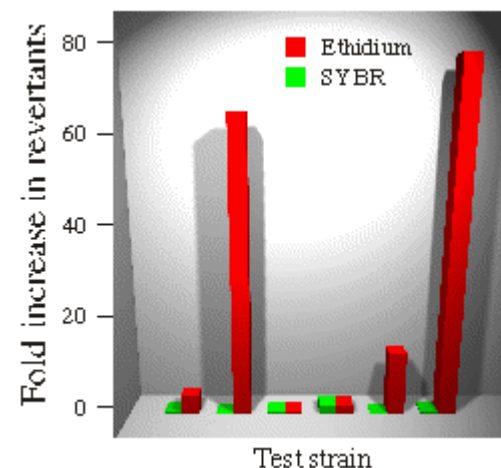
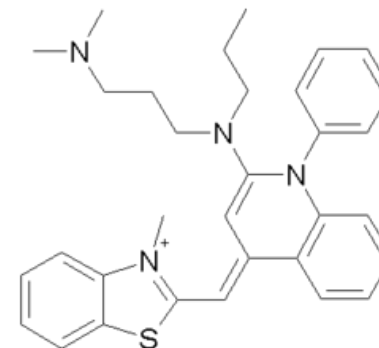
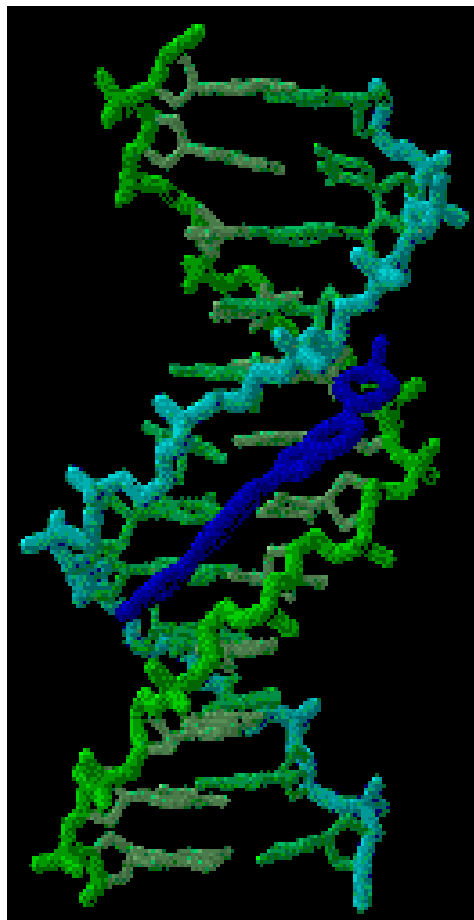
Ethidium bromide and SYBR Green I dye are two such reagents.

They bind to double-stranded DNA and emit light when illuminated with a specific wavelength.

SYBR Green I dye fluoresces much more brightly than ethidium.

Measuring DNA: SYBR Green I

SYBR Green I



Ames test results from Molecular Probes
Singer et al., Mutat. Res. 1999, 439: 37- 47

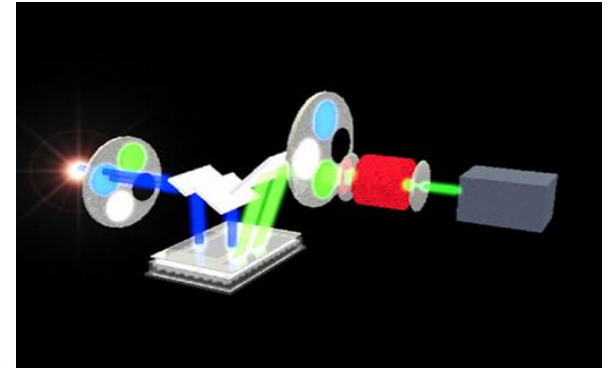
What Type of Instruments are used with Real-Time PCR?

Real-time PCR instruments consist of **THREE** main components:

1. Thermal Cycler (PCR machine)
2. Optical Module (to detect fluorescence in the tubes during the run)
3. Computer (to translate the fluorescence data into meaningful results)

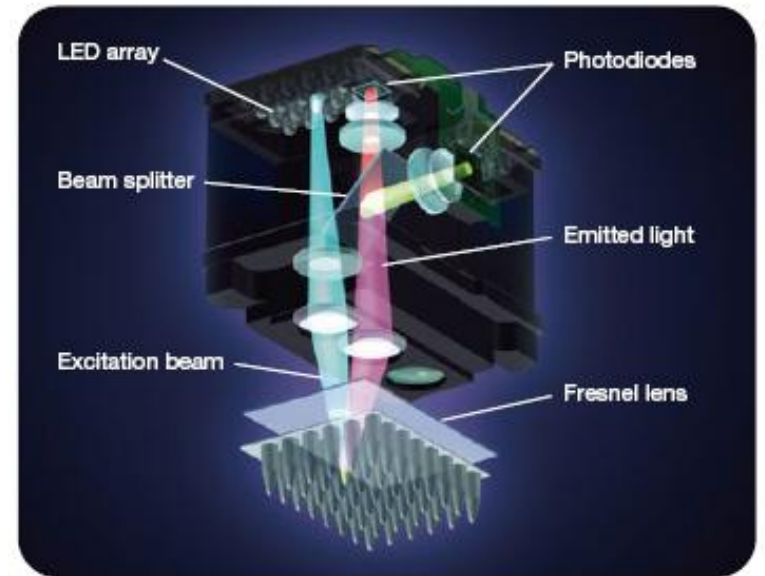
What Type of Instruments are used with Real- Time PCR?

An example of such an instrument is the Bio-Rad iQ5 real-time PCR instrument.



What
Type of
Instruments
are
used with
Real-
Time
PCR?

Another example is the MiniOpticon real-time instrument.



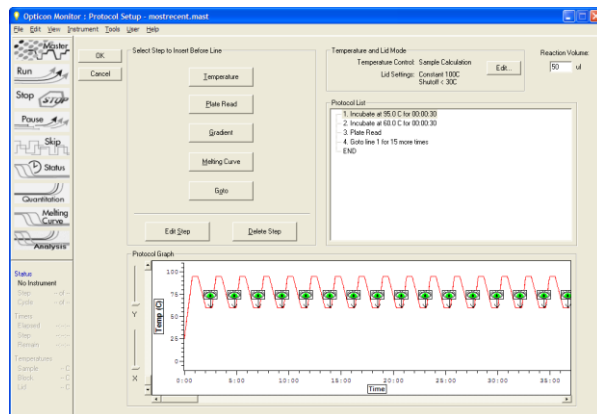
What Type of Software is used with Real-Time PCR?

The real-time software converts the fluorescent signals in each well to meaningful data.

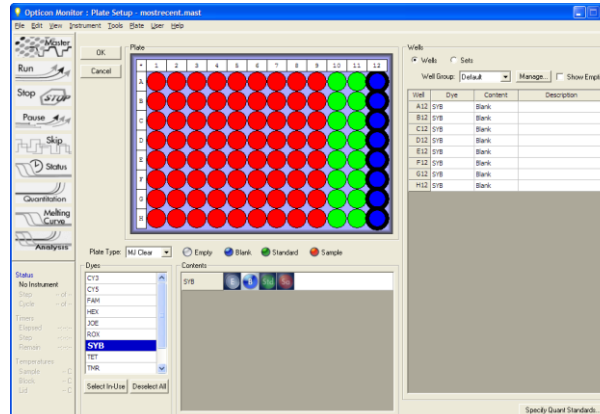
1. Set up PCR protocol.
2. Set up plate layout.
3. Collect data.
4. Analyze data.



