EXPERIMENT: 1

Centrifugation

Centrifugation is a process which separates or concentrates materials suspended in a liquid medium. The theoretical basis of this technique is the effect of gravity on particles in suspension. 2 particles of different masses will settle in a tube at different rates in response to gravity. The centrifugal force is proportional to the rotation rate of the rotor. The centrifuge consists of a rotor and closed in a refrigerated chamber buy an electric motor.

Definition of Centrifugation

It is a unit operation working for separation separating the consequent present in a dispersion with the help of centrifugal force for example centrifugal force includes the earth revolves around the sun. It is a technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

Principle of Centrifugation

1) The centrifuge involve the s principle of sedimentation.

2) The principle of the centrifugation technique is to separate the particles suspended in liquid media under the influence of a centrifugal field. These are placed either in tubes or bottles in a rotor in the centrifuge.

3) Sedimentation is a phenomenon where suspended material settles out of the fluids by gravity. The suspended material can be particles such as clay or powder. Example, tea leaves falling to the bottom in a teacup.

4) The particles having size more than 5 micrometres are separated by simple filtration process while the particles having size 5 micrometre or less do not sediment under gravity. The central force is useful to separate those particles.

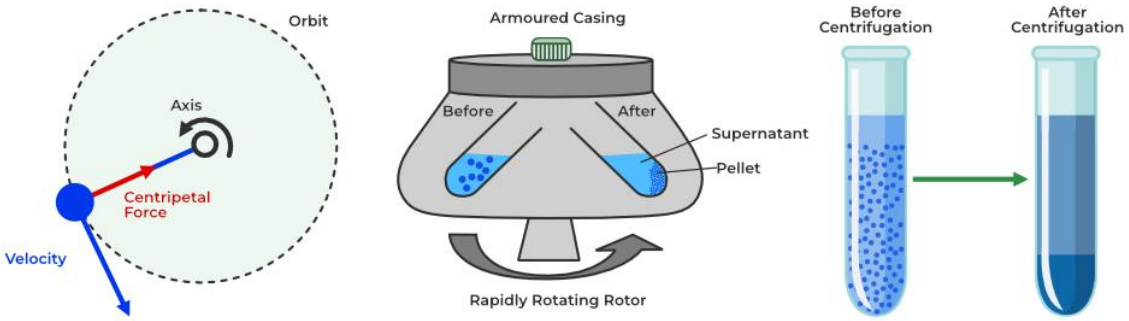


Fig: Principle of centrifugation

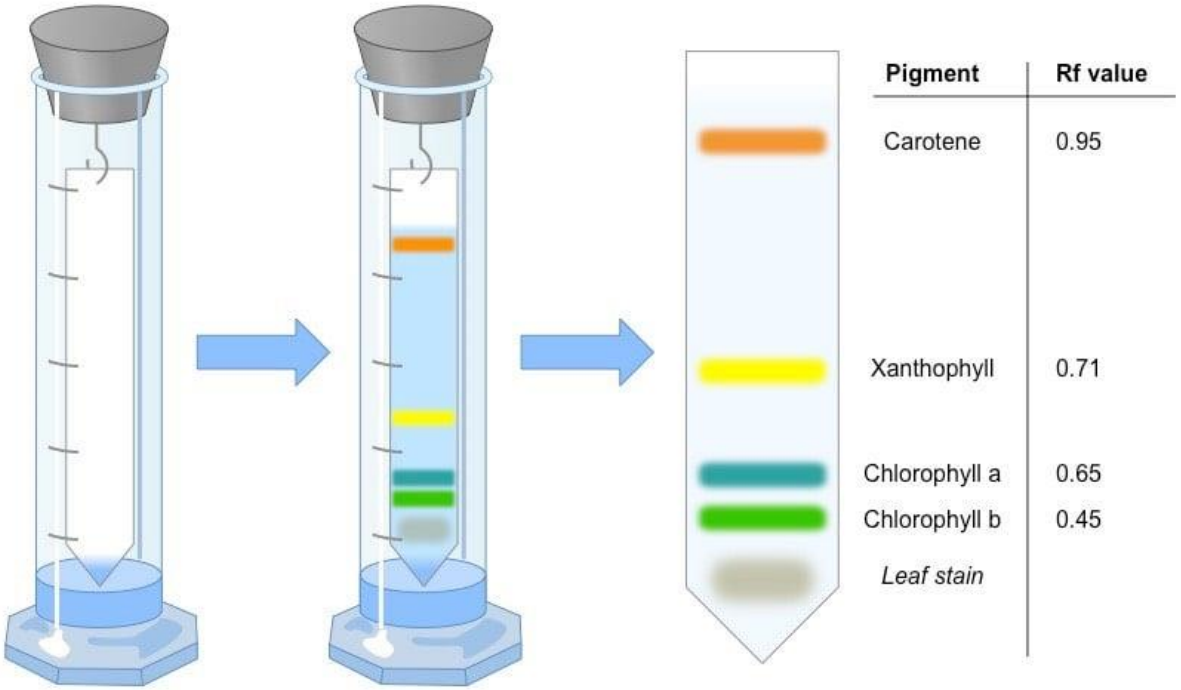
EXPERIMENT: 2

