جامعة بغداد/ كلية العلوم

قسم التقنيات الاحيائية

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مبادئ الوراثة المناعية/ الجزء العملي

Practical immunogenetics

تدريس

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BASIC IMMUNE REVIEW

Basic concepts in Immunology

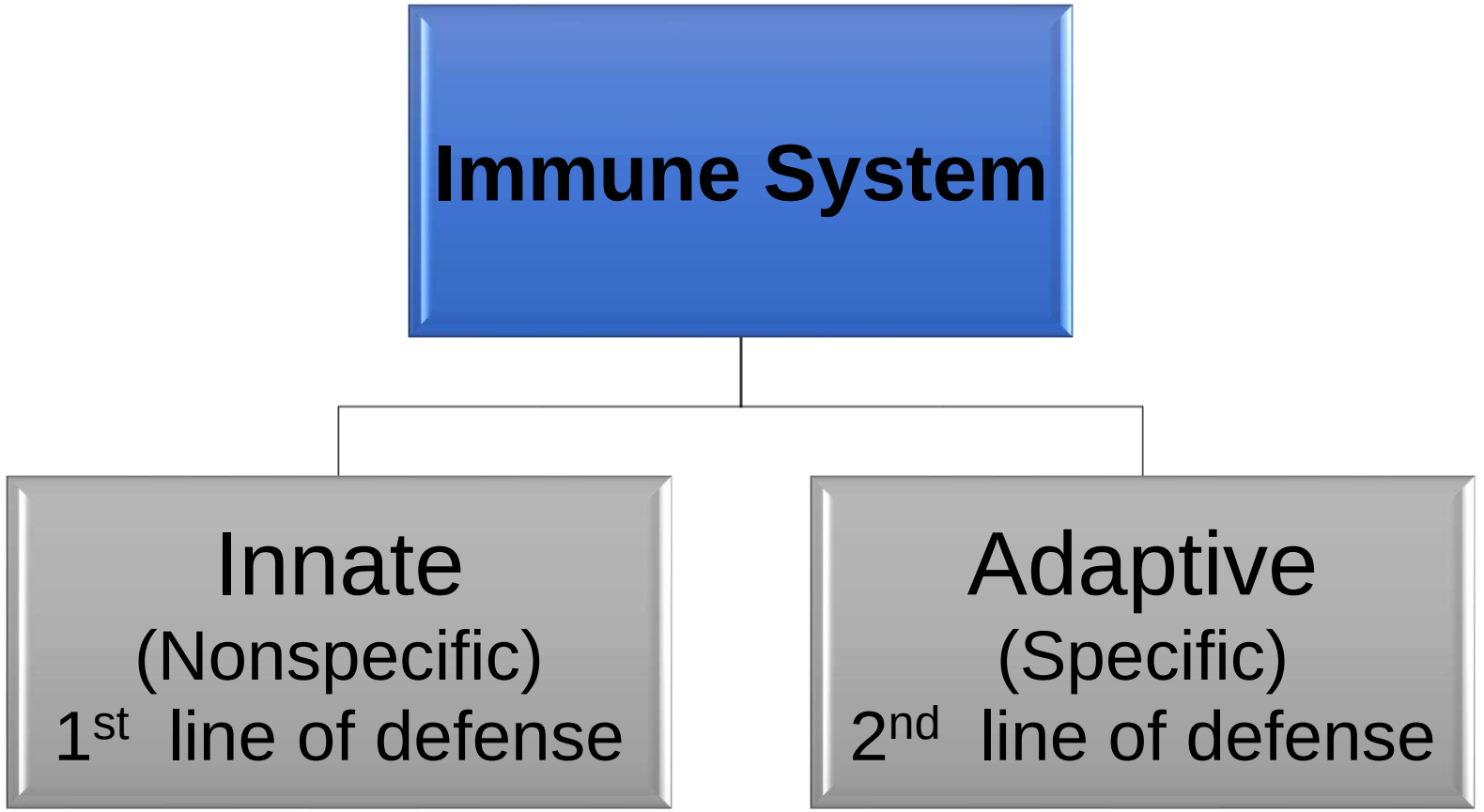
Lab #1

The immune system

Complex system, includes:

- Skin - **Physical barrier**
- Lining of mucus membranes - **Physical barrier**
- Secretions (ex. tears, mucus, etc.) - **Antimicrobial**
- Blood cells and vasculature (WBCs)
- Bone marrow
- Liver - makes complement proteins
- Lymphatic system and lymphoid organs
- Most tissues - have resident immune cells

Overview of the Immune System



Interactions between the two systems

Immune system (comparation)

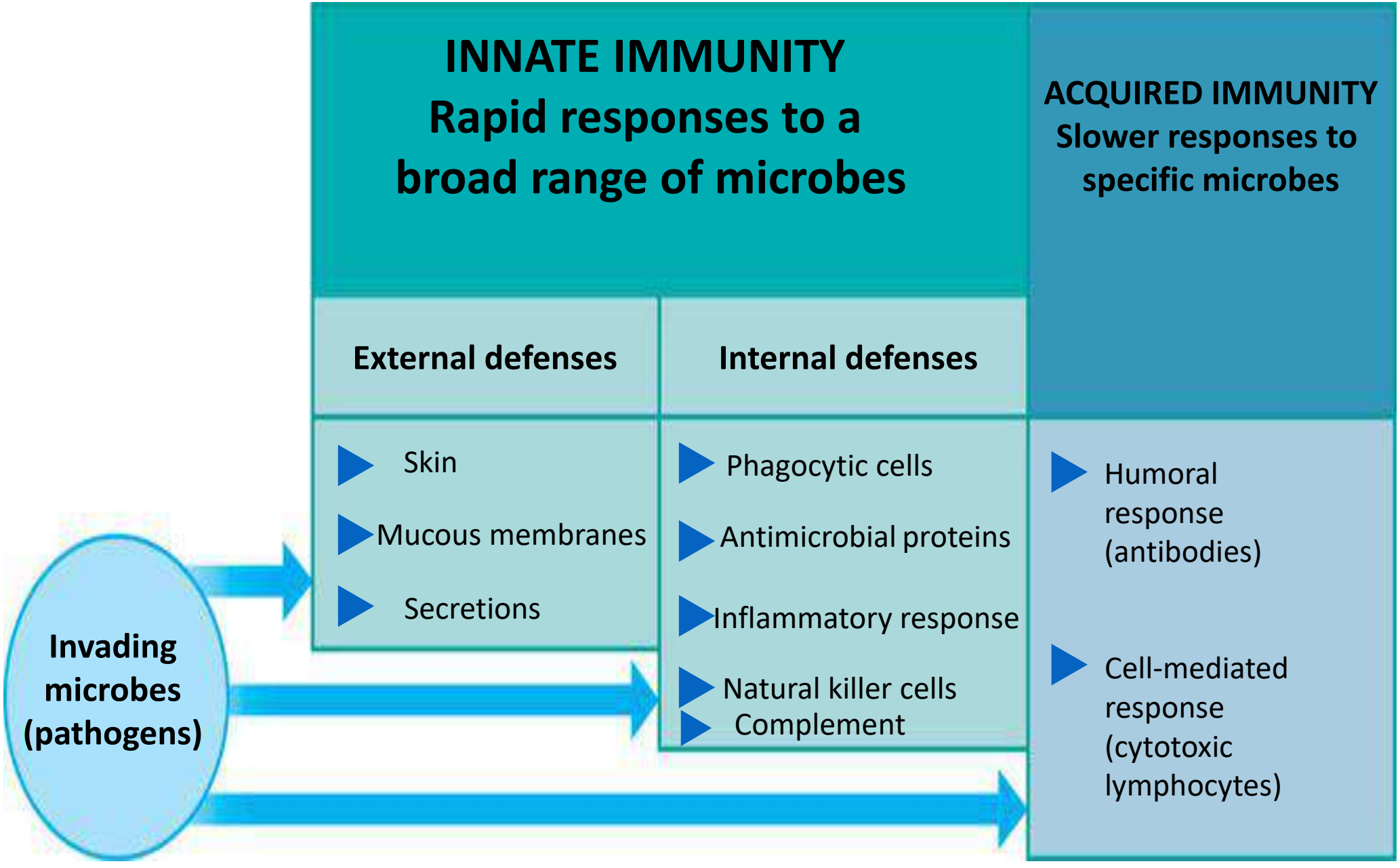
Innate (natural) immune system

- Not as sufficient as adaptive
- Immediate
- Present at birth
- Defense against any pathogen
- Accomplished by physical barriers(skin “largest barrier”, tears, saliva, mucus), cells, chemical mediators (lysozyme, mucus membranes, histamine interferons),and inflammatory response.

Adaptive (acquired) immune system

- Efficient
- Specialist cells, cytokines, antibodies
- Specific and has memory
- Specialized mucosal lymphoid tissue
- Lasting long compare to innate

A typical immune response



Main functions of immune response

The immune system recognizes infection and induces protective responses:

a) Immunological recognition

b) Effector functions

c) Immune regulation

d) Immunological memory

Immune Cells

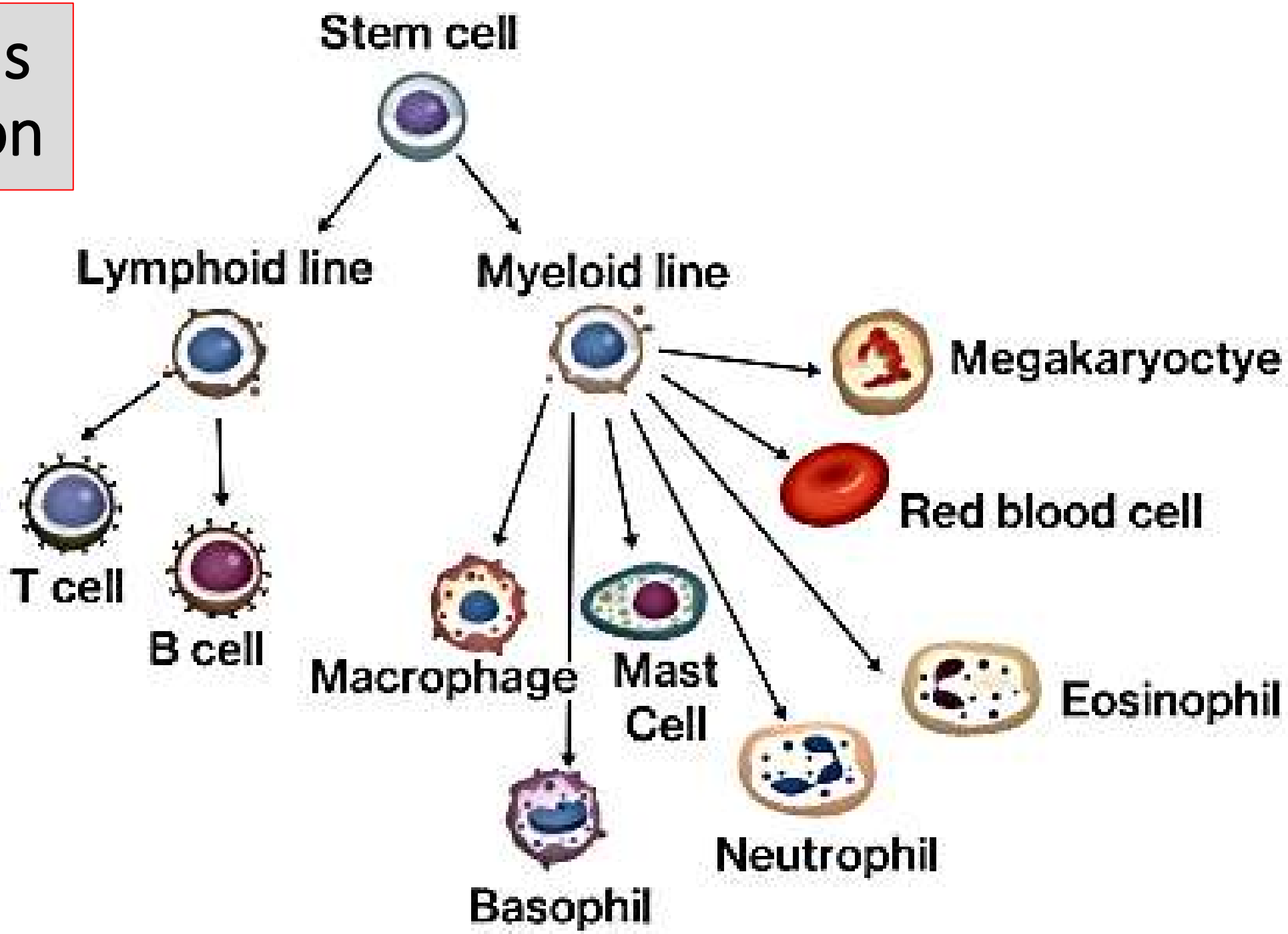
- White blood cells:

Produce in red bone marrow and lymphatic tissue that fight foreign substances

- Phagocytic cells:

- Ingest and destroy foreign substances
- Ex. neutrophils and macrophages

Immune cells differentiation



Immune cells types

Neutrophil

- First to respond to infection but die quickly

Macrophage

- Monocytes (in blood) Migrate into the tissues and become Macrophages
- Protect lymph in lymph nodes and blood in spleen and liver
- Given specific names for certain areas of body (ex. Kupffer cells in liver)

Basophil

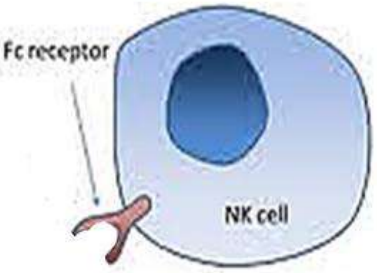
- Made in red bone marrow
- Leave blood and enter infected tissues
- Can release histamine

Mast cell

- Made in red bone marrow
- Found in skin, lungs, gastrointestinal tract, urogenital tract
- Can release leukotrienes

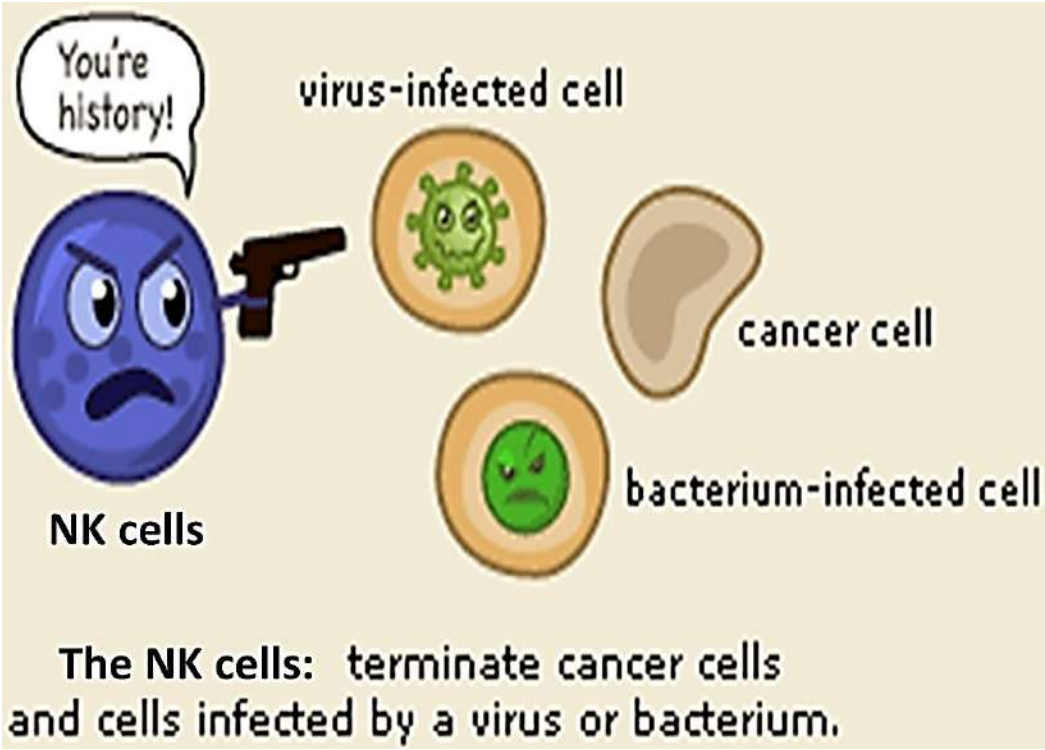
Eosinophil

- Release chemicals to reduce inflammation



Natural killer (NK) cells

- Important part of the innate immune system
- Type of lymphocyte
- Release chemicals to lysis cells



Chemical Mediators

- **What are they?**

  chemicals that can kill microbes and prevent their entry into cells

- **Lysozyme:**

  found in tears and saliva to kill bacteria

- **Mucous membranes:**

  prevent entry of microbes

- **Histamine:**

  promote inflammation by causing vasodilation

- **Interferons:**

  proteins that protect against viral infections by stimulating surrounding cells to produce antiviral proteins

Questions?

Lab-2- **IMMUNOGENETIC**

DNA Extraction

- DNA isolation is a process of purification of DNA from sample using combination of physical and chemical methods
- In general ,they aim to separate DNA present in the nucleus of the cell from other cellular component
- Isolation of DNA is needed for genetics analysis which is used for scientific ,medical or forensic purpose

Sources for DNA isolation :

- DNA can be extracted from almost any intact cellular tissue
- Skin
- Blood
- Saliva
- Mucus
- Muscle tissue
- Bone marrow

Basic Steps in DNA Extraction

- There are three basic steps in a DNA extraction the details of which may vary depending on the type sample and any substances that may interfere with the extraction and subsequent analysis

1-Cell lysis : Break the cells and remove membrane lipids

2-Protein precipitation: Remove cellular and histone proteins bound

to the DNA ,by adding a protease , by precipitation with sodium or ammonium acetate ,or by using phenol/chloroform extraction step

3-DNA purification : Precipitate DNA in cold ethanol or isopropanol ,this step also removes salts

- # Nucleic Acid Preparation Applications

- **Medical studies**

  Understanding genetic disorders at molecular level

  Rapid detection of genetic disorders in a patient

- **Agricultural studies**

  Plant and animal breeding

- **Criminology/Paternity testing**

  DNA fingerprinting to identify individuals

- # Blood Collection

- Blood collected in disodium EDTA tube
- Samples can be stored at -20^{o}C or -70^{o}C
- Fresh samples are kept in freezer for a few hours to facilitate RBCs hemolysis
- Allow samples to thaw before starting the extraction

- # Isolation Procedure

To isolate the DNA, the recommendations of manufacturer were adopted, and outlined as the following:

1. After a gentle mixing, 200 µl of EDTA blood was dispensed into the micro-centrifuge tube followed by adding 20 µl of proteinase K and 200 µl of lysis buffer. The contents were gently vortexing for 10 seconds, and then the tube was incubated for 10 minutes in a water bath at 56°C. This was followed by adding 250 µl of binding buffer, and the tube was gently vortexed for 10 seconds.
2. The binding column of ReliaPrep™ was allocated into an empty collection tube, and to which the tube contents of step 1 were

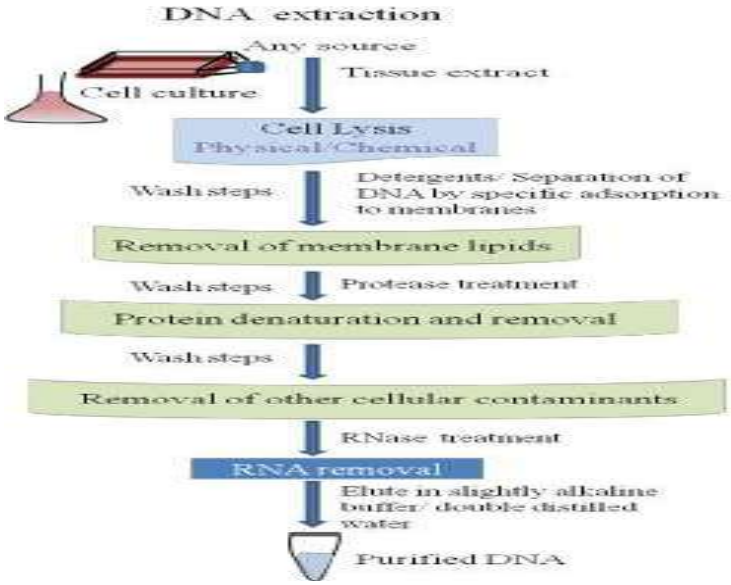
transferred and centrifuged (13000 rpm) for a minute. A re-centrifugation was carried out if the lysate was not completely crossed the membrane.

3. The flow- through containing collection tube was detached and allocated into a new collection tube, and to which, a column wash solution (500 µl) was added. The tube was centrifuged (13000 rpm) three minutes, and the washing step was two-times repeated.

4. The column was again allocated in a new 1.5ml micro-centrifuge tube; to which, nuclease-free water (200 µl) was added, and centrifuged (13000 rpm) for a minute. Then, the binding column was detached and the eluted DNA was subjected for concentration and purity assessment.

Concentration and Purity Assessment of DNA

NanoDrop 2000c spectrophotometer was used to assess DNA concentration, which was given as ng/µl. The concentration was in the range of 20-90 ng/µl. The NanoDrop 2000c was also used check DNA purity, in which the absorbance of DNA sample was read at 260 and 280nm wavelengths. A260/A280 ratio was within a range 1.7- 1.9, which suggested that the examined DNA sample was pure.



Phenol-chloroform method of DNA extraction from blood samples

Whole blood samples are one of the main sources used to obtain DNA, and there are many different protocols available to perform nucleic acid extraction on such samples. Bellow protocol is one of the organic DNA extraction methods (Chemical-based DNA extraction method).

Materials and chemicals

Tris-EDTA (TE) Buffer (10 mM Tris-HCl, 1 mM Na2EDTA, pH=8)

Prepared by dissolving 0.2422gm of Tris-Base, 0.0744gm of EDTA in D.W, pH was adjusted to 8.0, volume completed with D.W to 100 ml, sterilized by autoclaving and stored at 4°C.

TBS buffer (20 mM Tris-HCl, pH= 8, 150 mM NaCl)

Prepared by dissolving 0.1211gm of Tris-Base, 0.4383gm of NaCL in D.W, pH was adjusted to 8.0, volume completed with D.W to 50 ml, sterilized by autoclaving and stored at

4°C.