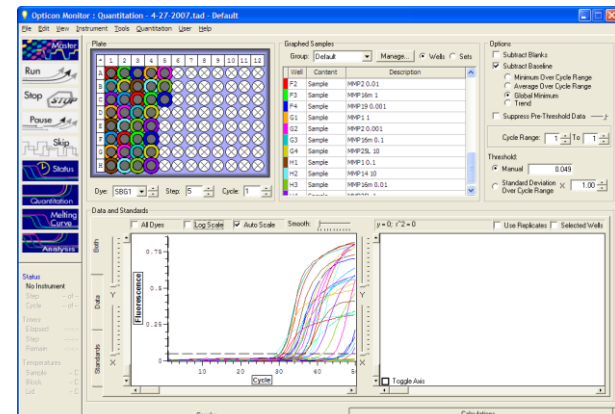
1



2



3,4



Cystic fibrosis

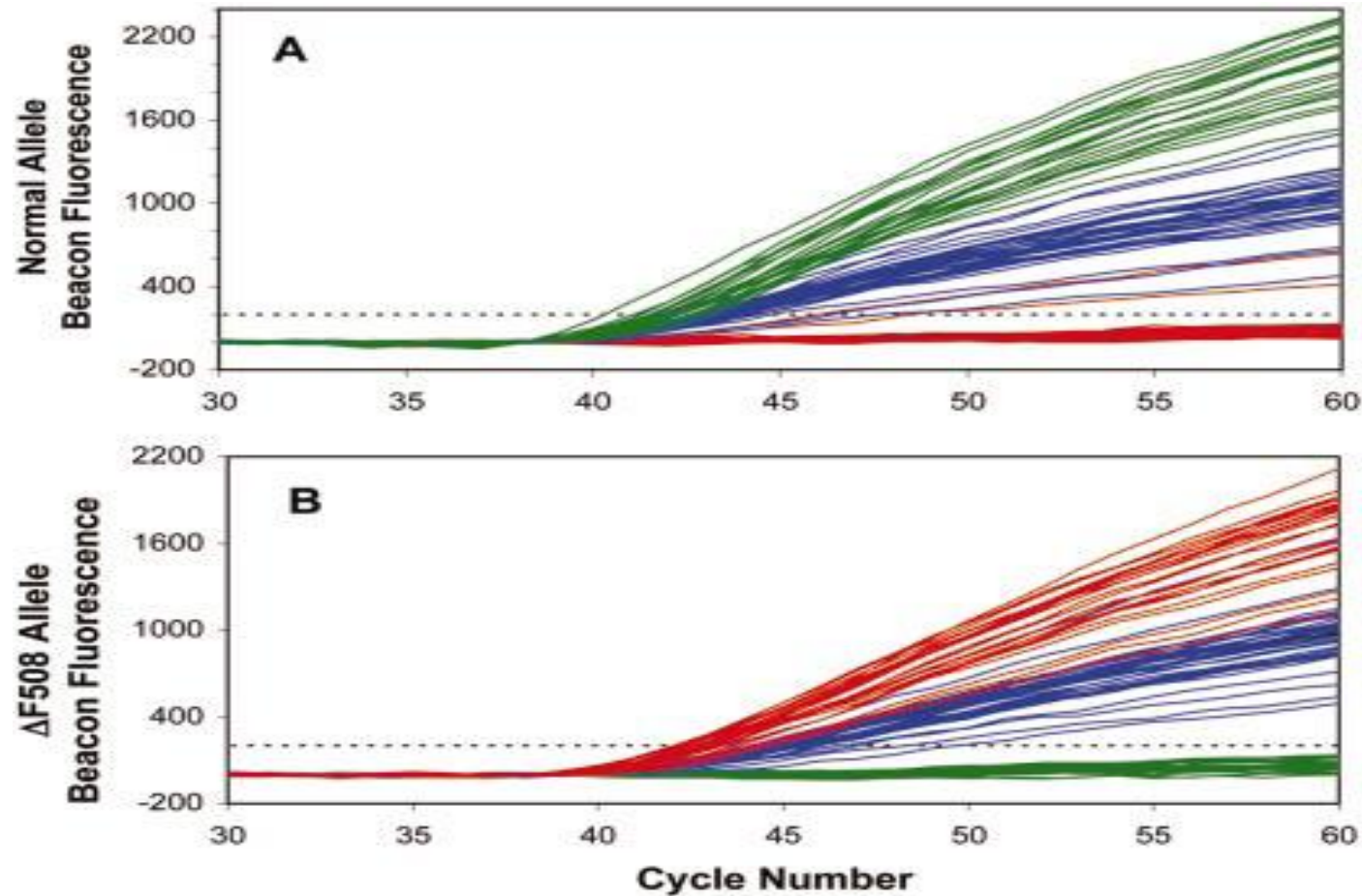
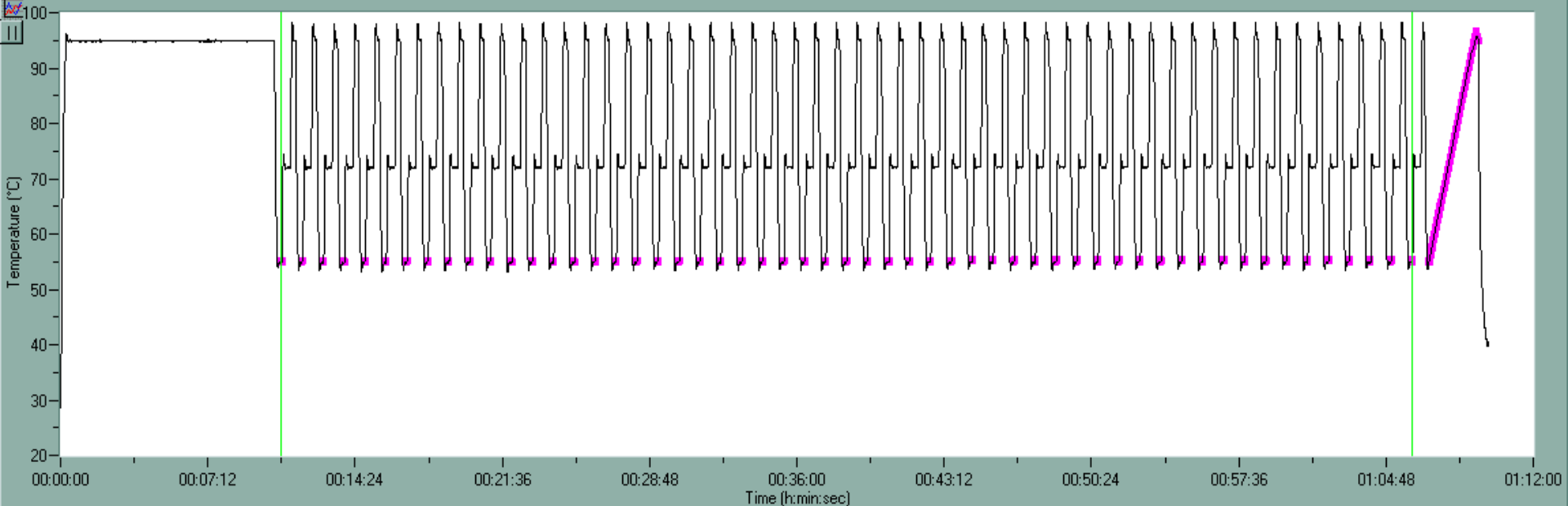
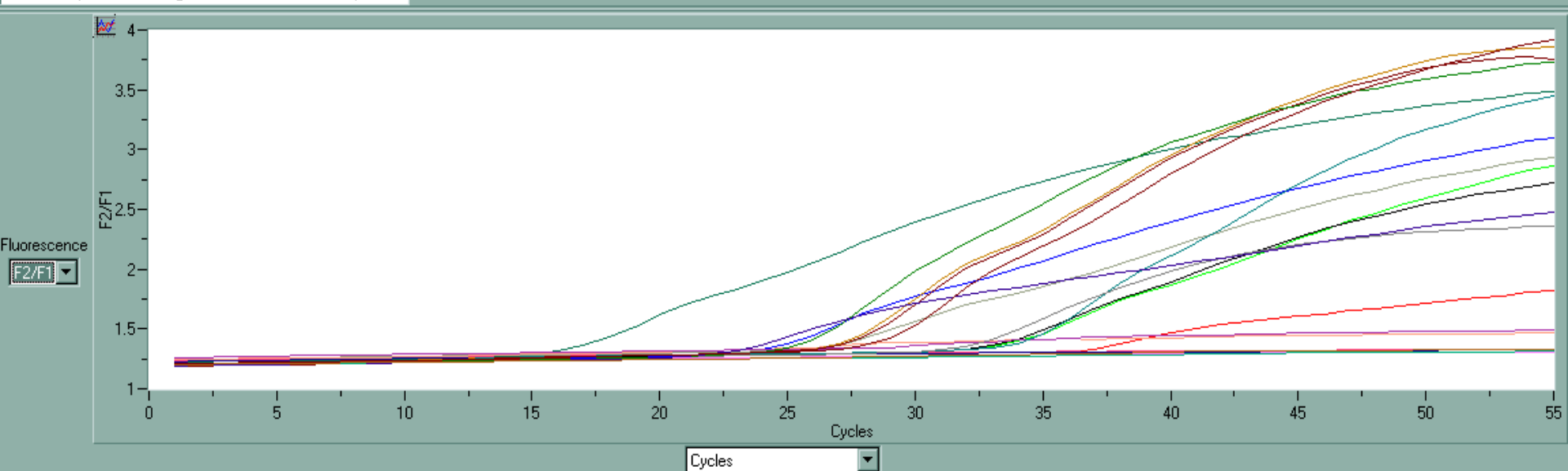
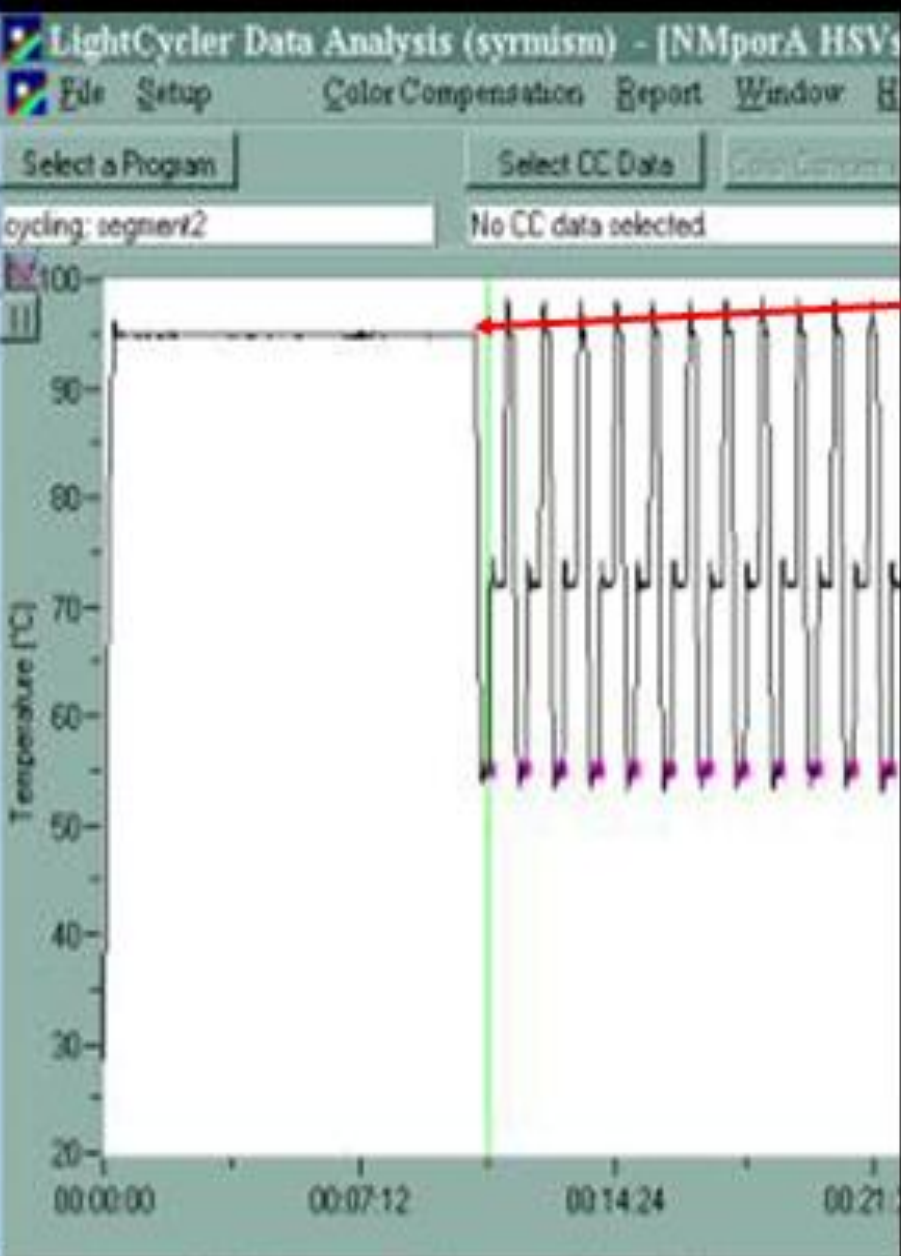