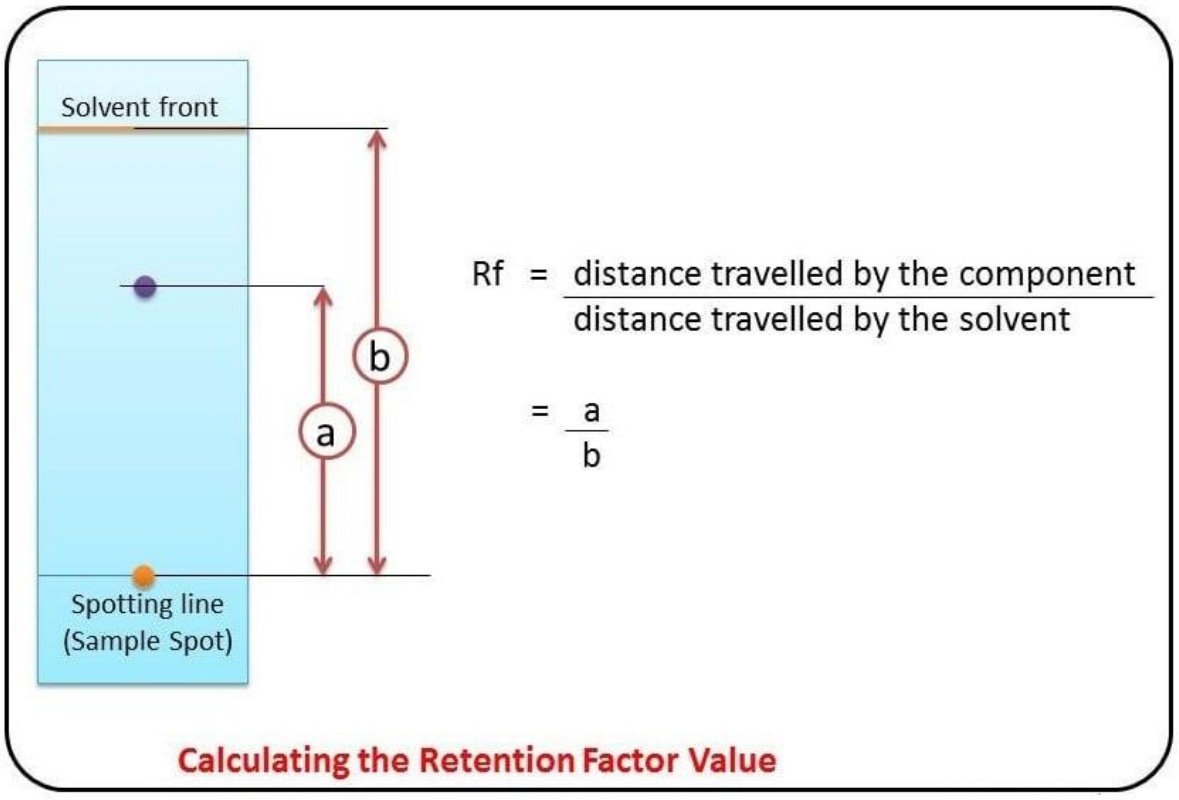
What Is Paper Chromatography?

Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.





Paper Chromatography Procedure

1. Selecting a suitable type of development: It is decided based on the complexity of the solvent, paper, mixture, etc. Usually ascending type or

radial paper chromatography is used as they are easy to perform. Also, it is easy to handle, the chromatogram obtained is faster and the process is less time-consuming.

2. Selecting a suitable filter paper: Selection of filter paper is done based on the size of the pores and the sample quality.
3. Prepare the sample: Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
4. Spot the sample on the paper: Samples should be spotted at a proper position on the paper by using a capillary tube.
5. Chromatogram development: Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
6. Paper drying and compound detection: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

Paper Chromatography Applications

There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals.

Types of paper chromatography:

1. Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
2. Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards, hence the name descending paper chromatography.
3. Ascending – Descending Paper Chromatography – In this version of paper chromatography, movement of solvent occurs in two directions after a

particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.

4. Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.
5. Two Dimensional Paper Chromatography – Substances which have the same r_f values can be resolved with the help of two-dimensional paper chromatography.