B buffer (400 mM Tris-HCl, 100 mM Na2EDTA, pH=8, 1% SDS)

Prepared by dissolving 2.4228gm of Tris-Base, 1.86gm of Na2EDTA, in D.W, pH was adjusted to 8.0, volume completed with D.W to 50 ml, sterilized by autoclaving then added 0.5gm of SDS

Protocol steps:

1- Prepare the work area by sterilizing surfaces. Wait until the surfaces dry completely before proceeding.

2- Using a sterile syringe collect 3-5 ml of blood from a donor and place it in an EDTA tubes.

3- Mix the sample with a volume of TE buffer to reach 10 ml/ tube, invert the tubes several times.

4- Centrifuge the mix at 10000 rpm for 10 minute at 4C.

5- Discard the supernatant by using a pipette and avoid losing the pellet.

6- Wash the pellet with 5 ml of TE buffer by repeating steps 3-5 until it is pink.

7- Discard the supernatant using a plastic pipette and avoid losing the pellet.

8- Resuspend the pellet in 1 ml TBS buffer.

9- Add 1 ml of Lysis buffer B and 100 µl of proteinase K solution.

10- Incubate tubes in water bath at 55C for 60 min with agitation

11- After incubation take the tubes out of the waterbath and add 1 ml of saturated phenol, and 1 ml of the mixture cloroforme: isoamyle alcohol (24:1).

12- Shake the tubes for 5 min by inverting them.

13- Centrifuge the tubes at 10000 rpm for 10 min.

14- Transfer the upper phase with plastic pipette to a new tube, the bottom phase should not be touched.

15- Add 2 ml of chloroforme: isoamyl alcohol (24:1).

16- Shake the tubes for 5 min by inverting them.

17- Centrifuge the tubes at 10000 rpm for 10 min.

18- Transfer the supernatant to a new tube (the bottom phase should not be touched).

19- Add Ammonuime acetate solution to a final concentration of 2.5 M and then 2.5volumes of cold (20C) 95% ethanol.

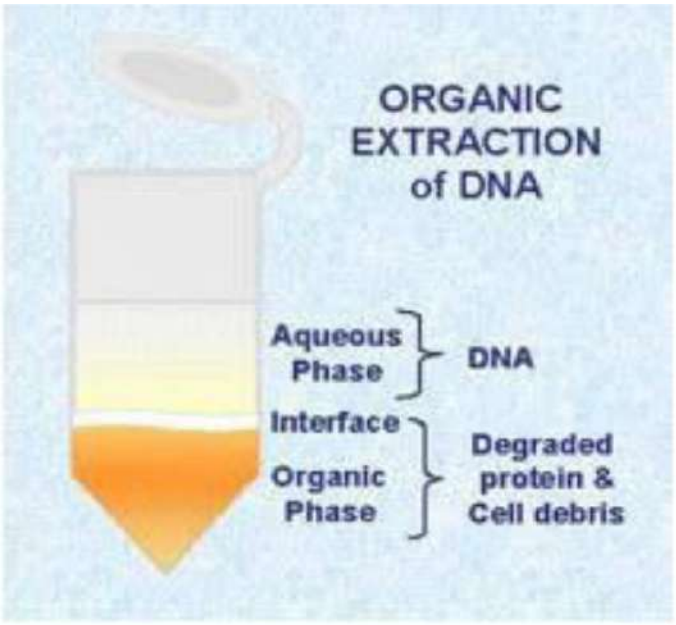
20- Invert the tubes several times until DNA appeared as a white precipitate.

Note: If DNA concentration is high, DAN strands would form a visible precipitate, which can be collected into a compact mass of material that can easily be removed from the tube by spooling the DNA mass on a pasture pipette. The DAN in this manner can be spooled, by this step the DNA will be separate from the bulk of RNA which remained in solution. This eliminated the need to add exogenous Rnase, which may be contaminated with nucleases.

21- Let the DNA to dry at room temperature (should not be over dried, otherwise it would be difficult to be resuspended) and then resuspend it in 2 ml of TE in 5 ml tube. Once the DNA is release from the pipette tip, let the tubes in the room temperature for hours to allow the DNA dissolving in the buffer, and then store it in deep freezer.

Note: For the samples which do not form a visible precipitate, the DNA should recover by 30 min of centrifugation and resuspend in TE buffer as described before.

Brief roles of some chemicals used in this protocol



Chemical	Role in DNA extraction
Tris	It maintains the pH of the solution and also permeabilizes the cell membrane.
EDTA	It is a chelating agent and blocks the activity of DNase enzyme.
SDS	It is an anionic detergent which helps in denaturation of cell membrane protein.
NaCl	Prevents the denaturation of DNA

Lab-3-

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Principle of electrophoresis

Electrophoresis is a common lab technique used to identify, quantify, and purify nucleic acid fragments. Samples are loaded into wells of an agarose or acrylamide gel and subjected to an electric field, causing the negatively charged nucleic acids to move toward the positive electrode. Shorter DNA fragments will travel more rapidly, whereas the longest fragments will remain closest to the origin of the gel, resulting in separation based on size.

- **Equipment**

Casting tray

Well combs

Voltage source

Gel box

UV light source

Microwave

- **Reagents**

TAE Agarose

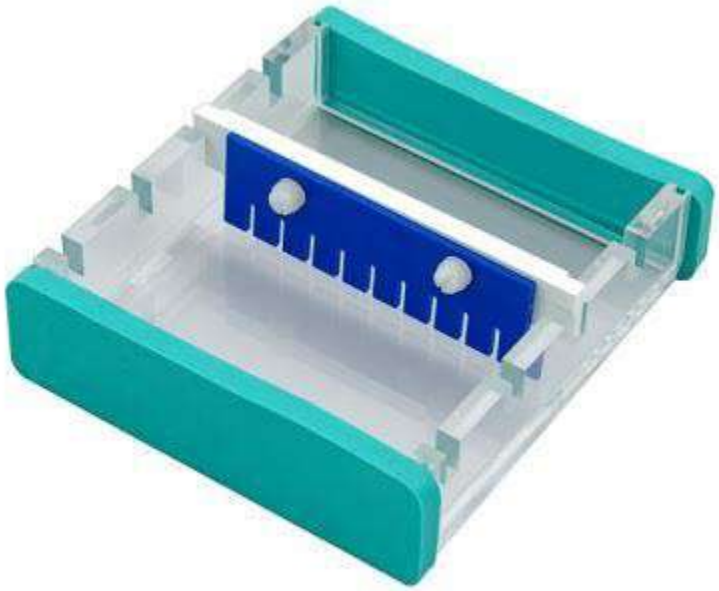
Ethidium bromide

Gel types

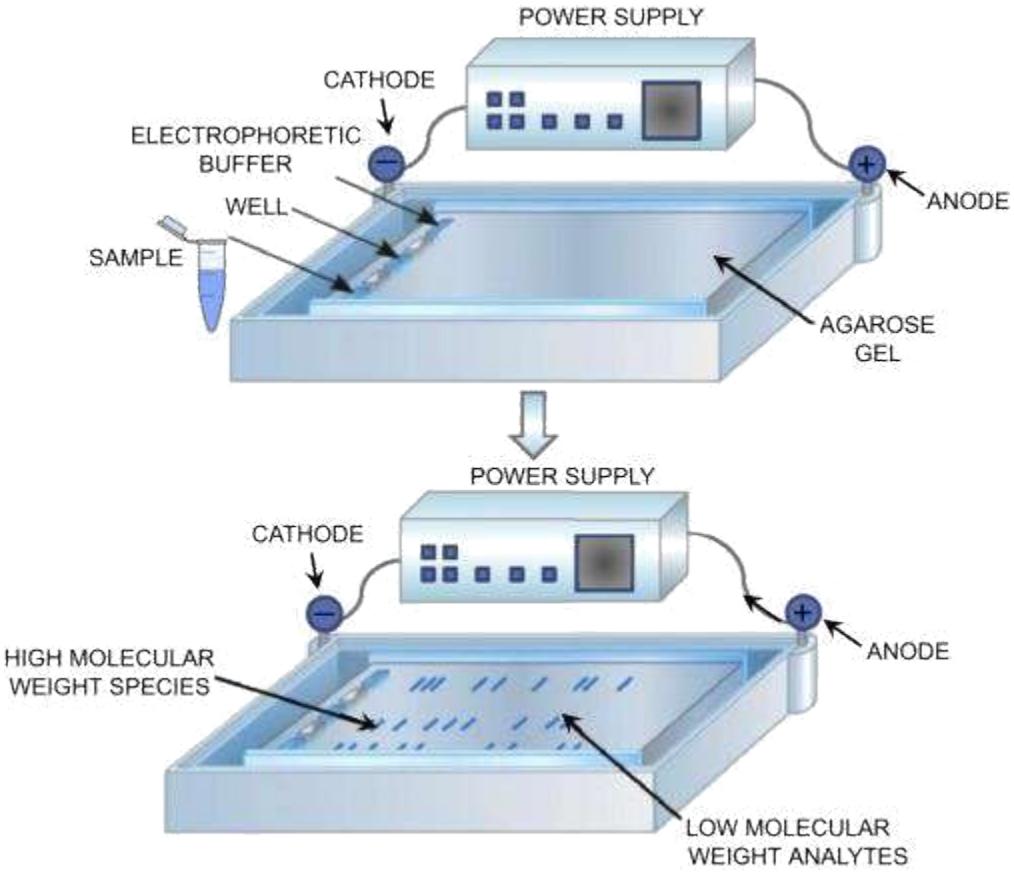
In general the macromolecules solution is electrophoresed through some kind of matrix. The matrix acts as a molecular sieve to aid in the separation of molecules on the basis of size. The kind of supporting matrix used depends on the type of molecules to be separated and on the desired basis for separation: charge, molecular weight or both .The most commonly used

materials for the separation of nucleic acids and proteins are agarose and acrylamide.

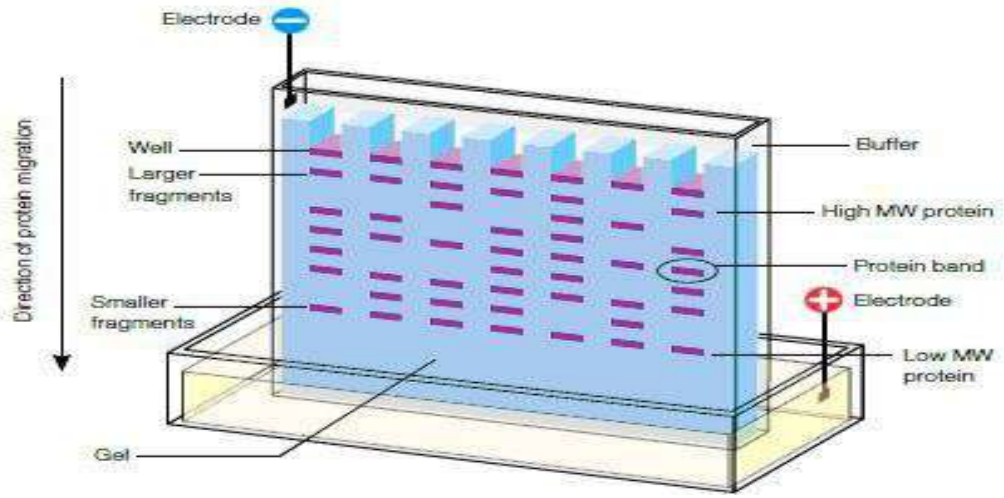
Agarose	polyacrylamide
Substance ,which is the main Constituent of agar used especially In gels for electrophoresis	synthetic resin made by polymerization acrylamide which is a water soluble polymer used to form a stabilized gel
A complex polysaccharide derived from seaweed	Made by the digestion of acrylonitrile by nitrile hydratase
Consist of many molecules	Contain one large molecules
A horizontal gel	A vertical gel
The pore size of the agarose gel becomes smaller with the increasing concentration	The ratio of acrylamide to bisacrylamide determines the pore size
Important in separation of much larger DNA fragments such as the products of PCR	Important for separation of protein as well as nucleic acid such as oligonucleotides ,tRNA etc
Separate DNA about50-20000bp in size	Separate DNA about 5-500bp in size
Separate DNA in double strand form	Separate DNA in single strand