Figure 1. Examples of specific molecular beacon fluorescence increase during real-time PCR in samples containing single lymphoblasts homozygous normal for CF (green), heterozygous DF508 (blue), or homozygous DF508 (red). (A) Fluorescent signal from the molecular beacon detecting the normal allele. (B) Fluorescent signal from the molecular beacon detecting the DF508 allele. Dashed lines indicate the threshold of 200 units (~ 10 SD above baseline readings) used for determining CT values.



— Temperature Tracing ■ Fluorescence Acquisition

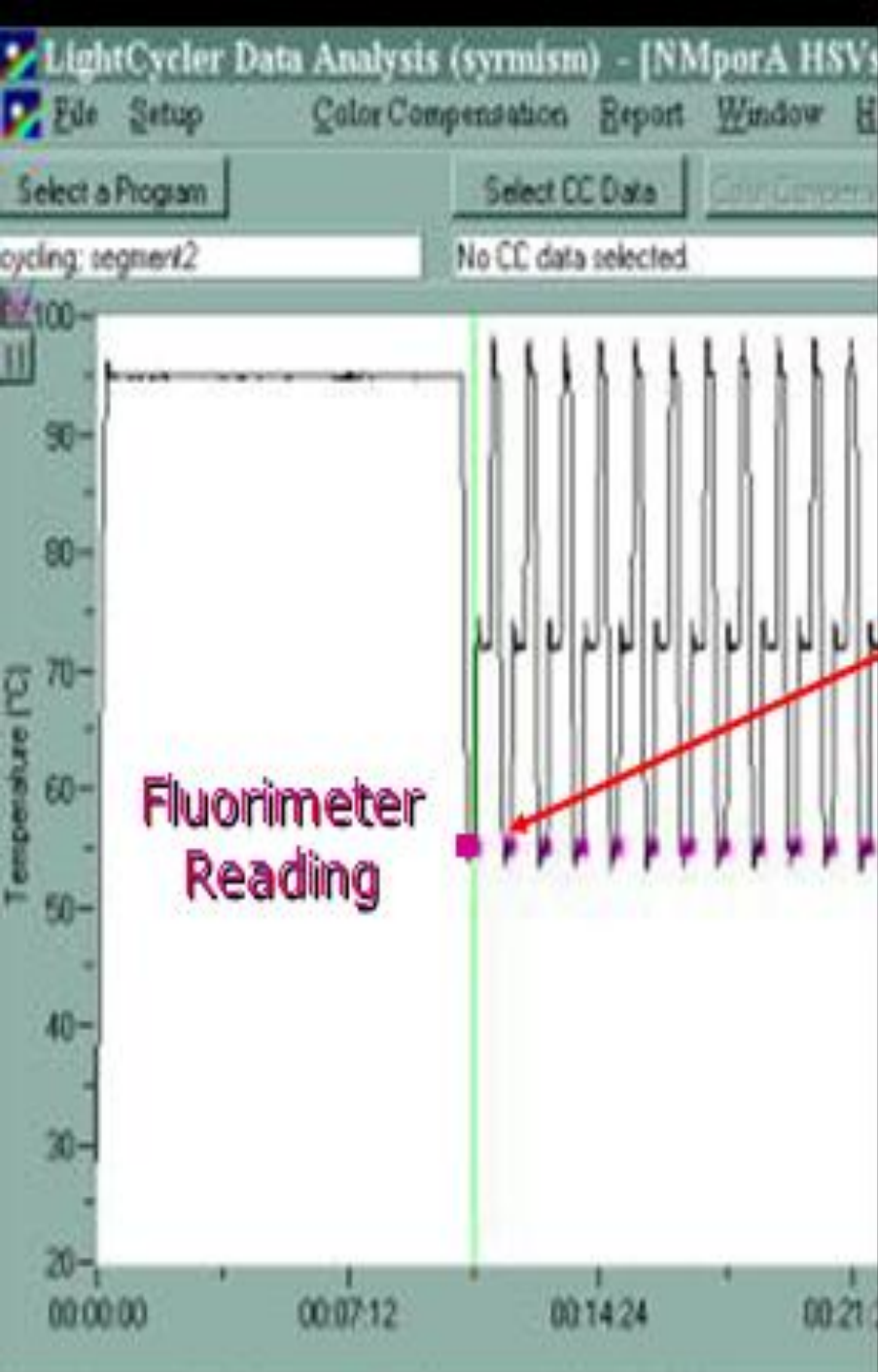




95°C



Denatürasyon



T_m

55°C

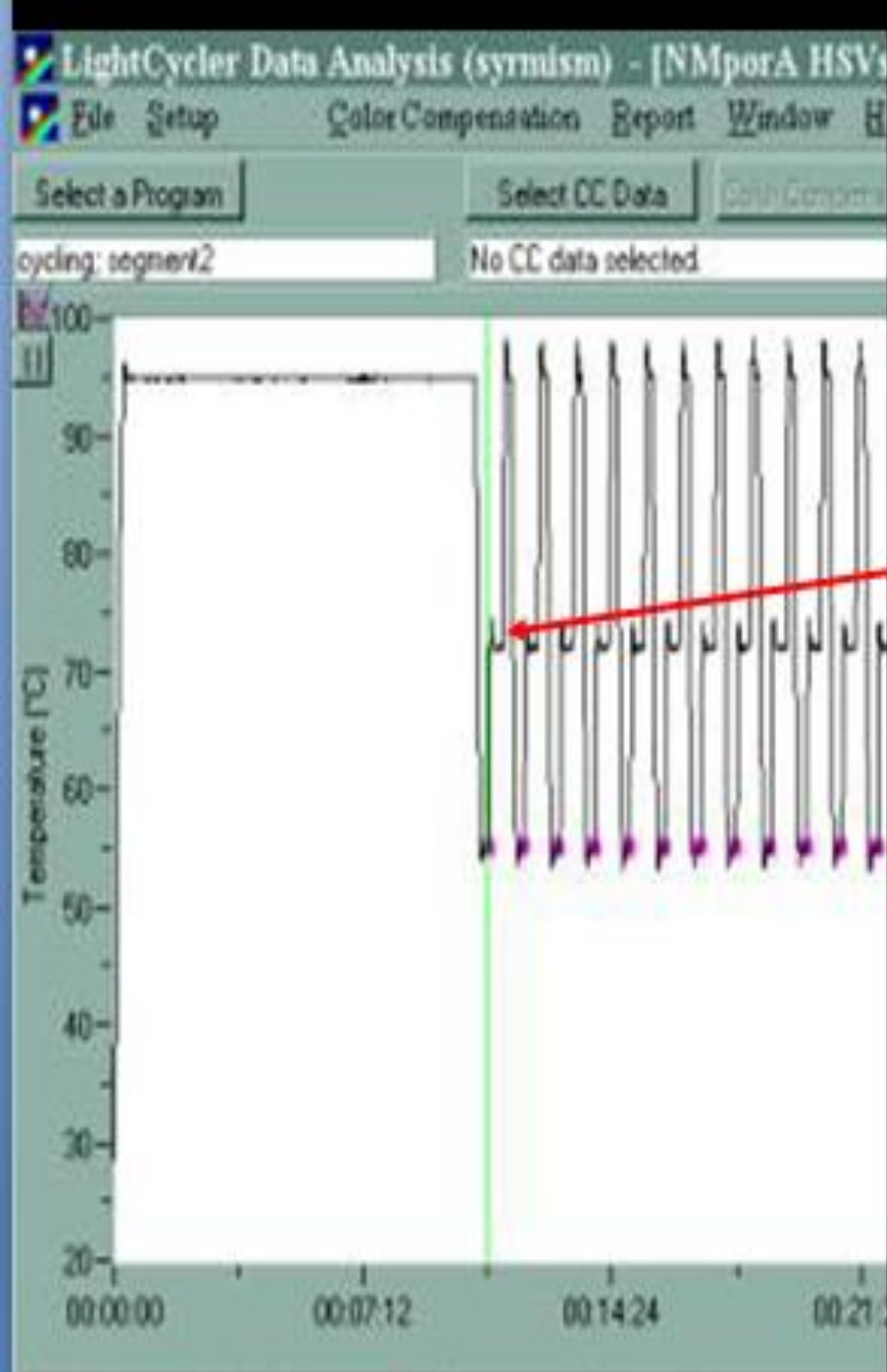
Uyarım

FRET

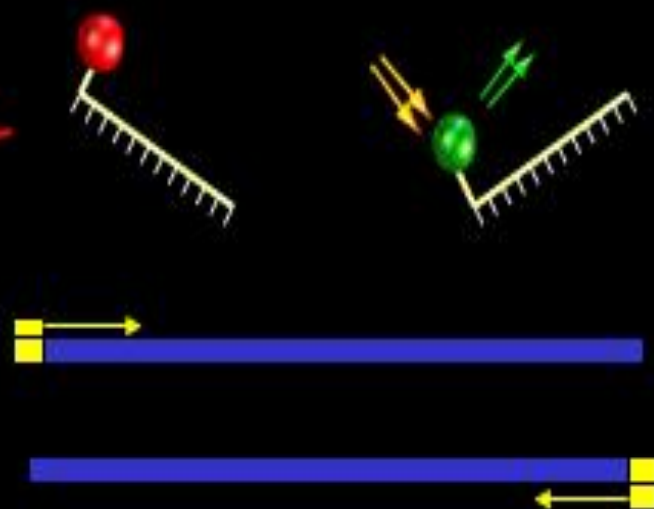
İşinım



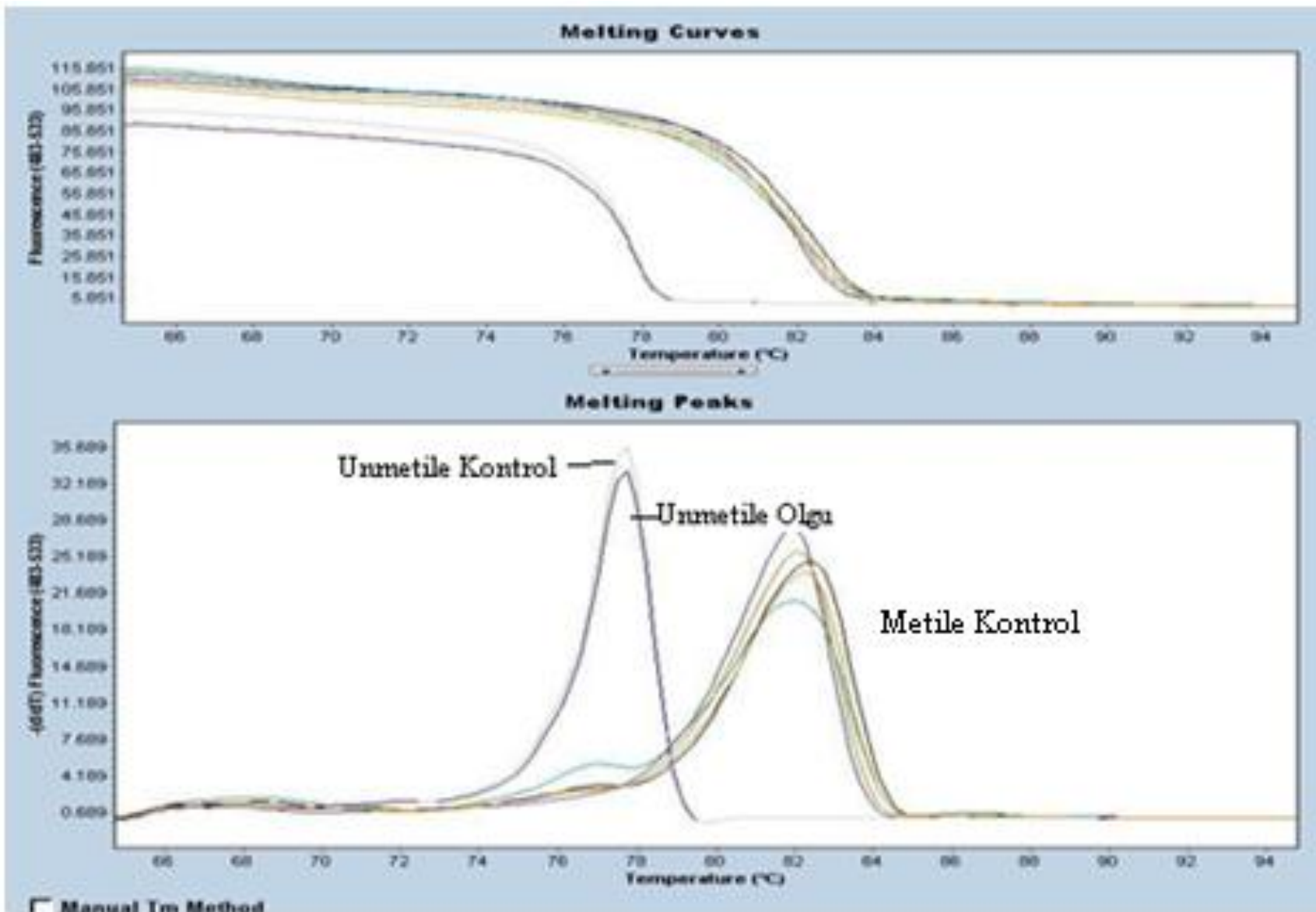
Primer/Probe Bağlanma



72°C



Primer Uzama

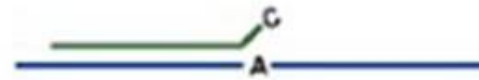