EXPERIMENT: 3

Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

spectrophotometer is an instrument that measures the amount of photons (the intensity of light) absorbed after it passes through sample solution. With the spectrophotometer, the amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected. Depending on the range of wavelength of light source, it can be classified into two different types:

- UV-visible spectrophotometer: uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
- IR spectrophotometer: uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.

In visible spectrophotometry, the absorption or the transmission of a certain substance can be determined by the observed color. For instance, a solution sample that absorbs light over all visible ranges (i.e., transmits none of visible wavelengths) appears black in theory. On the other hand, if all visible wavelengths are transmitted (i.e., absorbs nothing), the solution sample appears white. If a solution sample absorbs red light (~700 nm), it appears green because green is the complementary color of red. Visible spectrophotometers, in practice,

use a prism to narrow down a certain range of wavelength (to filter out other wavelengths) so that the particular beam of light is passed through a solution sample.

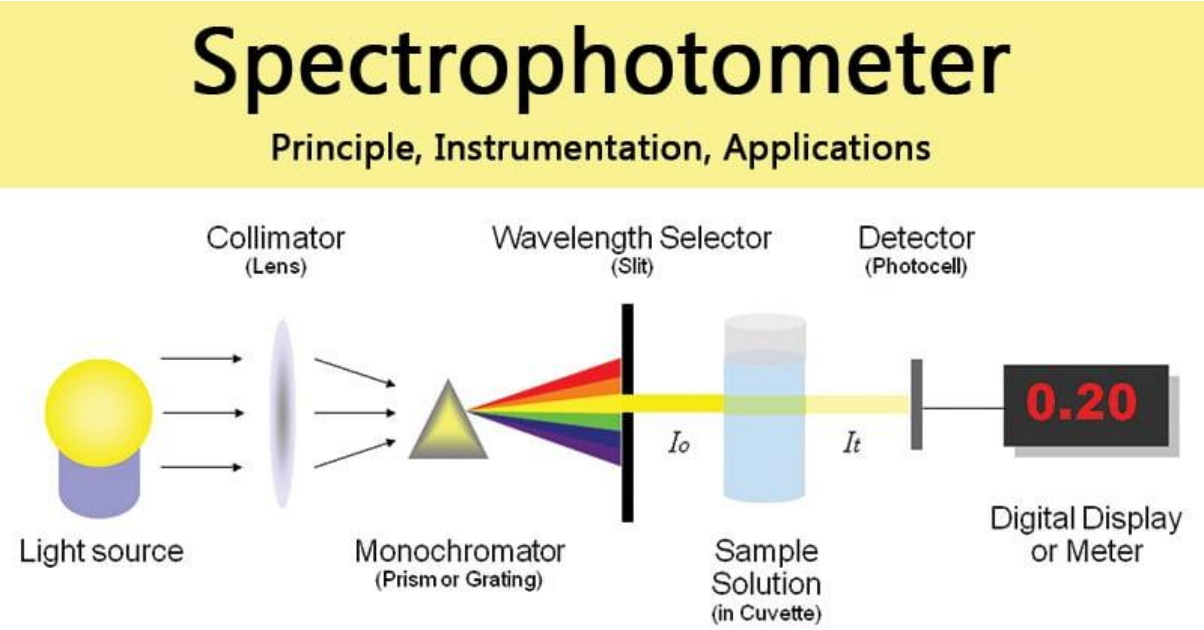


Fig. Spectrophotometer

A spectrophotometer, in general, consists of two devices; a spectrometer and a photometer. A spectrometer is a device that produces, typically disperses and measures light. A photometer indicates the photoelectric detector that measures the intensity of light.

- Spectrometer: It produces a desired range of wavelength of light. First a collimator (lens) transmits a straight beam of light (photons) that passes through a monochromator (prism) to split it into several component wavelengths (spectrum). Then a wavelength selector (slit) transmits only the desired wavelengths, as shown in Figure 1.
- Photometer: After the desired range of wavelength of light passes through the solution of a sample in cuvette, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display, as illustrated in Figure 1.