Casting tray and well comb



Agarose Gel electrophoresis



Polyacrylamide Gel electrophoresis

Preparation of standard agarose gel

The agarose-gel was prepared at a concentration of 1% (w/v). Agarose (two grams) was dissolved in 200 ml of 1X TAE buffer (Tris-acetate-EDTA) by heating until the agarose was completely melted, and then 3.1 µl of ethidium bromide (101 mg/ml) was added. The agarose was mixed with stirring in order to avoid formation of bubbles. After cooling at 55 - 60°C, the agarose-gel solution was casted into the tray of gel that both edges were sealed with a cellophane tape. The agarose was left to solidify for 30 minutes at room temperature. After a careful removal of the comb, the solidified gel was allocated in the tank of gel electrophoresis and covered with the buffer 1X TAE (3 - 5 mm over the gel surface). DNA products (5 µl) were loaded into each well, and in addition, 15 µl of a DNA marker (100bp ladder) was loaded in one-sided single well. The electrophoresis was performed at 5 V/cm2 for 45 minutes, and the bands were visualized using a system of gel-imaging.

Applications

- **Used for estimation of molecular weight of proteins and nucleic acid**
- **Determination of subunit structure of proteins**
- **Purification of isolated proteins**
- **Monitoring changes of protein content in body fluids**
- **Identifying disulfide bonds between protein**
- **Quantifying proteins**
- **Blotting Applications**

References

file:///C:/Users/PC/Downloads/35088%20(1).pdf

https://www.addgene.org/protocols/gel-electrophoresis/

Polymerase chain reaction (PCR)

lab#5
by
Haneen Moayad

Haneen M. I. Biotech. Dept

1

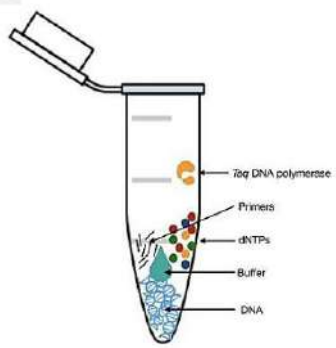
Polymerase chain reaction (PCR)

A technique used to rapidly make numerous copies (millions to billions) of a specific segment of DNA sample.

What we need to perform a PCR?

➢ **Reaction mixture which contains:**

- Template DNA : the DNA that contains the sequence of interest to be replicated
- Taq polymerase
- Primers
- The four deoxyribonucleoside triphosphates (dNTPs)
- $MgCl_2$
- Buffer solution



➢ **An automated thermal cycler (Thermocycler)**

2

Taq polymerase

- Allows replication.
- Purified from *Thermus aquaticus* lives in hot springs and resists temperatures above 100°C.
- The optimum temperature for Taq polymerase activity is 72°C.

BY Haneen M. I. Biotech. Dept

3

PCR primers

Primers are:

- Single-stranded DNA.
- Synthesized chemically
- Complement and hybridize the sequences flanking the sequence of interest to be replicated
- Short sequences (10 - 30 nt) to guarantee a sufficiently specific hybridization of the primers on the sequences of interest of the template DNA

Forward primer: Designed to recognize complementarily a sequence located upstream of the fragment 5′–3′ strand DNA of interest.

Reverse primer: Designed to recognize complementarily a sequence located upstream complementary strand (3′–5′) of the same fragment DNA.

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PCR steps

Denaturation (94 - 96°C): It is the separation of the two strands of DNA. The hydrogen bonds cannot be maintained at a temperature higher than 80°C and the double-stranded DNA is denatured into single stranded DNA.

Annealing (40-70°C): Also called **primer hybridization** temperature. Decreasing the temperature allows the hydrogen bonds to reform and thus the complementary strands to hybridize. Short single-strand sequences complementary to regions that flank the DNA to be amplified.

Since primers are short, they hybridize (anneal) more easily than long strand template DNA in annealing step.

Extension/Elongation (72°C): It is the synthesis of the complementary strand. At 72°C, Taq polymerase binds to primed single-stranded DNAs and catalyzes replication using the dNTPs present in the reaction mixture.

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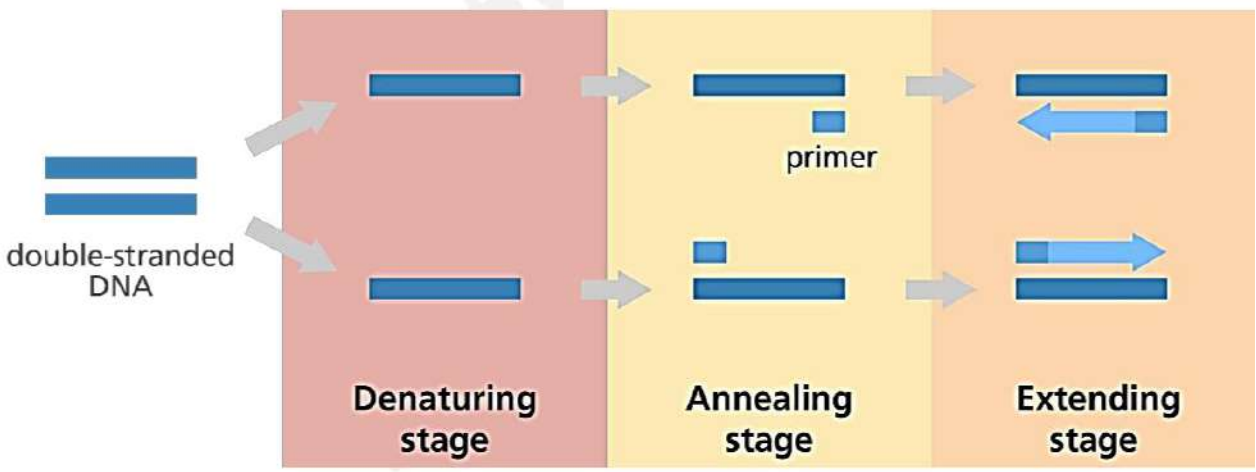
PCR steps



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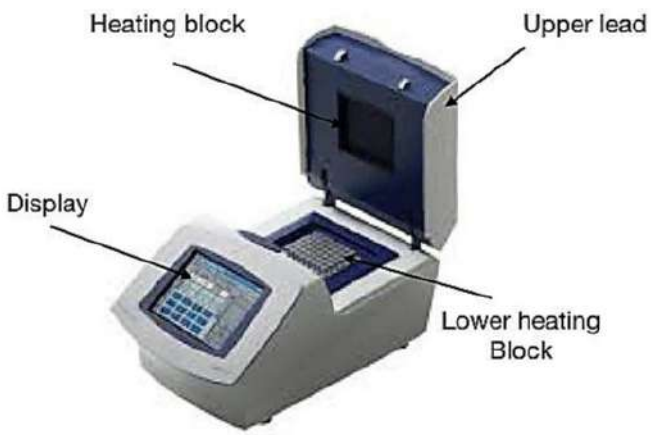
The PCR machine (thermocycler)

A heating block which provides the constant temperature and rapidly changes between two temperature states. This machine contains:

- Lower block of metal having deep wells for putting PCR tubes.
- Heating block present on the upper side of the lid to maintain the inner environment Temperature.
- The display, power on and off switch, and cooling assembly.
- Has the ability to heat and cool the PCR tube in a short period of time.



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The traditional polymerase chain reaction machine With the set up of 3 different water baths

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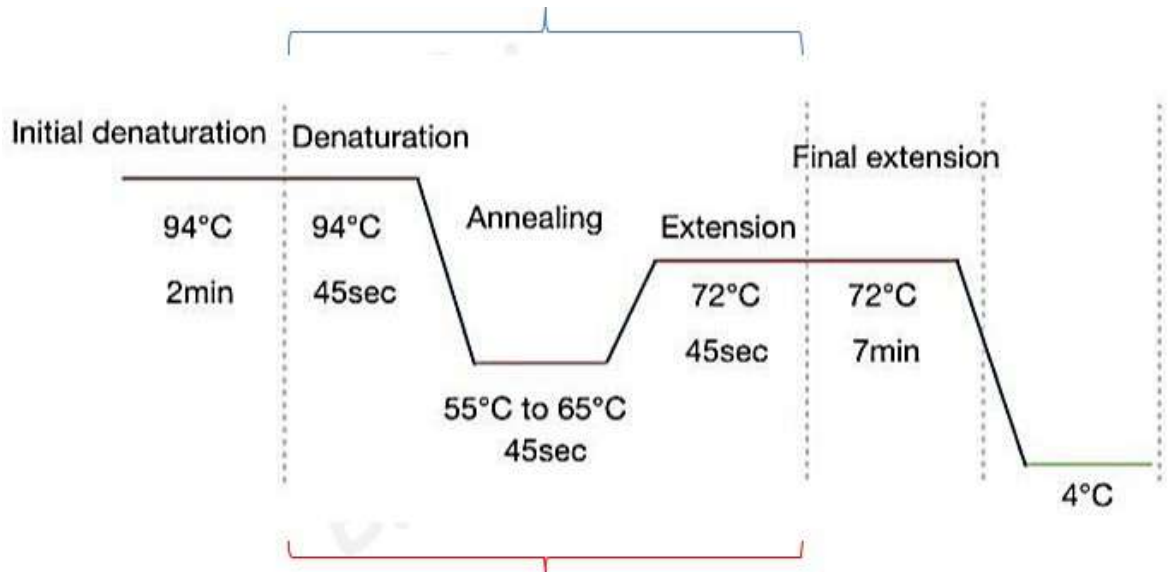
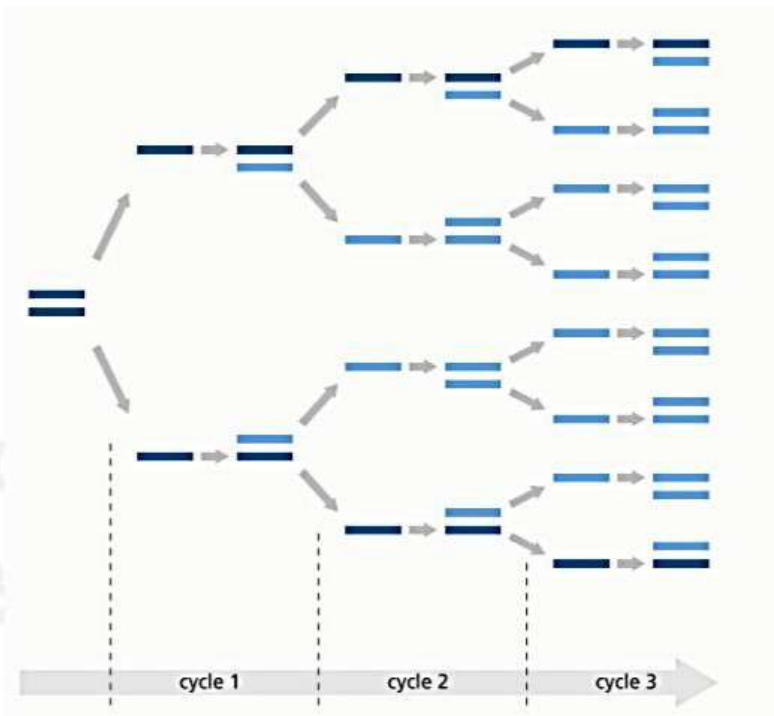


Cycle number ( )

9

An illustration showing how PCR technique produces lots of copies of DNA.