Allele-specific PCR

- Allele-specific PCR is used to identify or utilize single nucleotide polymorphisms (SNPs: single base differences in DNA).
- It requires prior knowledge of a DNA sequence, including differences between alleles and uses primers whose 3' ends encompass the SNP.
- PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.



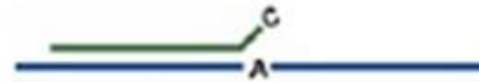
Perfect match



Mismatch



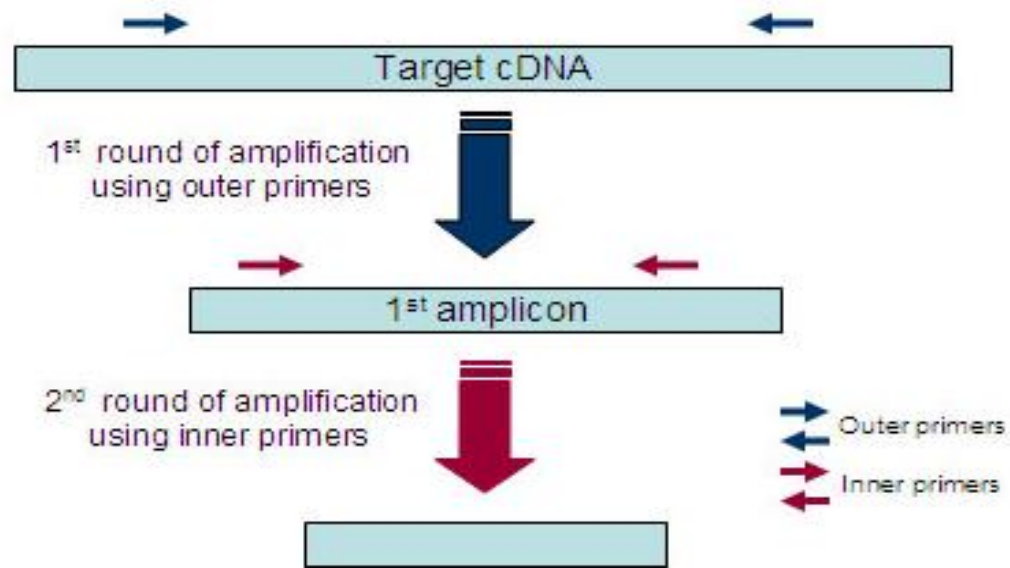
Primer extended



Primer not extended

Nested PCR

- Nested PCR increases the specificity of DNA amplification by reducing background due to non-specific amplification of DNA.
- Two sets of primers are being used in two successive PCR.
 - In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.
 - The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction.
- Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