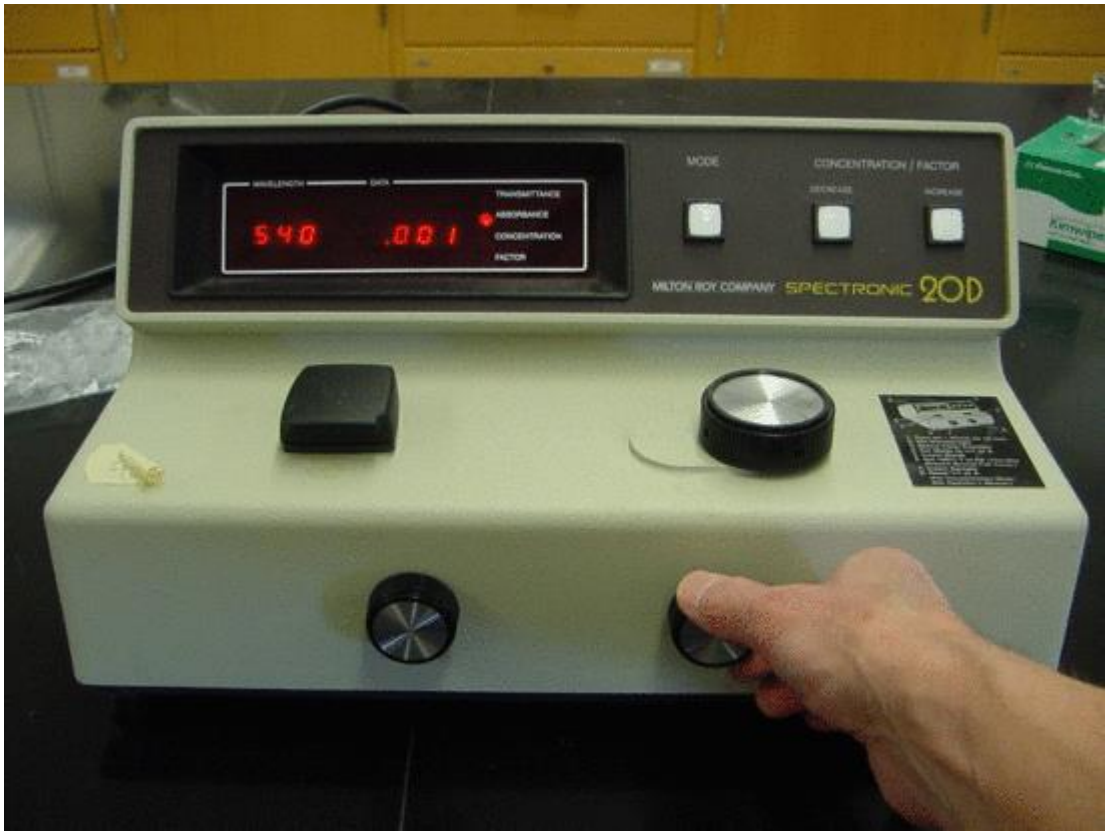


Fig 2: A single wavelength spectrophotometer

You need a spectrometer to produce a variety of wavelengths because different compounds absorb best at different wavelengths. For example, p-nitrophenol (acid form) has the maximum absorbance at approximately 320 nm and p-nitrophenolate (basic form) absorb best at 400nm, as shown in Figure 3.

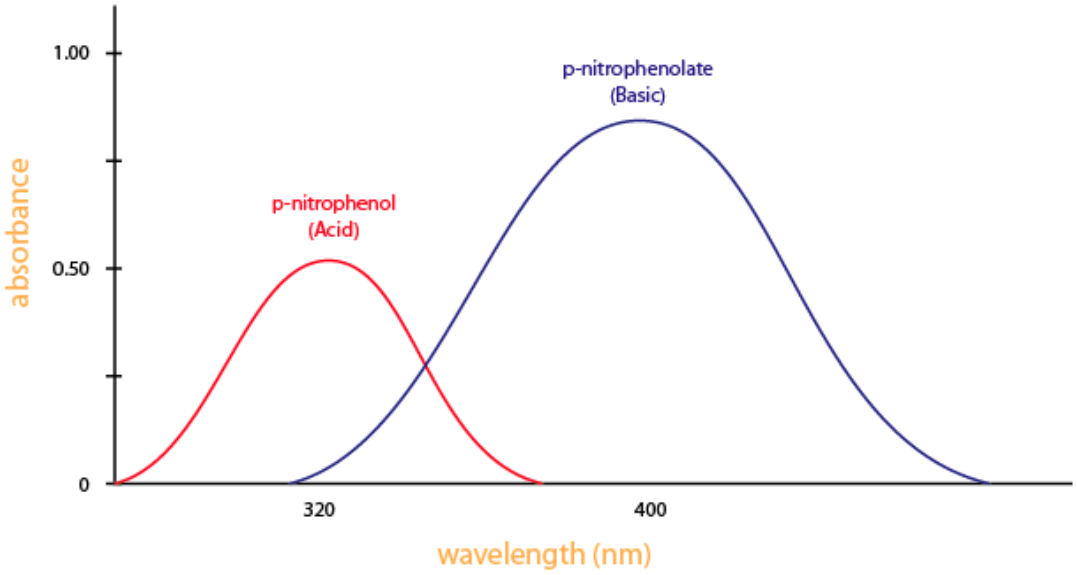


Figure 3: Absorbance of two different compounds

Looking at the graph that measures absorbance and wavelength, an isosbestic point can also be observed. An isosbestic point is the wavelength in which the absorbance of two or more species are the same. The appearance of an isosbestic point in a reaction demonstrates that an intermediate is NOT required to form a product from a reactant. Figure 4 shows an example of an isosbestic point.