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PCR protocol and calculation :

- The entire hood work surface, as well as pipettes, should be sterilized.
- Calculate the components and reaction mix volumes that you need to prepare by making a table of reagents that will be added to the reaction mixture (will be explained in the lab).
- label PCR tubes with permanent marker.
- Reaction volumes will vary depending on the concentrations of the stock reagents, you will need to calculate the final concentrations for a typical 50 µl reaction.

Protocol: In a traditional PCR protocol, reaction components are assembled as described below. The final volume should be 50 µL.

1) Prepare a master mix based on volumes you calculated and add reagents in following order: water, buffer, Mg CL_2, dNTPs, primers, and Taq polymerase. Mix well and briefly centrifuge.

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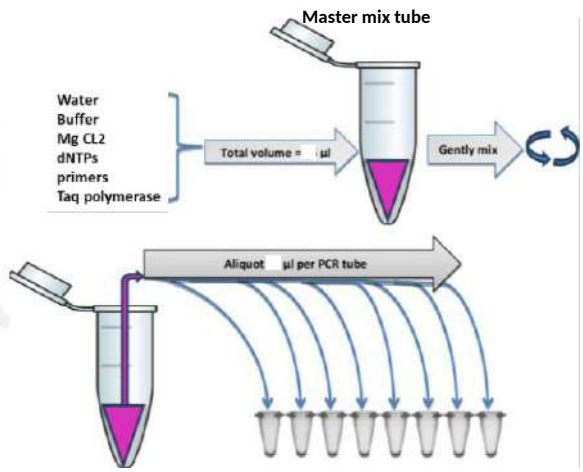
2) Distribute reaction mix into the 0.2 mL PCR tubes and then add template DNA for each tube to reach 50µL/tube. Gently mix by tapping tubes and briefly centrifuge to settle tubes contents.
3) Prepare negative control reaction **without template DNA.**
4) If possible, prepare positive control reaction **with template of known size and appropriate primers.**
5) Analyze the results of your PCR reaction via gel electrophoresis.



Master mix tube

12

Applications of PCR

- Detection of genetic diseases.
- Detection of infectious diseases
- Forensics
- Biomedical research
- DNA sequencing

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14

Immunogenetics - Lab #6

Immunoassays

By
Haneen Moayad

Biotechnology department

By Haneen M. I. Biotech. Dep.

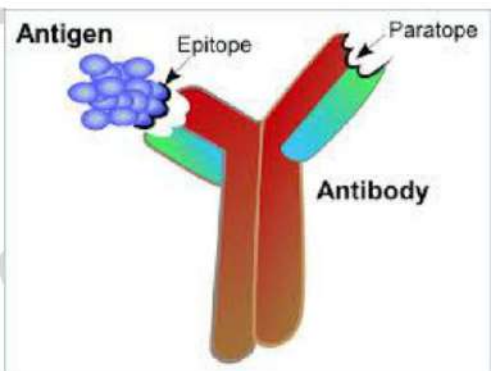
1

In immunology:

An **antibody** is a protein produced by the body's immune system

An **antigen** is a particular macromolecule bound by an antibody

An **epitope** is the small site on an antigen to which a complementary antibody may specifically bind.



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Comparison Chart on Differences Between Antigen & Antibody

	Antigen	Antibody
Overview	Substance that can induce an immune response	Proteins that recognize and bind to antigens
Molecule type	Usually proteins, may also be polysaccharides, lipids or nucleic acids	Proteins
Origin	Within the body or externally	Within the body
Specific binding site	Epitope	Paratope
Image		

From: https://www.technologynetworks.com/immunology/articles/antigen-vs-antibody-what-are-the-differences-293550

3

Immunoassays

- A procedure for detecting or measuring specific proteins or other substances through their properties as antigens or antibodies.
- An immunoassay capitalizes on the specificity of the antibody-antigen binding found naturally in the immune system.
- Immunoassay principle: Rely on the ability of an antibody to recognize and bind a specific antigen.
- Examples:
  - ELISA
  - Western blot

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ELISA: Enzyme-linked immunosorbent assay

- An immunological assay commonly used for detecting and quantifying antibodies, antigens, proteins and glycoproteins in biological samples.
- Described by Engvall and Perlmann (1971).
- In ELISA, an enzyme conjugated with an antibody reacts with colorless substrate to generate a colored product. Such substrate is called **chromogenic substrate**.
- A number of enzymes have been used for ELISA such as **horse radish peroxidase (HRP).**
- The **chromogenic substrate** is hydrolysed by enzyme (ex. HRP) to give colored end product.
- **Sample types:** Cell culture, Biological fluids (Plasma, Serum, Urine), Purified recombinant protein in solution

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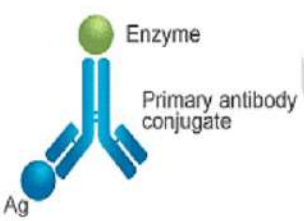
Types of ELISA : The four main types of ELISA are:

1. Direct ELISA

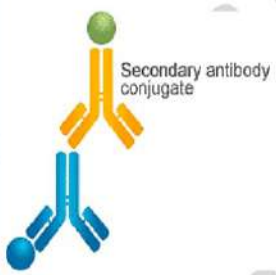
Suitable for determining the amount of high molecular weight antigens



antigen-coated plate

2. Indirect ELISA

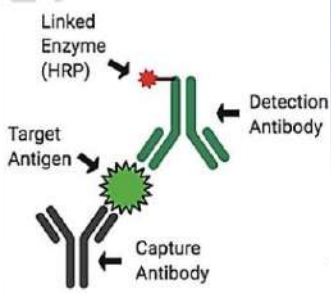
Useful for screening for specific antibodies



antigen-coated plate

3. Sandwich ELISA

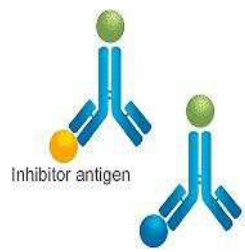
Detect or quantitate soluble antigens



antibody-coated plate

4. Competitive ELISA

Detect or quantitate soluble antigens (possible for low molecular weight antigens with a limited number of epitopes)



inhibitor antigen-coated plate

Note: some ELISA procedures can be modified to measure antigens and/or antibodies

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96 well plate

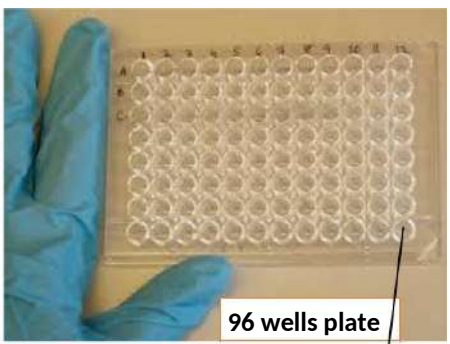
Multichannel pipette

ELISA plate reader

7

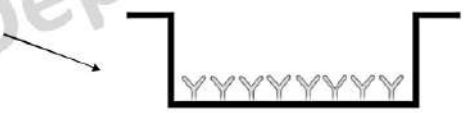
A basic procedure of ELISA

- In ELISA a plate with 96 wells (the plate made of **polystyrene**) is used, and wells are coated with antibodies (depends on ELISA type). These antibodies are called "**capture antibodies**", the role of which is to capture the target antigen molecules in the sample.



- ELISAs begin with a coating step, where the first layer, an antibody, is adsorbed to a well in an ELISA plate. Coating is carried out by adsorption on the surface (usually the bottom area of plate wells)

One well for explanation



Note: Coating step is followed by blocking steps by using blocking buffer. A blocking buffer is a solution of irrelevant protein, mixture of proteins that passively adsorbs to all remaining (empty) binding surfaces of the plate.

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One well of 96 well plates for explanation

1. Standard solutions or assay samples are added to the antibody-coated wells, and incubated for several hours so as to the antigen molecules are captured by "capture antibody".

2. After this binding reaction, the reaction mixture is discarded, and wells are washed to remove excessive materials.

3. The second antibody which recognizes another epitope in antigen is added. This second antibody has been labeled with an enzyme such as horseradish peroxidase (HRP).

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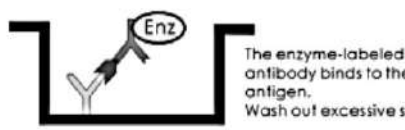
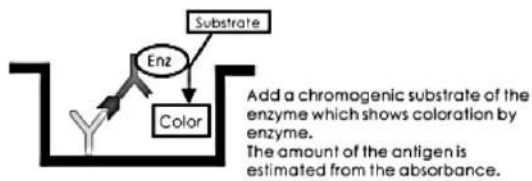
4. The enzyme-labeled second antibody will bind to the antigen which is bound to the capture antibody on the bottom area of wells. This means that the enzyme (HRP) is also fixed on the bottom of wells. The amount of the antigen captured is **proportional** to fixed enzyme.



5. Enzyme activity is measured by adding a chromogenic substrate of this enzyme. In the case of HRP enzyme, tetramethylbenzidine (TMB) is often used as a substrate.
   - After incubation for some period, the chromogenic substrate is changed to a colored product.



6. The reaction is stopped by adding a reaction stopper, e.g. diluted sulfuric acid, and absorbance is measured using a **plate reader**.

Note:

Chromogen: a chemical alters color as a result of an enzyme interaction with substrate (color reaction used as signal, ex: tetramethylbenzidine (TMB)

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7. The standard curve is prepared from the concentration of standard solutions and their absorbance. And the <u>sample assay values are obtained from the absorbance using the standard curve</u> (calibration curve).

Note: The standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range of concentrations near the expected unknown concentration.

Importance of repeated washing steps:

Since the assay uses surface binding for separation, several washes are repeated between each ELISA step **to remove unbound materials**. During this process it is important to remove excess liquid in order to prevent the dilution of the solutions added in the next step.

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ELISA results

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Applications of ELISA

There are many applications of ELISA, below are some examples:

1. Detecting and quantifying antibodies, antigens, proteins and glycoproteins in biological samples
2. Determination of serum antibody concentrations in a virus test (ex. diagnosis of HIV infection).
3. Used in food industry when detecting potential food allergens.
4. Applied in disease outbreaks- tracking the spread of disease e.g. Bird flu, cholera, HIV, etc.