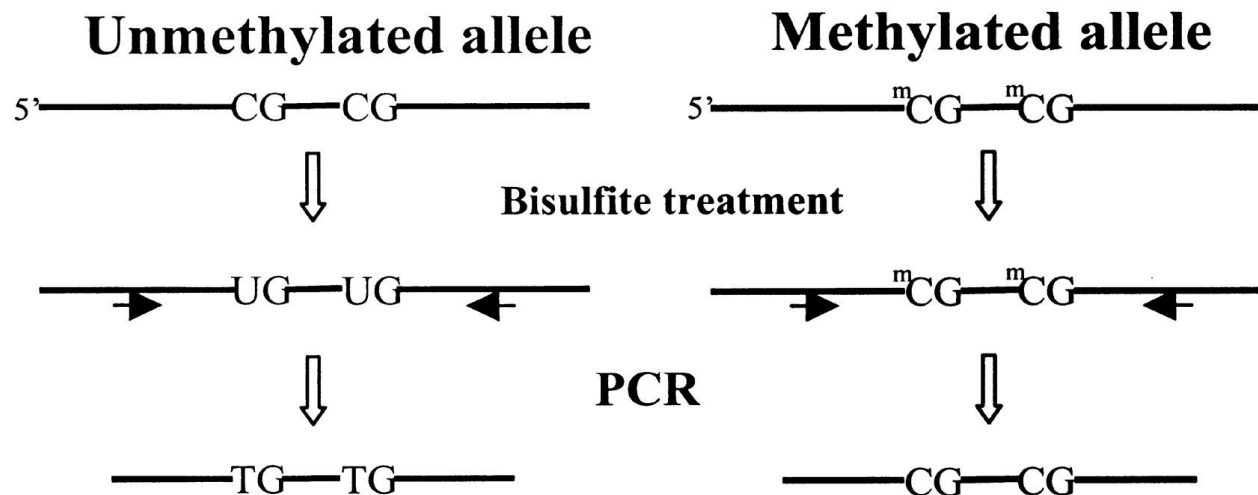


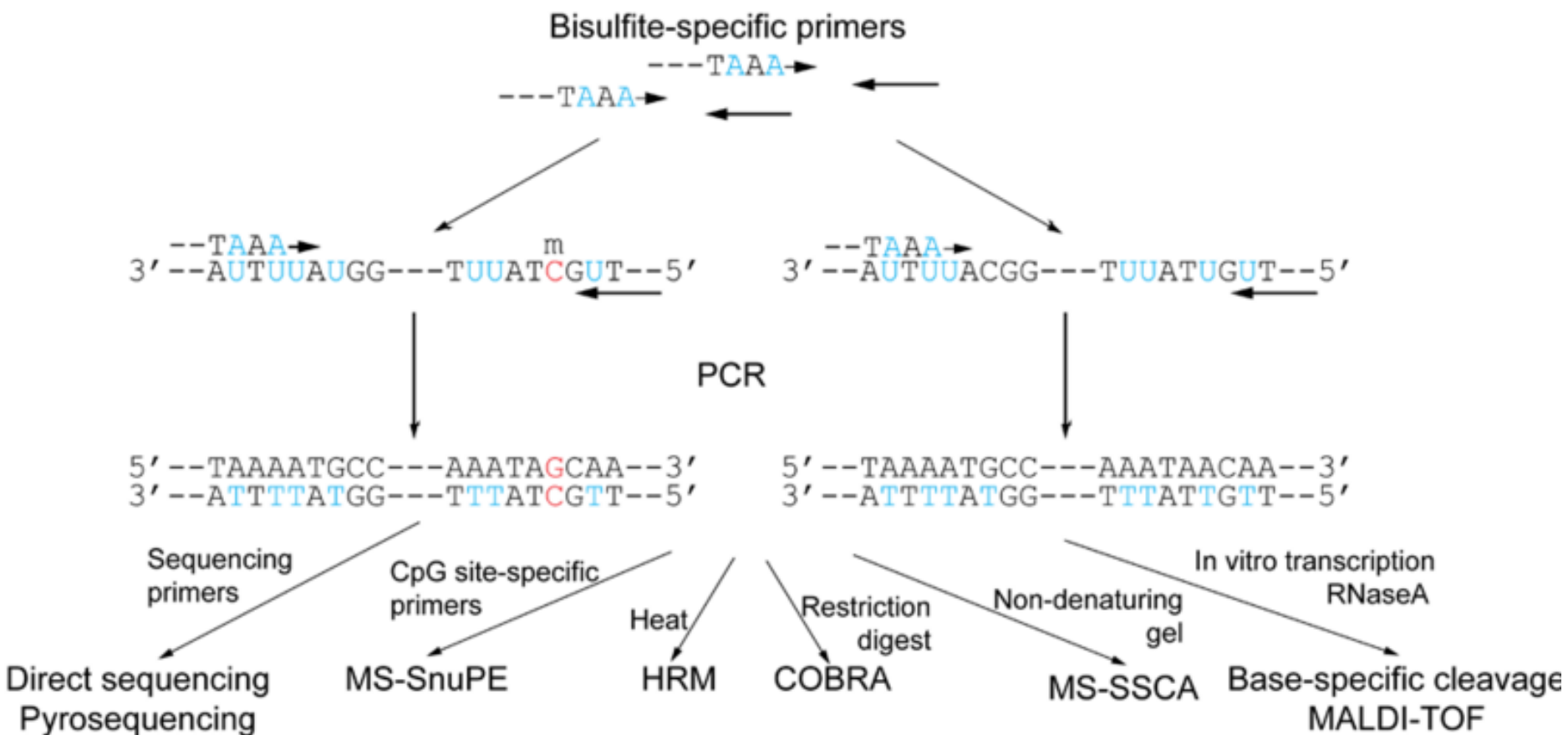
Methylation-specific PCR (MSP):

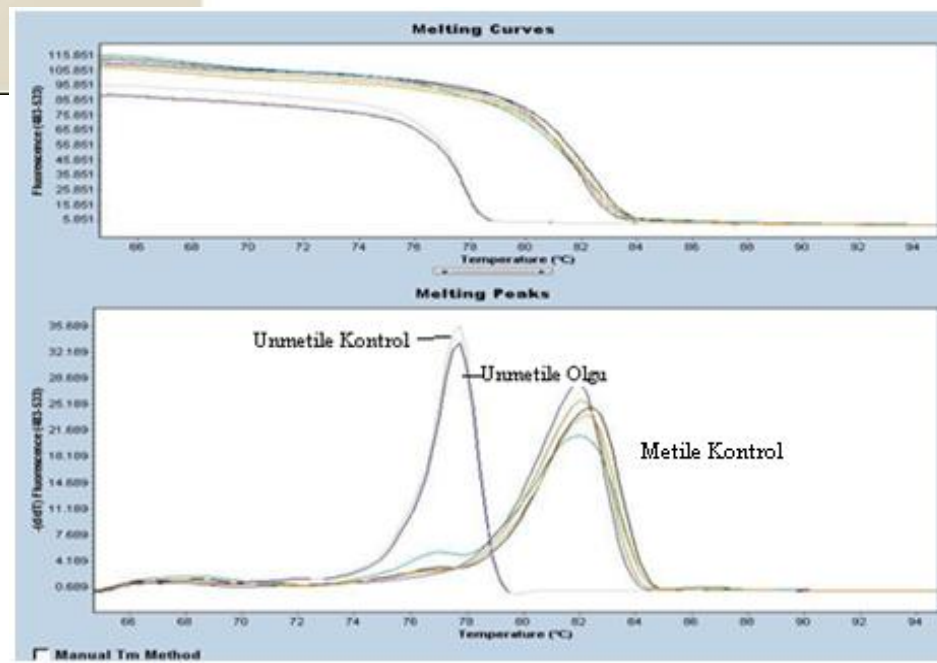
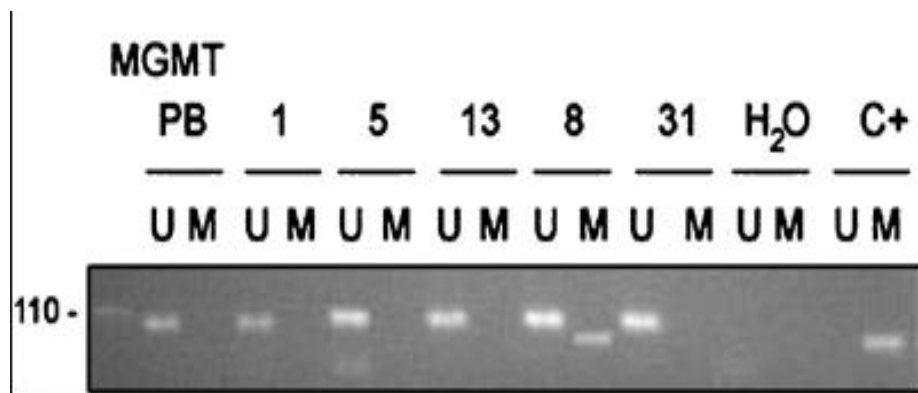
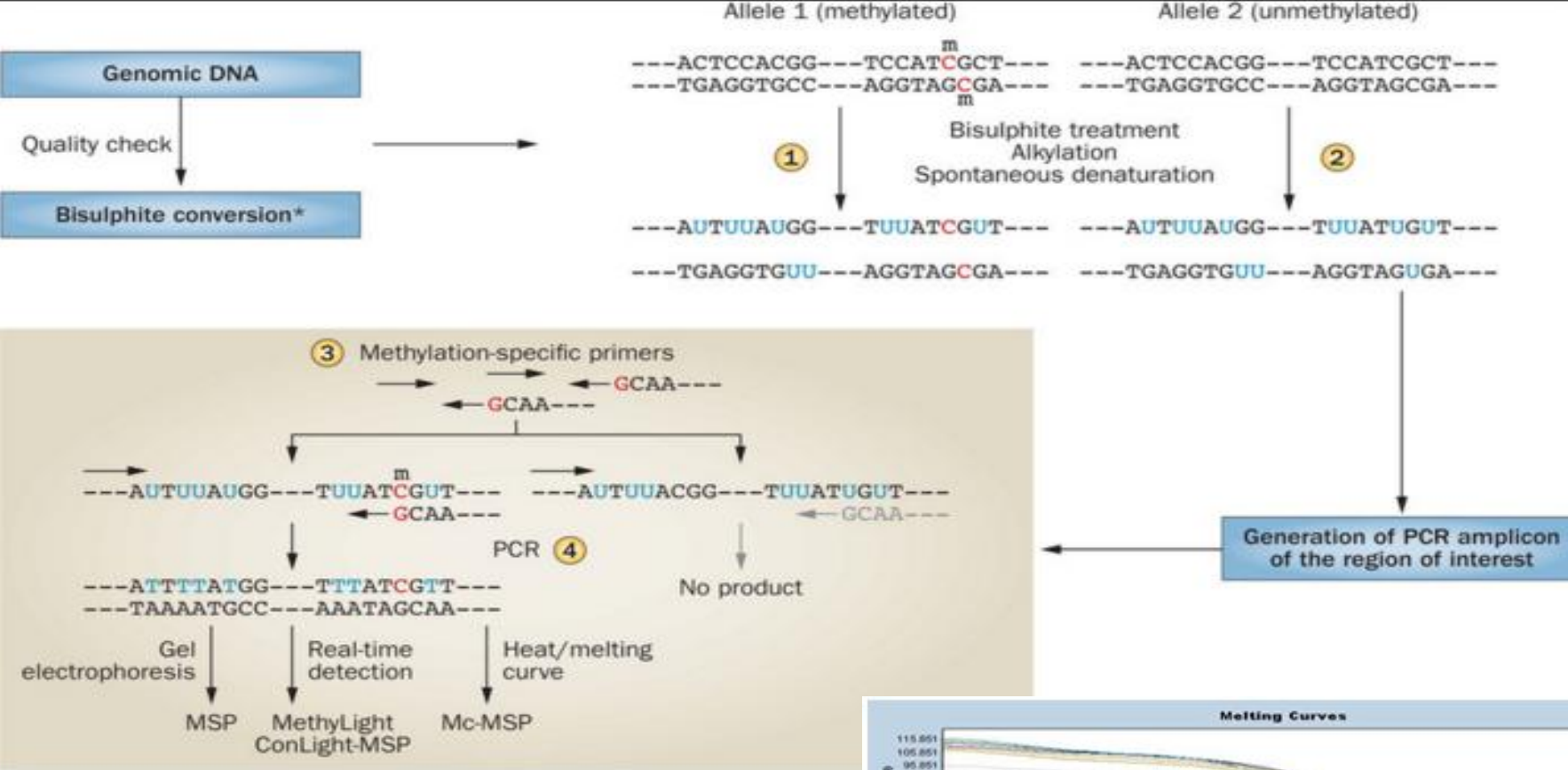
- MSP is used to detect methylation of CpG islands in genomic DNA.
- DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine.
 - Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences.