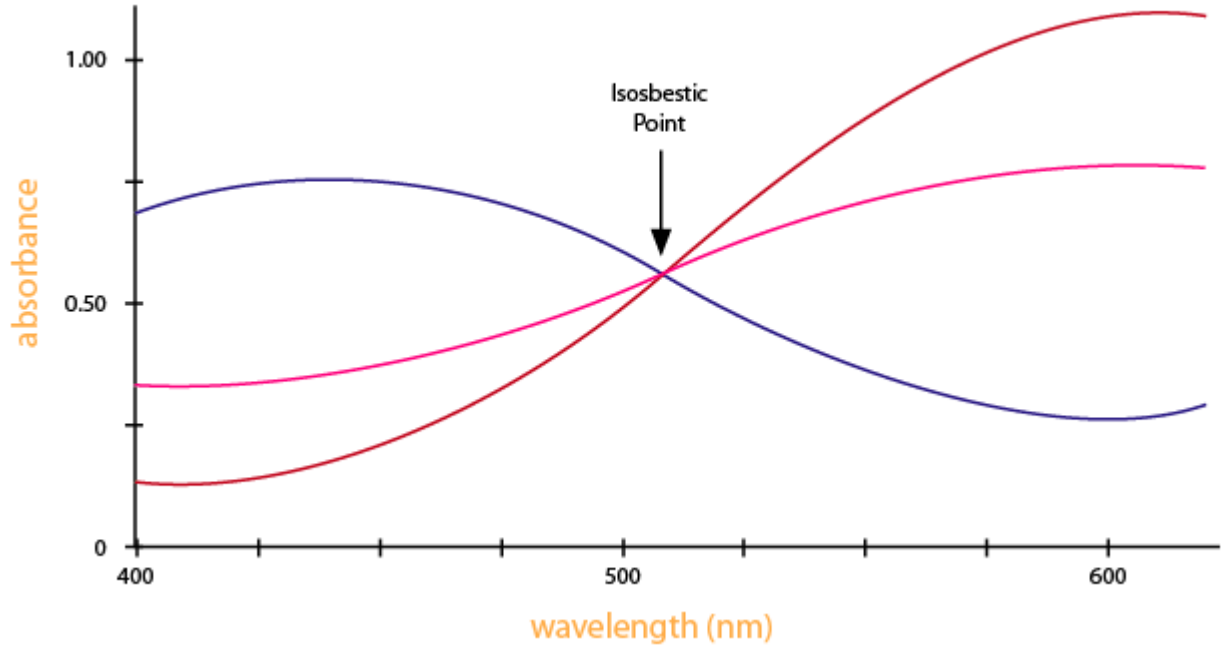


Fig 4. An example of isosbestic point

Referring back to Figure 1 (and Figure 5), the amount of photons that goes through the cuvette and into the detector is dependent on the length of the cuvette and the concentration of the sample. Once you know the intensity of light after it passes through the cuvette, you can relate it to transmittance (T). Transmittance is the fraction of light that passes through the sample. This can be calculated using the equation:

Transmittance(T) = It/Io

Where I_t is the light intensity after the beam of light passes through the cuvette and I_o is the light intensity before the beam of light passes through the cuvette. Transmittance is related to absorption by the expression:

Absorbance(A) = −log(T) = −log(It/Io)

Where absorbance stands for the amount of photons that is absorbed. With the amount of absorbance known from the above equation, you can determine the unknown concentration of the sample by using Beer-Lambert Law. Figure 5

illustrates transmittance of light through a sample. The length l is used for Beer-Lambert Law described below.