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https://www.cusabio.com/c-20659.html

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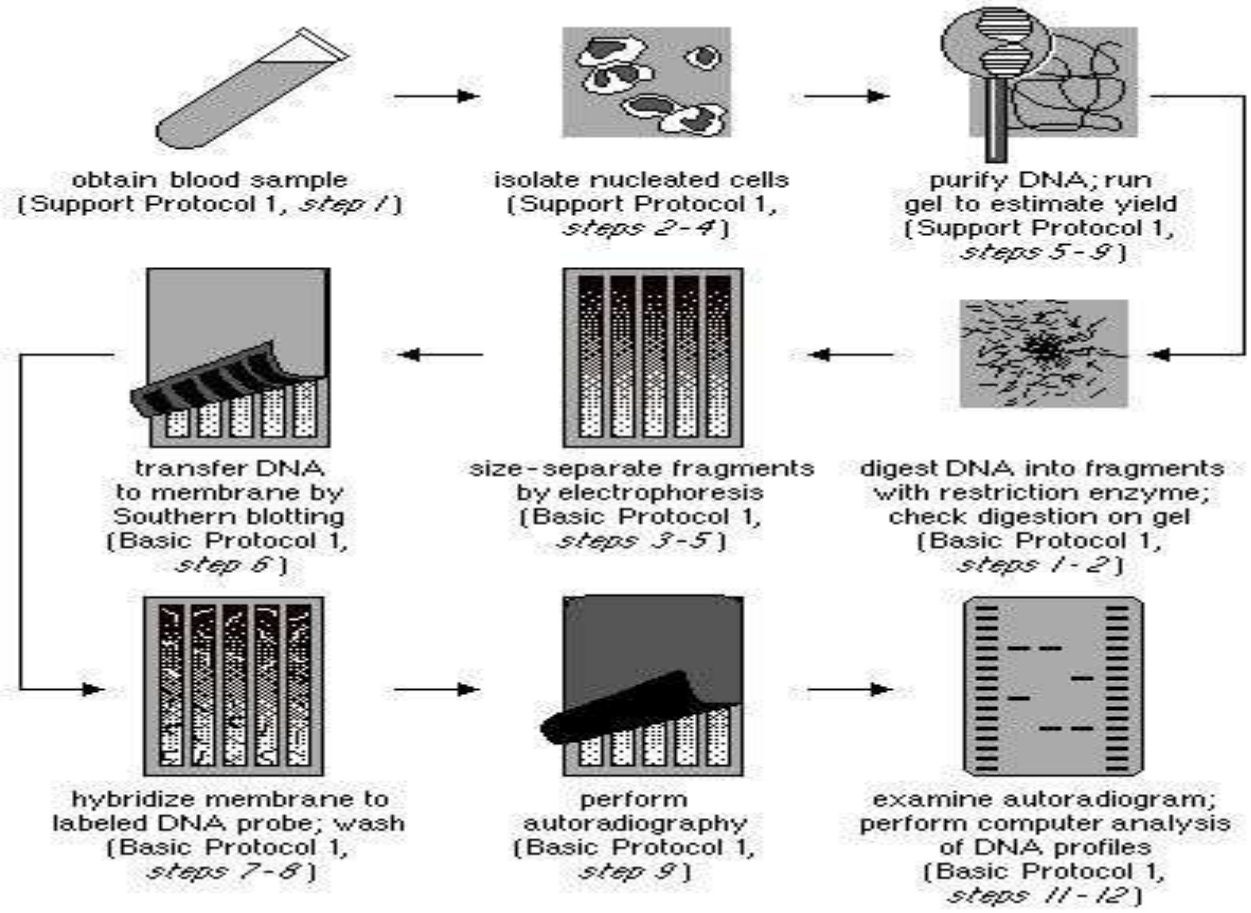
Restriction Fragment Length Polymorphism (RFLP):

- RFLP is an enzymatic procedure for separation and identification of desired fragments of DNA. Using restriction endonuclease enzymes fragments of DNA is obtained and the desired fragment is detected by using restriction probes. Southern hybridization using restriction endonuclease enzymes for isolation of desired length of DNA fragments is an example of RFLP.

Procedures or steps of RFLP test:



Step I: Restriction digest

Extraction of desired fragments of DNA using restriction endonuclease (RE).

The enzyme RE has specific restriction site on the DNA, so it cut DNA into fragments. Different size of fragments are generated along with the specific desired fragments.

Step II: Gel electrophoresis

The digested fragment are run in polyacrylamide gel electrophoresis or Agarose gel electrophoresis to separate the fragments on the basis of length or size or molecular weight.

Different size of fragments form different bands.

Step III: Denaturation

The gel is placed in sodium hydroxide (NaOH) solution for denaturation so that single stranded DNA are formed.

Step IV: Blotting

The single stranded DNA obtained are transferred into charge membrane ie. Nitrocellulose paper by the process called capillary blotting or electro-blotting.

Step V: Baking and blocking

The nitrocellulose paper transferred with DNA is fixed by autoclaving.

Then the membrane is blocked by using bovine serum albumin or casein to prevent binding of labelled probe nonspecifically to the charged membrane.

Step VI: Hybridization and visualization

The labelled RFLP probe is hybridized with DNA on the nitrocellulose paper.

The RFLP probes are complimentary as well as labelled with radioactive isotopes so they form color band under visualization by autoradiography.

Application of RFLP test:

Genome mapping: helps in analysis of unique pattern in genome for organism identification and differentiation. It also helps in determining recombination rate in the loci between restriction sites.

Genetic disease analysis: After identification of gene for particular genetic or hereditary disease, that gene can be analyzed among other family members.

To detect mutated gene.

DNA finger printing (forensic test): It is the basis of DNA finger printing for paternity test, criminal identification etc.

DNA sequencing

Immunogenetics Lab#8

By: Haneen M.

1

DNA sequencing

- The determination of sequence of nucleotide bases (A , T , C , and G) in a piece of DNA called a DNA sequencing.
- One of the most common methods of DNA sequencing is the **Sanger method.**
- 1977, Fredrick Sanger suggested the first method for sequencing the DNA, named as **a chain termination method**.

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DNA sequencing steps (Sanger sequencing steps)

Sanger sequencing steps:

1- **DNA extraction**: using any of the DNA extraction protocols

2- PCR amplification of target sequence: DNA synthesis reactions in four separate tubes. Also using modified, radioactive nucleotide called ddNTPs.

3- Denaturation of the resulting DNA fragments into single stranded DNA (ssDNA)

4- Identification of the amplified fragments: The denatured fragments are separated by gel electrophoresis and the sequence is determined. Also, an automated machine called **sequencer** can be used to analyze the sequence.

3

DNA sequencing steps (Sanger sequencing steps)

In the second step (the PCR step):

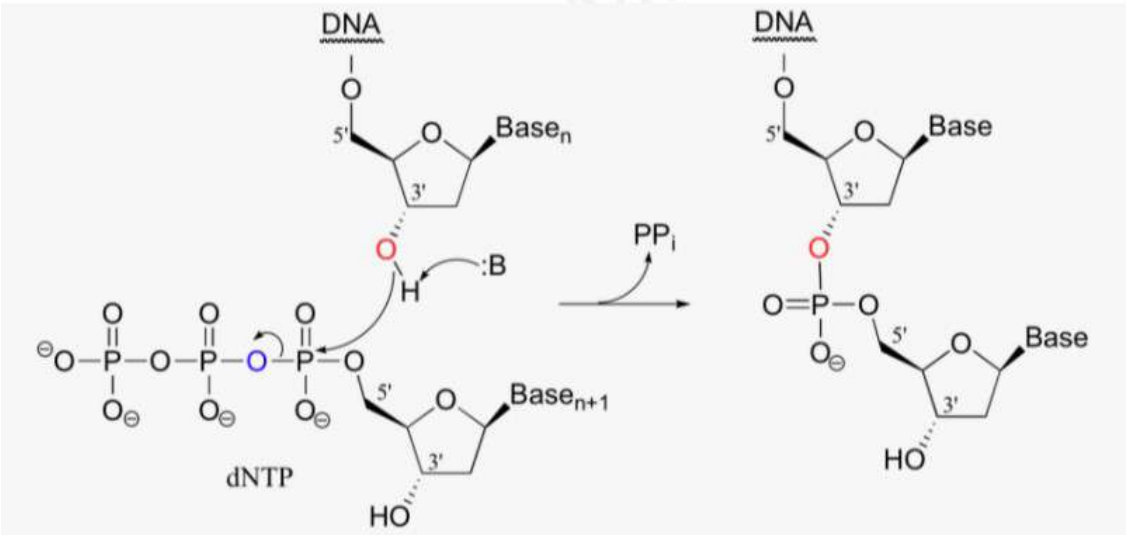
PCR amplification of target sequence: DNA synthesis reactions in 4 separate tubes, each tube contains:

- Specific primers
- dNTPs (for all four reactions): normal DNA nucleotides (deoxynucleotides, dATP, dGTP, dTTP and dCTP)
- Taq DNA polymerase
- PCR buffer
- Other PCR components
- DNA template
- **ddNTPs (only one type per a reaction)**: a mixture of **radioactive** dideoxynucleotides (ddATP, ddGTP, ddTTP and ddCTP).

Note : ddNTP also called terminator because it terminates the elongation step

4

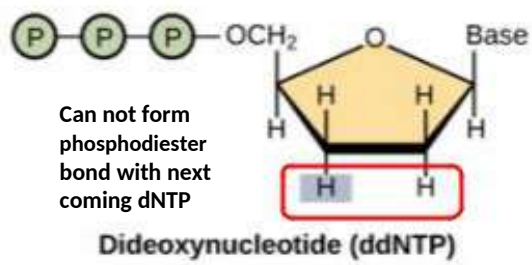
Phosphodiester bond formation in DNA

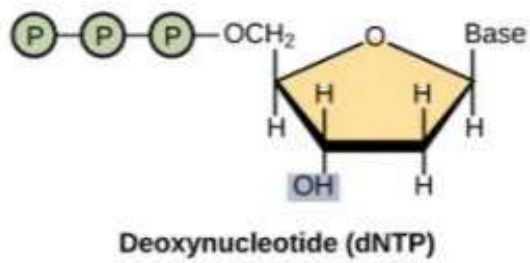


https://www.pngitem.com/middle/mwiwiJ_image130-phosphodiester-bond-formation-in-dna-hd-png/

dNTPs vs. ddNTPs

- The dideoxynucleotides are just like ordinary DNA nucleotides except that one hydroxyl (OH) group has been chemically changed to a hydrogen (H).
- With normal DNA nucleotides, one nucleotide can be attached to another and so on, forming a chain.
- Because of chemical change in a dideoxynucleotide, the dideoxynucleotide (ddNTPs) can not form phosphodiester bond with next coming dNTP.