At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA

One set recognizes DNA with uracil or thymine to amplify unmethylated DNA.

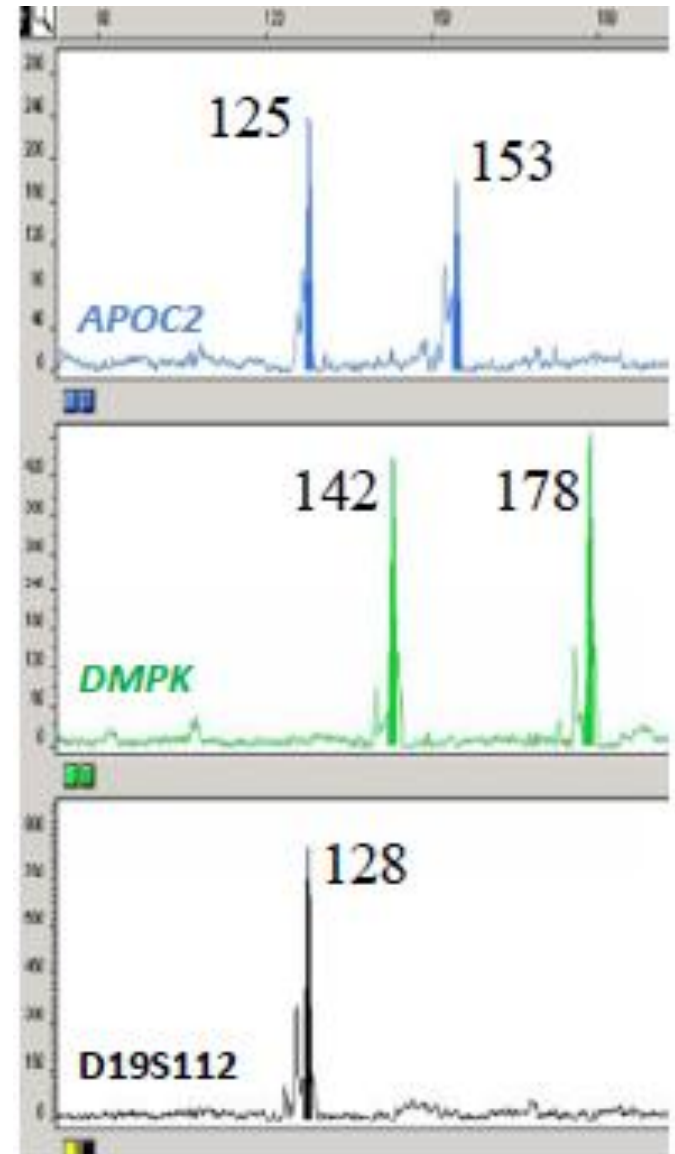






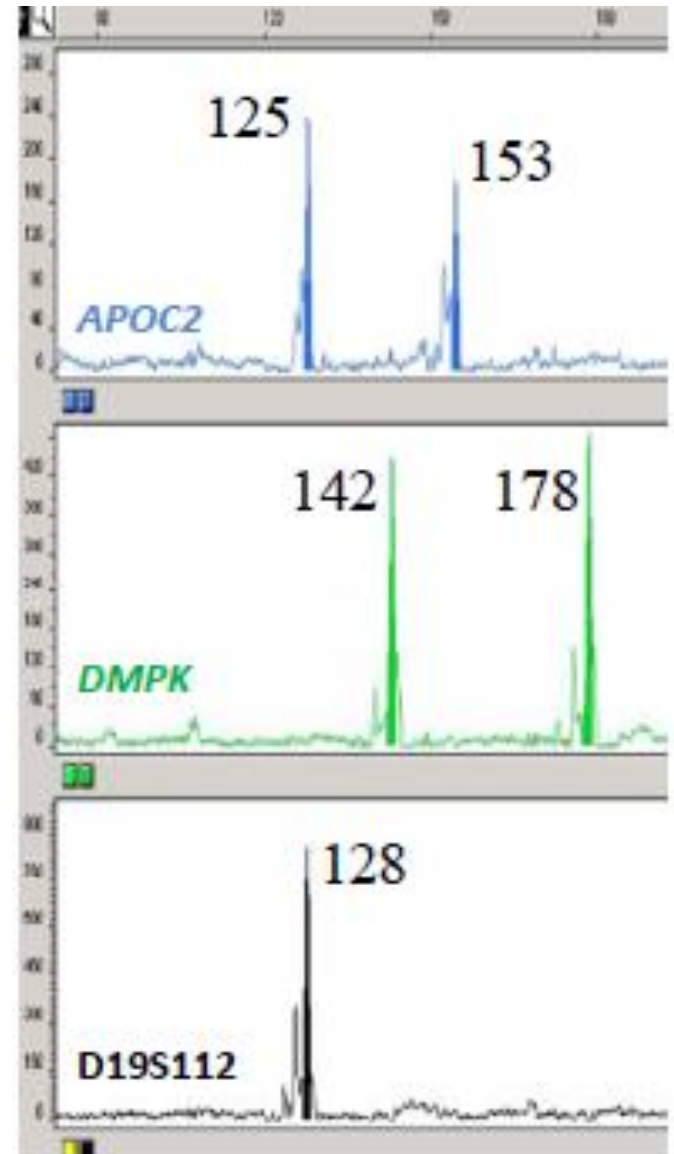
Fluorescent PCR

- Primers labelled at 5'terminus with fluorescent molecule



Flourescent PCR

- PCR Products detected by laser analysis system
- Sizing of amplicon to single nucleotide accuracy
- Can use more than one colour of fluorescence → **multiplex PCR**
- Can differentiate same sized but different products
- **Compatible with mutation analysis techniques**