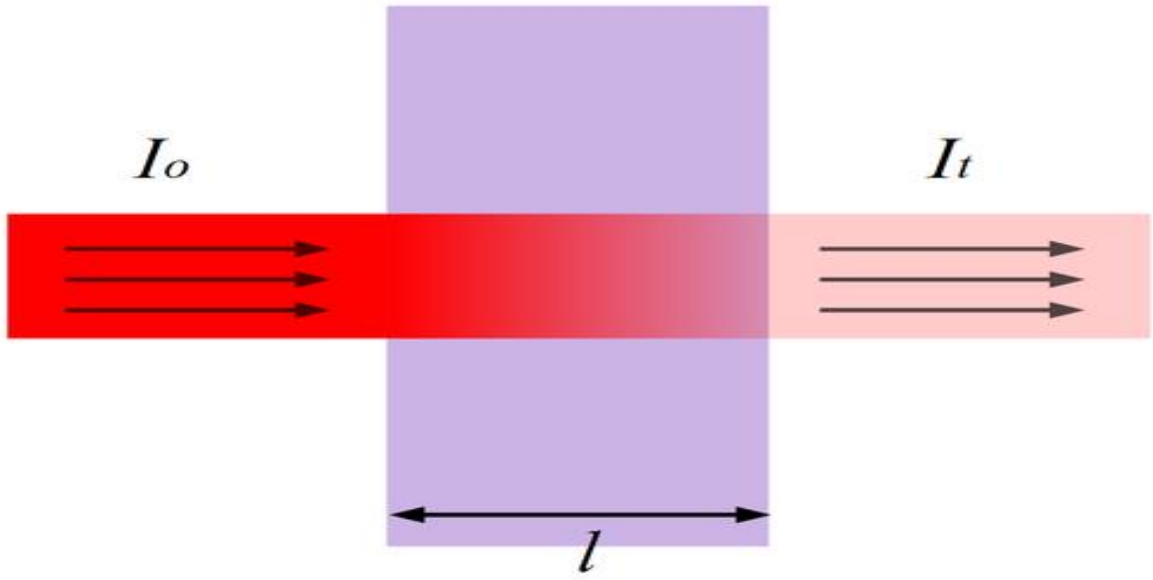


Fig. 5 Transmittance

Beer-Lambert Law

Beer-Lambert Law (also known as Beer's Law) states that there is a linear relationship between the absorbance and the concentration of a sample. For this reason, Beer's Law can *only* be applied when there is a linear relationship. Beer's Law is written as:

$A=\epsilon lc$

where

- A is the measure of absorbance (no units),
- ϵ is the molar extinction coefficient or molar absorptivity (or absorption coefficient),
- l is the path length, and
- c is the concentration.

The molar extinction coefficient is given as a constant and varies for each molecule. Since absorbance does not carry any units, the units for ϵ must cancel out the units of length and concentration. As a result, ϵ has the units: $L \cdot mol^{-1} \cdot cm^{-1}$. The path length is measured in centimeters. Because a standard spectrometer uses a cuvette that is 1 cm in width, l is always assumed to equal 1 cm. Since

absorption, ϵ, and path length are known, we can calculate the concentration c of the sample.

EXPERIMENT: 4

Many biological applications that use cells, such as microbiology, cell culture, and blood work, require that we determine cell concentration for our experiments.

Cell counting is actually quite straightforward and requires a counting chamber called a hemocytometer, a device invented by the 19th-century French anatomist Louis-Charles Malassez to perform blood cell counts.

A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle. The grid has specified dimensions so that the area covered by the lines is known, which makes it possible to count the number of cells in a specific volume of solution.

The most common type of hemocytometer has an "H" shape engraved in the middle that encloses two separate mirror-like polished grid surfaces and provides the coverslip mounting area

Materials

- 3% Acetic Acid with Methylene Blue or Trypan Blue
- 96-well plate or microcentrifuge tube
- Hemocytometer and coverslip
- 70% Ethanol
- Pipettor Protocol

Part I: Sample Preparation

Option 1: Cell Dilution for Total Nucleated Cell Counts with 3% Acetic Acid with Methylene Blue

1. Prepare an appropriate dilution of the well-mixed single-cell suspension using phosphate-buffered saline or serum-free medium. For an accurate representation of concentration, use at least 20 µL of cell suspension to make the dilution.

   Example: Preparing a 10-fold dilution

   a.Add 180 µL of 3% Acetic Acid with Methylene Blue to a

microcentrifuge tube or well of a 96-well plate.

b.Mix the cell suspension and add 20 µL to the tube or well containing 3% Acetic Acid with Methylene Blue.

Option 2: Cell Dilution for Viable Cell Counts by Trypan Blue Dye Exclusion

1. Ensure that the cell suspension to be counted is completely resuspended. Before the cells settle, place a suitable volume of a cell suspension (20 - 200 µL) in a centrifuge tube.
2. Add an equal volume of 0.4% Trypan Blue and gently mix.
3. Incubate the mixture at room temperature (15°C – 25°C) for 5 minutes.

Part II: Cell Counting with a Hemocytometer

1. Prepare a hemocytometer by cleaning the chamber surface with 70% ethanol. Wipe dry. Position the coverslip over the chambers.
2. Resuspend the cell mixture and place 10 µL of stained cells into the hemocytometer chamber using a 20 µL pipettor.