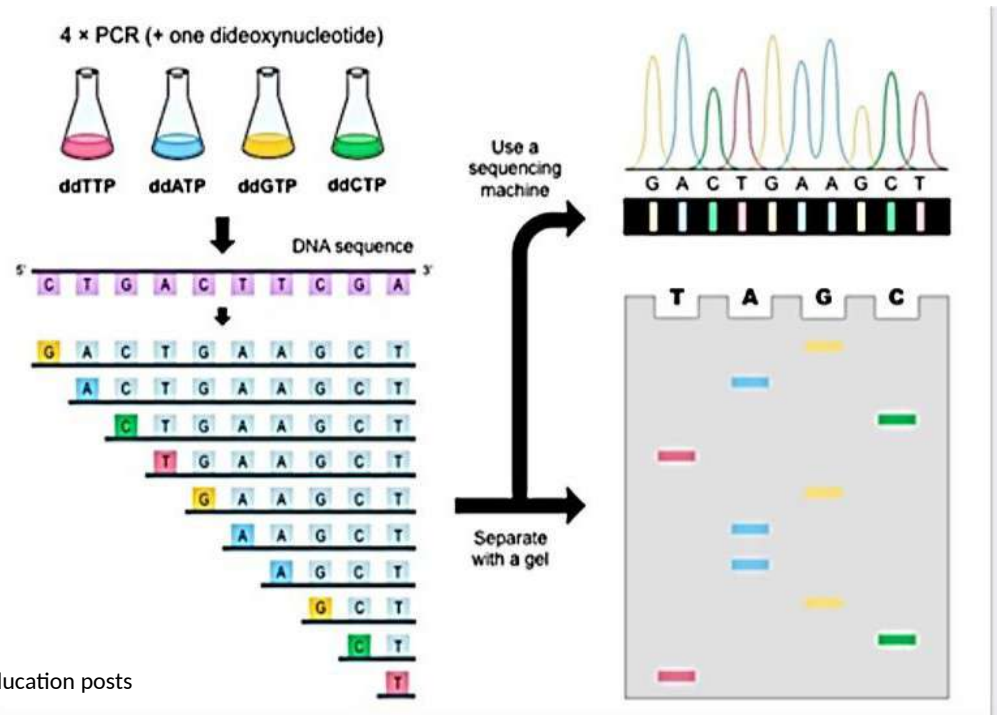
https://www.cd-genomics.com/blog/sanger-sequencing-introduction-principle-and-protocol/

DNA sequencing steps (Sanger sequencing steps)

- The new DNA strand is made by complementary base pairing with the original DNA template.
- Because all four ordinary DNA nucleotides are present in reaction mixture in a good amount, the chain elongation continues normally, until by chance a dideoxynucleotide (terminator) is added in the place of a normal DNA nucleotide , the elongation of strand is hold and terminated. And the last base in each of these fragments is known (radioactive).

7

Sanger sequencing method



From: the genome education posts

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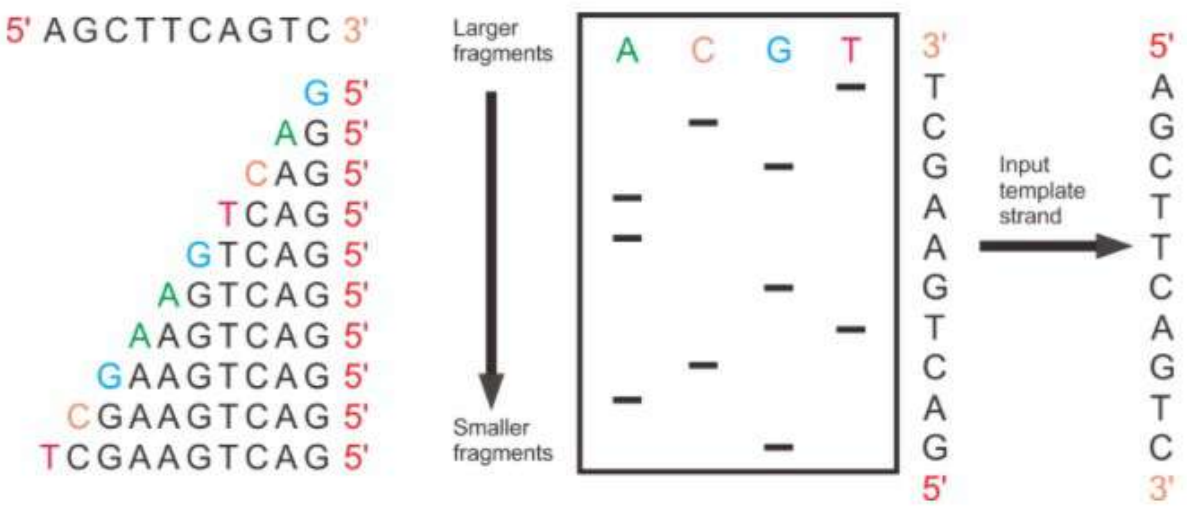

Sanger Sequencing scheme - Chain-termination methods

https://zhonglab.gitbook.io/3dgenome/chap0-preparation/0.2-sequencing-technologies

9

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10

Human leukocyte antigen (HLA)

Immunogenetics lab#9

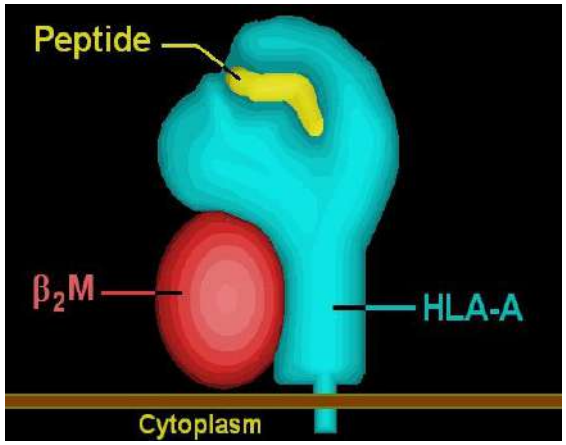
by

Wasan Wael

What is HLA System?

HLA = Human Leukocyte Antigen

- The HLA system represents the human major histocompatibility complex (MHC).
- It is a large genomic region or gene family found in most vertebrates.
- It is the most gene-dense region of the mammalian genome and plays an important role in the:
- **Immune system Functions**
- **Autoimmunity**
- **Reproductive success**



- surface of cells, and as serum proteins in all vertebrates, and display fragments of molecules from invading microbes or dysfunctional cells (e.g. tumor cells) to a particular type of leucocytes called a T cell that has the capacity to kill or co-ordinate the killing of the microbe, infected cell or malfunctioning cell.
- The HLA spans over a region of about 4 megabases on the short of chromosome 6 at the band 6p21.31, and it contains more than 200 genes.
- These genes are organized in Three Subregions:

  - **HLA-Class I**
  - **HLA-Class II**
  - **HLA-Class III**

HLA typing

- **HLA typing** is a kind of genetic test used to identify certain individual variations in a person's immune system. The process is critical for identifying which people can safely donate bone marrow, cord blood, or an organ to a person who needs a transplant .Typing is also used to identify markers for specific diseases.
- Donors antigens expressed on the surface of leukocyte or their genes are matched with that of the recipient.
- The closer the HLA antigens on the transplanted organ match the recipient ,the more likely that the recipient's body will not reject the transplant

What is HLA polymorphism?

gene is referred to as **polymorphic** when more than one allele is expressed in a population. **HLA** genes are extremely **polymorphic** but the same alleles are frequently associated in individual.

- Every person inherited each of the following antigen from each parent:

HLA-A antigen

HLA-B antigen

HLA-C antigen

HLA-DR antigen

HLA-DQ antigen

HLA-DP antigen

Methods Of HLA Typing

A -Phenotypic method:

- Serology: Microcytotoxicity
- Tissue typing: Mixed lymphocyte reaction

B-Genotypic methods:

- PCR detecting HLA genes
- PCR detecting –RFLP(restriction fragment length polymorphism)
- DNA sequence based typing

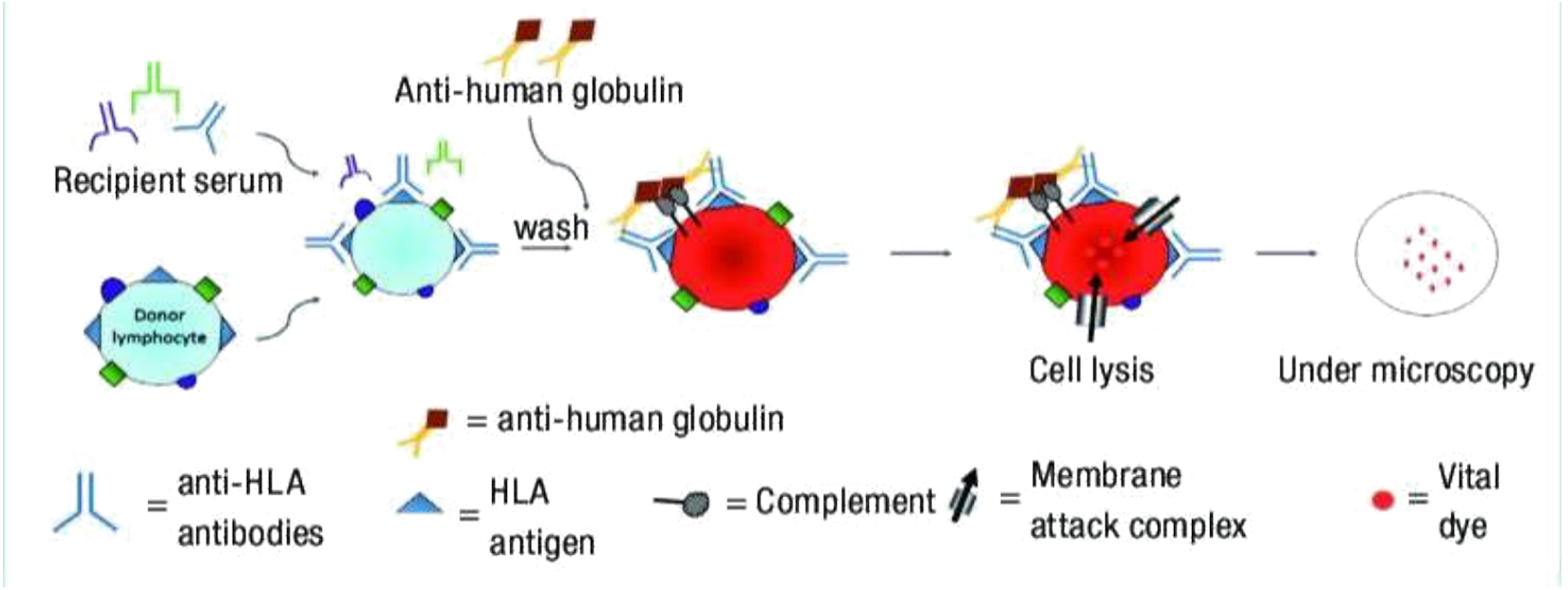
C-Lymphocyte cross-matching

Microlymphocytotoxic test:

1-Viable lymphocytes are incubated with HLA specific antibody. If the specific antigen is present on the cell the antibody is bond.

2-Rabbit serum as a source of complement is added ,incubate .If antibody is bound to the HLA antigen on the cell surface it activates the complement which damage the cell membrane making it permeable to vital stain.

3-Result are visualized by adding ethidium bromide ,if the reaction take place the EB enters the cell and binds to the DNA.