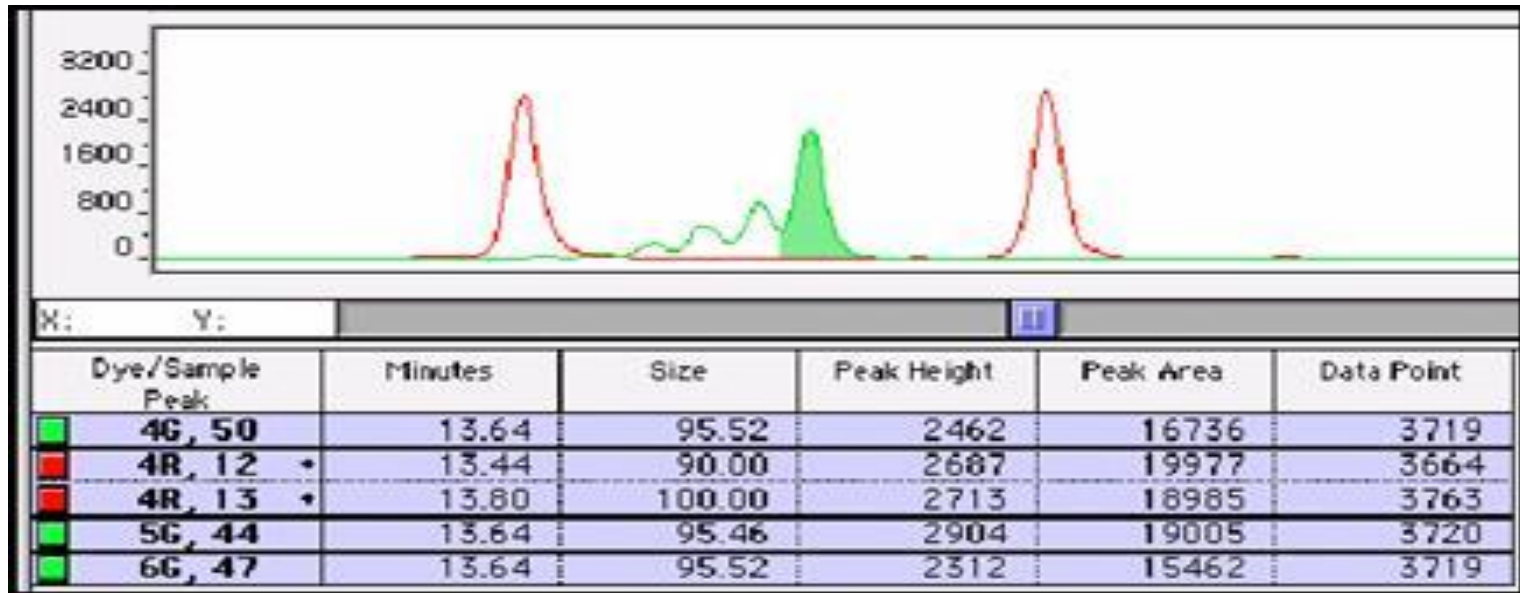


Detection of fluorescent PCR products by genetic analyser



Detection of fluorescent PCR products by genetic analyser

Amount

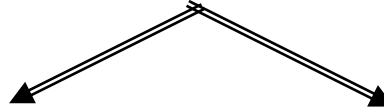


Short time/Short fragments

Long time/Long fragments

Time / Length

Applications of PCR



Basic Research

- Mutation screening
- Drug discovery
- Classification of organisms
- Genotyping
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- Bioinformatics
- Genomic cloning
- Site-directed mutagenesis
- Gene expression studies

Applied Research

- Genetic matching
- Detection of pathogens
- Pre-natal diagnosis
- DNA fingerprinting
- Gene therapy

Applications of PCR



Molecular Identification

- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- DNA fingerprinting
- Classification of organisms
- Genotyping
- Pre-natal diagnosis
- Mutation screening
- Drug discovery
- Genetic matching
- Detection of pathogens

Sequencing

- Bioinformatics
- Genomic cloning
- Human Genome Project

Genetic Engineering

- Site-directed mutagenesis
- Gene expression studies

Applications of PCR

- DNA amplified by PCR can be used for DNA sequencing, as a probe in Northern and Southern blotting, and to generate clones.
- **Inherited diseases** — PCR is used to amplify gene sequences, which can then be screened for disease-causing mutations (*e.g., in haemophilia and thalassaemia*)
- **Cancer research** — PCR has been widely used to identify mutations in oncogenes and tumour-suppressor genes.
- **Forensic science** — By amplifying repetitive sequences (*i.e., short tandem repeats or STRs*), PCR can be used to identify individuals from samples of their DNA.
- **Biotechnology** — PCR has played an important role in the production of recombinant proteins such as insulin and growth hormone, which are widely used as drugs, and in the development of recombinant vaccines such as that for the hepatitis B virus

PCR and Disease

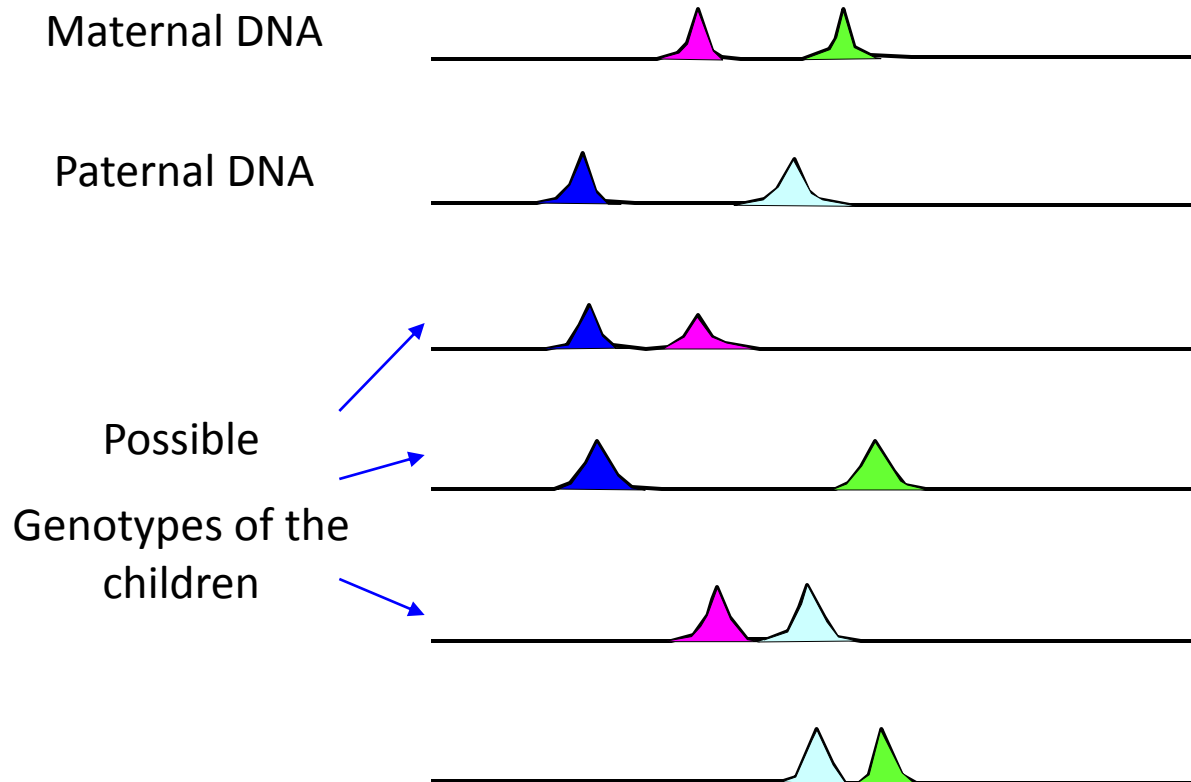
- Primers can be created that will only bind and amplify certain alleles of genes or mutations of genes
 - This is the basis of diagnostic tests and genetic counseling
 - PCR is used for diagnosis of genetic diseases.
- Some diseases that can be diagnosed with the help of PCR:
 - Huntington's disease
 - Cystic fibrosis
 - Human immunodeficiency virus
- Detection of Pathogenic Organisms in Clinical Samples

PCR and Forensic Science

- 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 is used to amplify highly variable regions of the human genome. These regions contain runs of short, repeated sequences (STRs).
 - 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.

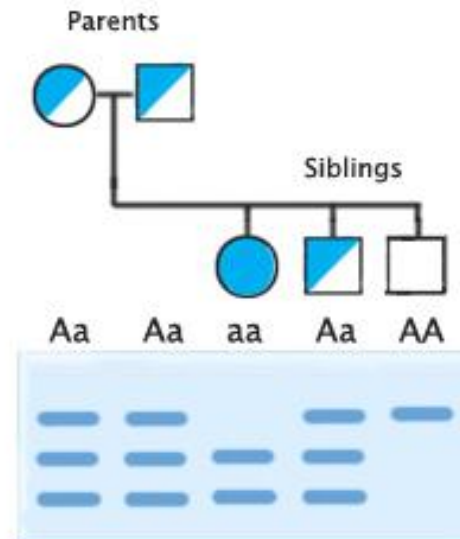
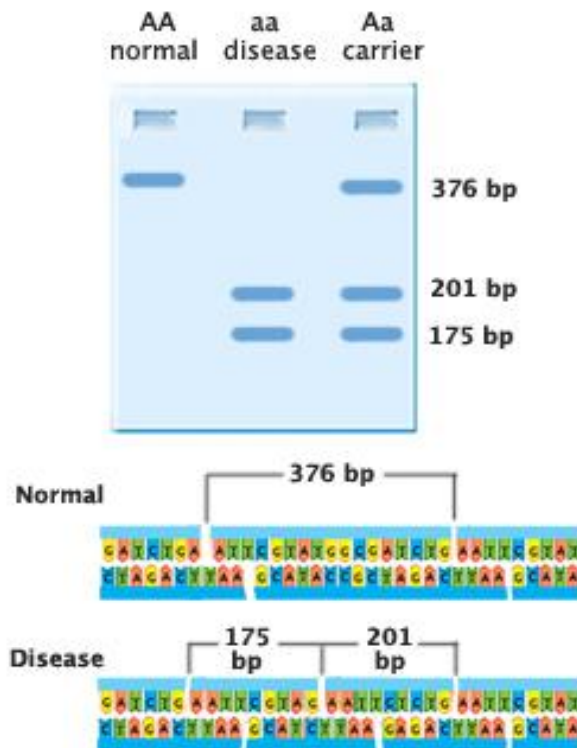
DNA Fingerprinting