Note: Be careful not to move the coverslip. Allow capillary action to draw the sample in.

3. Place the hemocytometer on the stage of a binocular light microscope. Adjust the microscope to 10X magnification and focus on the cells.
4. Using a hand tally counter, count the cells (stained nuclei) in each of the four outside squares of the hemocytometer (Figure 1A), including cells that lie on the bottom and left-hand perimeters, but not those that lie on the top and right-hand perimeters (Figure 1B). If 3% Acetic Acid with Methylene Blue was used in Part I, count the stained cell nuclei. If Trypan Blue was used in Part I, count the unstained viable cells.

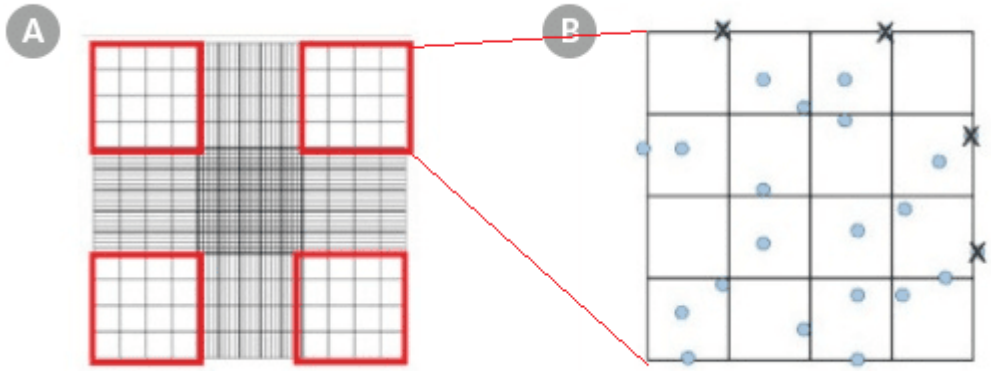


Figure 1. Hemocytometer Gridlines
Hemocytometer diagram indicating the (A) four sets in red and (B) the 16 squares within one of the sets that should be used for counting.

Note: The appropriate dilution factor will depend on the approximate number of cells present in the starting sample but should result in a cell concentration that gives 50 - 100 cells per square (i.e. large or major square) in the hemocytometer. If there are more or fewer than approximately 100 cells per major square on the hemocytometer, prepare a new diluted sample using a greater or smaller dilution factor.

5. Each of the nine major squares of the hemocytometer represents a total volume of 0.1 mm^3. Since 1 cm^3 is equivalent to 1 mL, the cell concentration can be determined using the following equation:

Total number of nucleated cells/mL = average cell count per square x dilution factor x 10^4

Example: If the cell counts for each of the four outer squares were 21, 15, 20, and 17 at a 100 dilution factor then the average cell count would be (21 + 15 + 20 + 17) ÷ 4 = **18.25**.

Therefore, the total concentration of cells in the original suspension would be: 18.25 x 100 x 10^4 = **18,250,000 cells/mL**