Anti-human globulin
Recipient serum
wash
Donor lymphocyte
Cell lysis
Under microscopy
= anti-human globulin
= anti-HLA antibodies
= HLA antigen
= Complement
= Membrane attack complex
= Vital dye

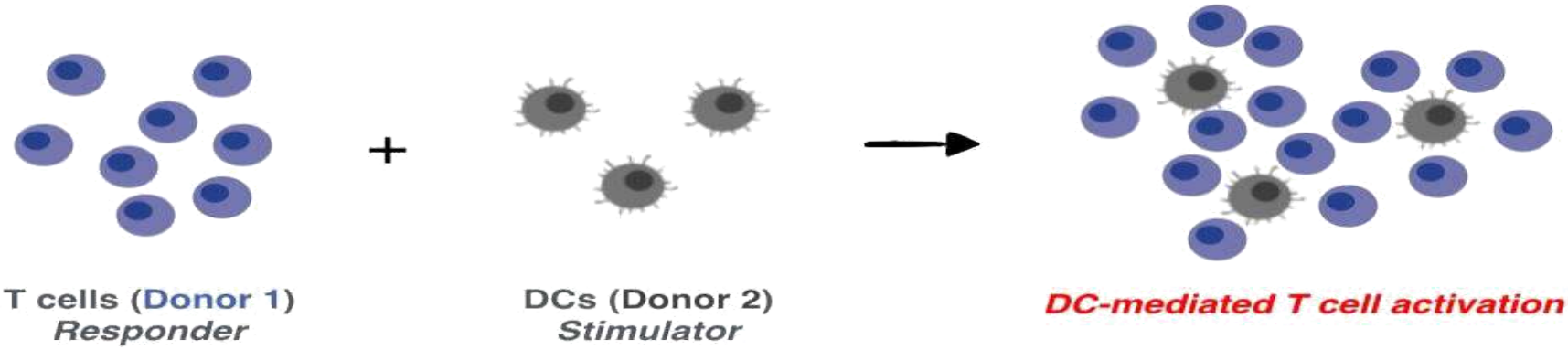
Cellular: Mixed lymphocyte culture (MLC)

- Used to quantity the degree of class II MHC compatibility between potential donor and recipients.

- It is based on the principle that if immunocompetent T-cells from one individual is incubated with APC's of a genetically different individual ,the T-cell of the first individual will proliferate .

- Proliferation of the recipient T-cell is measured by the uptake of thymidine into the cell DNA

- The test is important for determining histocompatibility between donor and recipient in the transplantation of bone marrow and organs.



Lymphocyte cross-matching: This step takes place when a donor is identified; the objective is to identify any antibody that, if present in the recipient, might be directed against antigens present on the donor's lymphocyte. In this test, serum from the intended recipient is mixed with T and B lymphocytes (white blood cells) from the donor to investigate

potential reactions (a positive test result) that might destroy white blood cells of the recipient

Importance of HLA Typing:

- Organ transplantation
- Hematopoietic stem cell transplant
- HLA types associated with specific immune diseases.
- Parental testing

An and Deshpande, The Human Leukocyte Antigen System … Simplified . Global Journal of Transfusion Medicine September 12, 2017, IP: 212.115.253.120

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Lab 10

The Comet Assay

by Dr. Aaiad Hameed

Comet Assay: Is a gel electrophoresis–based method, which can be used to measure DNA damage in single cells. It measures variation in DNA damage and repair capacity within a population of cells- (to measurement of DNA damage in specific genomic sequences). It can detect single or double strand breaks. This assay has become well known in the field of toxicology and drug discovery.

* Comet assay is based on the ability of denatured cleaved DNA fragments or damaged DNA, to migrate out of the cell under electrophoresis, creating a (comet tail) while, the undamaged DNA remains within the cell membrane creating the (comet head), so it is based on the capacity of negatively charged loops/fragments of DNA to be pulled through an agarose gel in response to an electric field, appearing like a 'comet.' Fig1

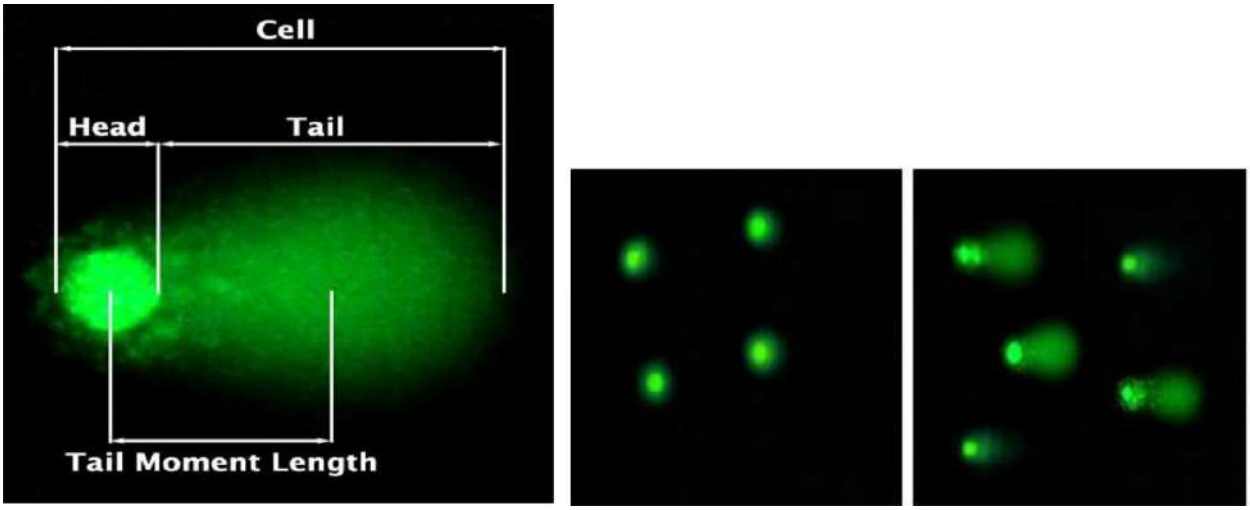


Fig1

The protocol: the alkaline comet assay

We need: Comet Slides, reagents, and a fluorescent dye to visualize cells under a fluorescence microscope.

3 Well Comet Slides

1. Cells are suspended in Comet agarose (generally at 37 °C), then coverslip is used to flatten out molten agarose layer, and the slides are often chilled during the process to enhance gelling (thickening) of the agarose.

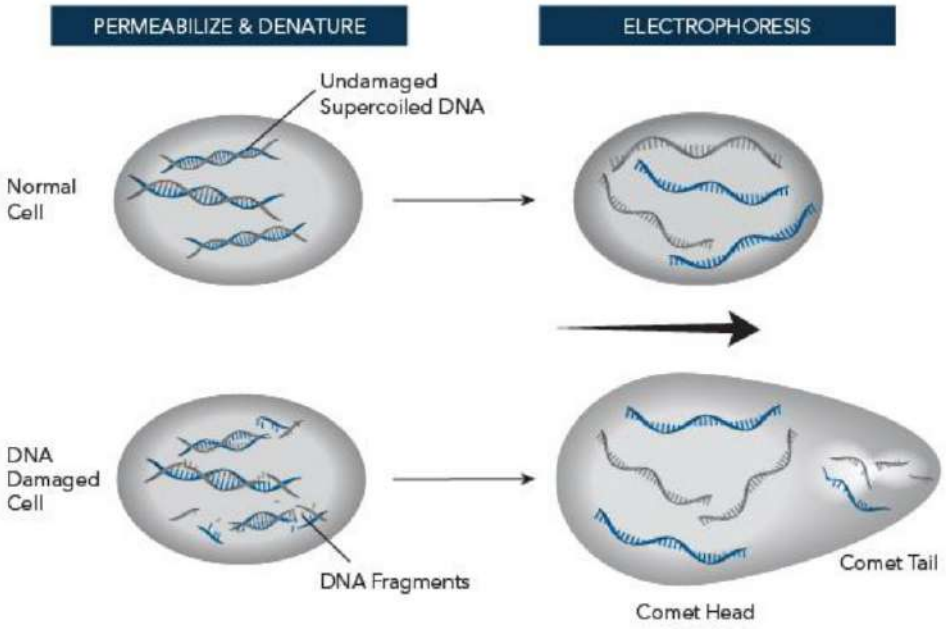
2. After, the slides are placed in a lysis solution consisting of high salts and detergents generally for at least 1 h. (The lysis solution is chilled prior to use- to maintain the stability of the agarose gel).

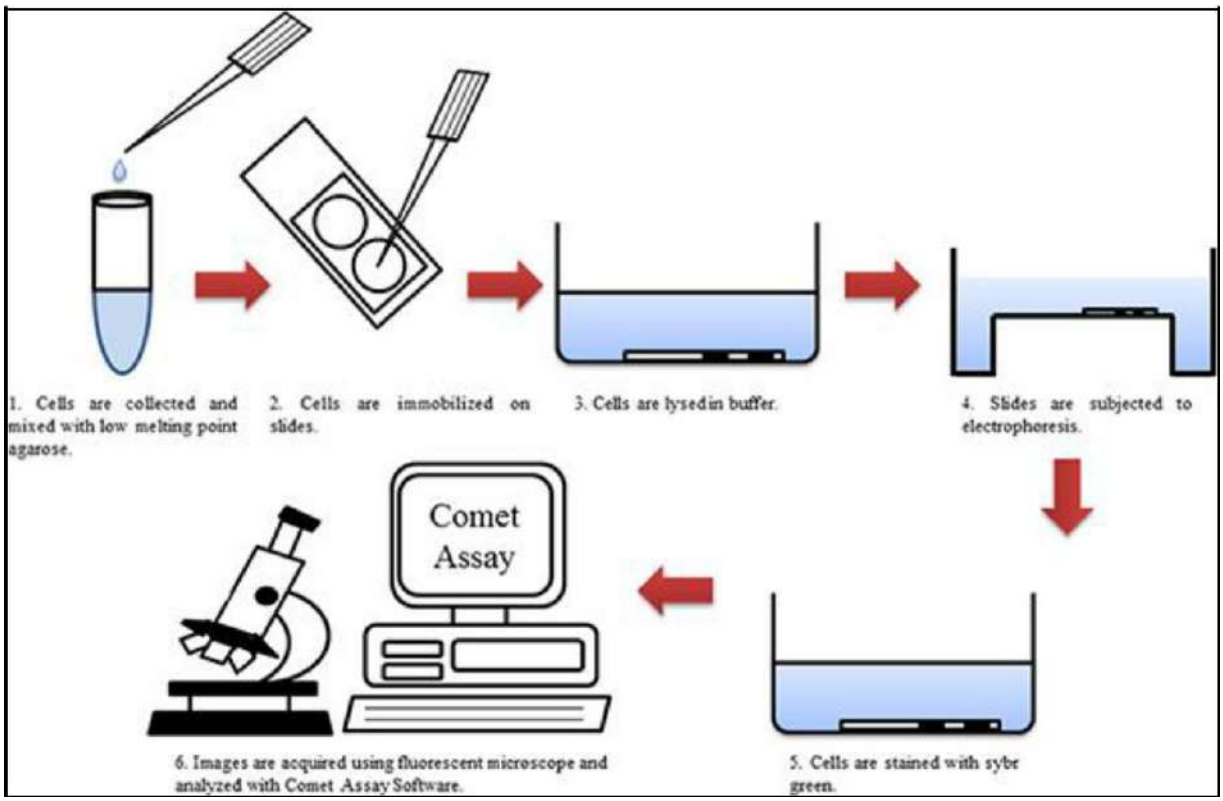
3. Prior to electrophoresis, the slides are incubated in alkaline (pH > 13) electrophoresis buffer to produce single-stranded DNA.The alkaline solution consists of EDTA and sodium hydroxide, pH > 13 for 20 min.

4. Electrophoresis is performed at RT.

5. After electrophoresis, the alkali in the gels is neutralized by rinsing slides with a suitable buffer (trizma buffer), 3 washes for 5 min each.

6. After neutralization, slides can be stained, the DNA is visualized by fluorescence microscopy after staining with a fluorescent DNA binding dye and comets can be scored.





The comet assay protocol steps

Applications of comet assay:

1. To measure DNA damage in a variety of cell types, including sperm.
2. Biomonitoring of environmental and occupational exposure.
3. Prediction of tumour radio and chemosensitivity and in male infertility.
4. Forensic applications.