Paternity testing



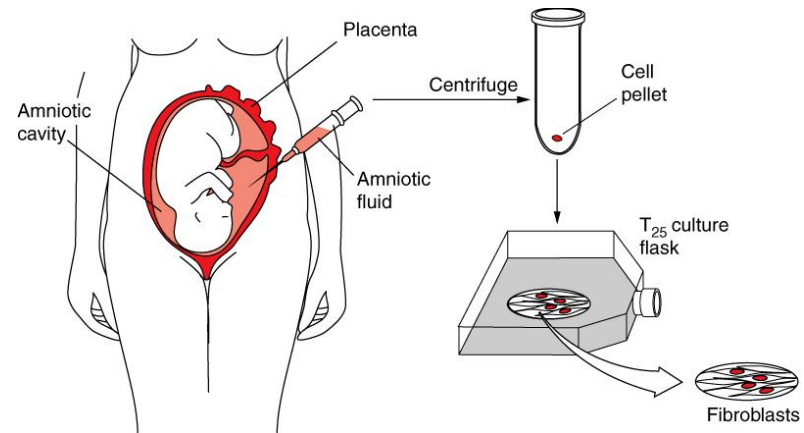
Applications

❖ Diagnosing Disease



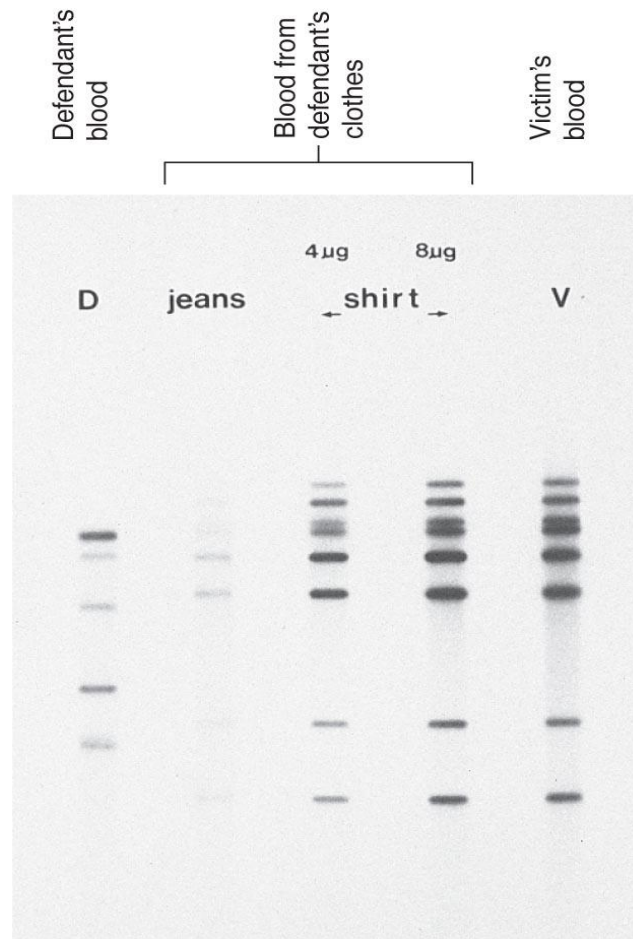
Applications

❖Paternity Testing

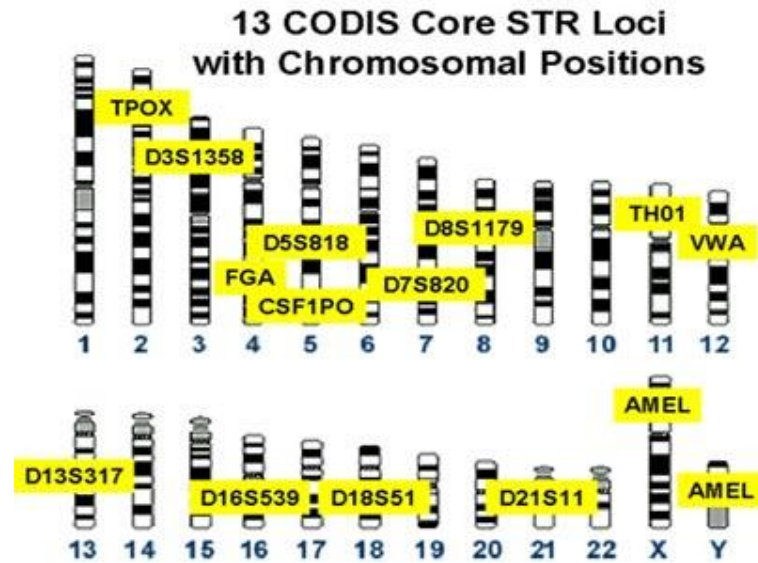


Applications

❖ Forensics

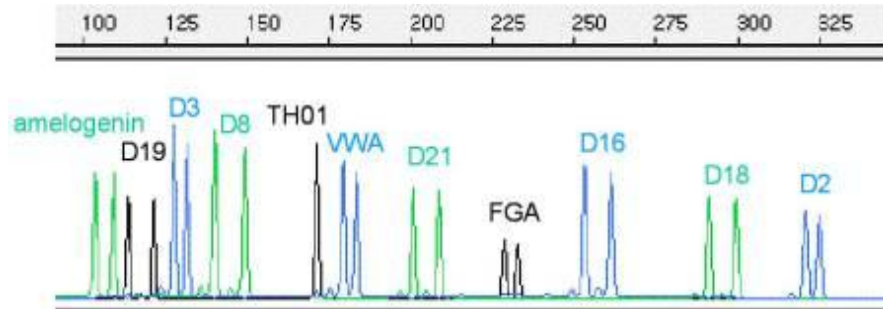


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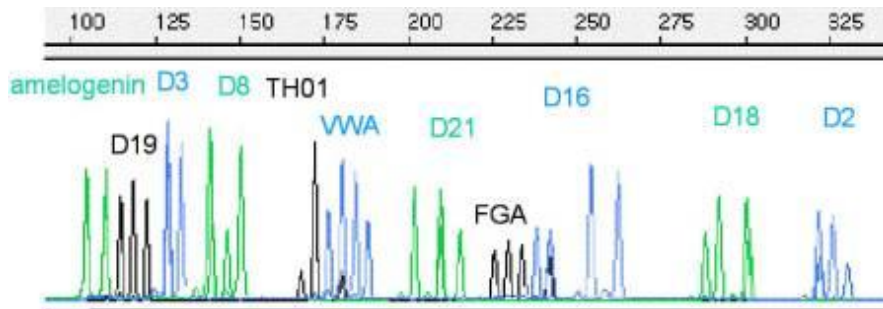


Applications

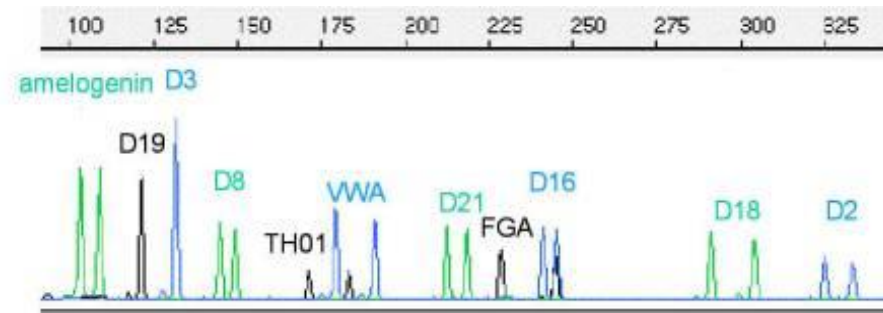
Victim



Crime
Scene



Suspect



Advantages of PCR

- Minute amounts of DNA template may be used from as little as a single cell.
- DNA degraded to fragments only a few hundred base pairs in length can serve as effective templates for amplification.
- Large numbers of copies of specific DNA sequences can be amplified simultaneously with multiplex PCR reactions.
- Contaminant DNA, such as fungal and bacterial sources, will not amplify because human-specific primers are used.
- Commercial kits are now available for easy PCR reaction setup and amplification.

Potential Pitfalls of PCR

- The target DNA template may not amplify due to the presence of PCR inhibitors in the extracted DNA
- Amplification may fail due to sequence changes in the primer binding region of the genomic DNA template
- Contamination from other human DNA sources besides the forensic evidence at hand or previously amplified DNA samples is possible without careful laboratory technique and validated protocols

Steps in DNA Analysis

Collection

Specimen Storage

Extraction

Quantitation

PCR Amplification

Genotyping

Interpretation
of Results

Database

Storage & Searching



Blood Stain

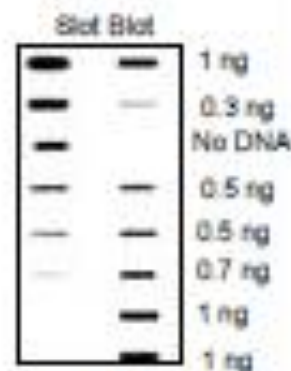


Buccal swab

Sample Collection
& Storage



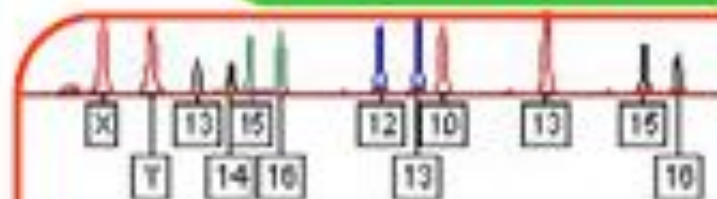
DNA
Extraction



DNA
Quantitation



Multiplex PCR Amplification



STR Typing

Male: 13,14-15,16-12,13-10,13-15,16

Interpretation of Results



DNA Database

DNA in the Cell