EXPERIMENT: 5

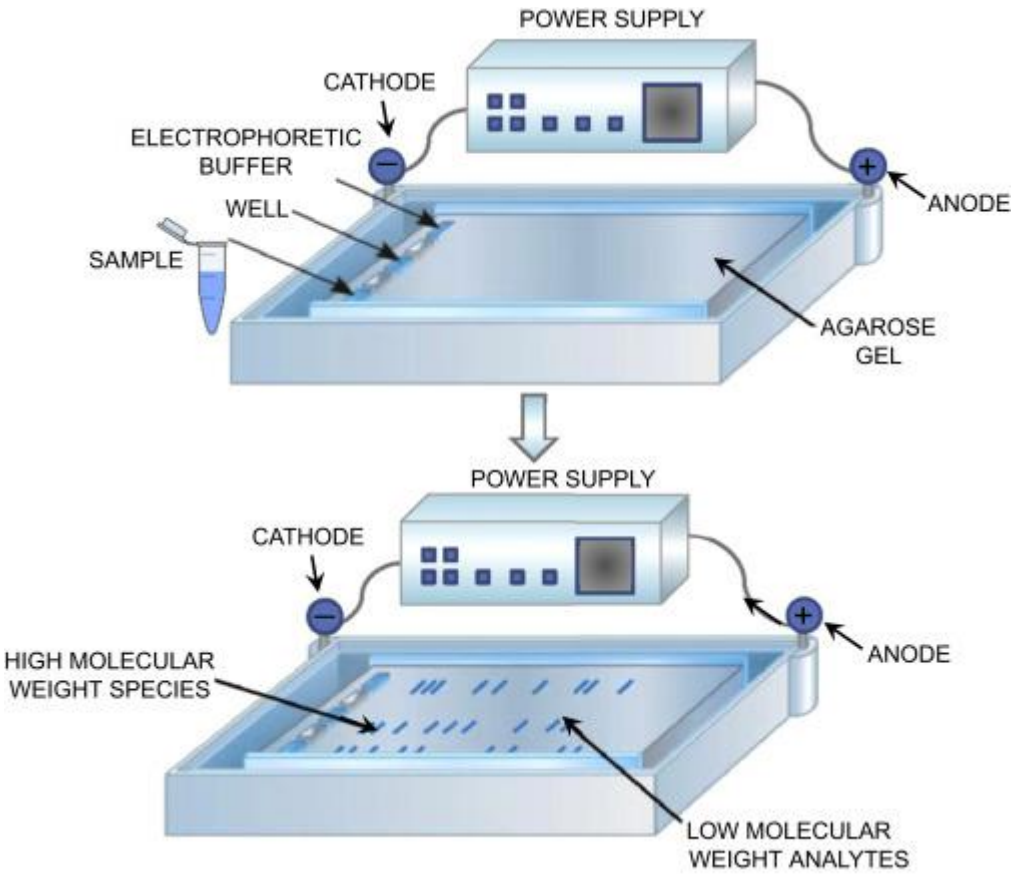


Fig. Agarose Gel Electrophorasis

1. Preparation of the Gel

1. Weigh out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask.

2. Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA) and TBE (45 mM Tris-borate, 1 mM EDTA).

3. Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove

the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.

4. Add ethidium bromide (EtBr) to a concentration of 0.5 μg/ml. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/ml EtBr for 15-30 min, followed by destaining in running buffer for an equal length of time. Note: EtBr is a suspected carcinogen and must be properly disposed of per institution regulations. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however EtBr remains the most popular one due to its sensitivity and cost.

5. Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath. Failure to do so will warp the gel tray.

6. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.

7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use (Fig. 1).

2. Setting up of Gel Apparatus and Separation of DNA Fragments

1. Add loading dye to the DNA samples to be separated (Fig. 2). Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.

2. Program the power supply to desired voltage (1-5V/cm between electrodes).

3. Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.

4. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.

5. Remove the lid. Slowly and carefully load the DNA sample(s) into the gel (Fig. 3). An appropriate DNA size marker should always be loaded along with experimental samples.

6. Replace the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Double check that the electrodes are plugged into the correct slots in the power supply.

7. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

3. Observing Separated DNA fragments

1. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.

2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.

3. Remove the gel from the gel tray and expose the gel to uv light. This is most commonly done using a gel documentation system (Fig. 4). DNA bands should show up as orange fluorescent bands. Take a picture of the gel (Fig. 5).

4. Properly dispose of the gel and running buffer per institution regulations.

4. Representative Results

Figure 5 represents a typical result after agarose gel electrophoresis of PCR products. After separation, the resulting DNA fragments are visible as clearly defined bands. The DNA standard or ladder should be separated to a degree that allows for the useful determination of the sizes of sample bands. In the example shown, DNA fragments of 765 bp, 880 bp and 1022 bp are separated on a 1.5% agarose gel along with a 2-log DNA ladder.

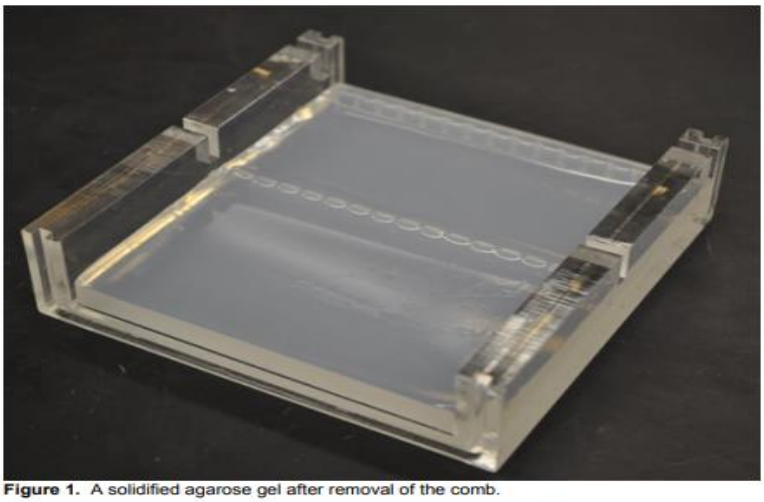


Figure 1. A solidified agarose gel after removal of the comb.

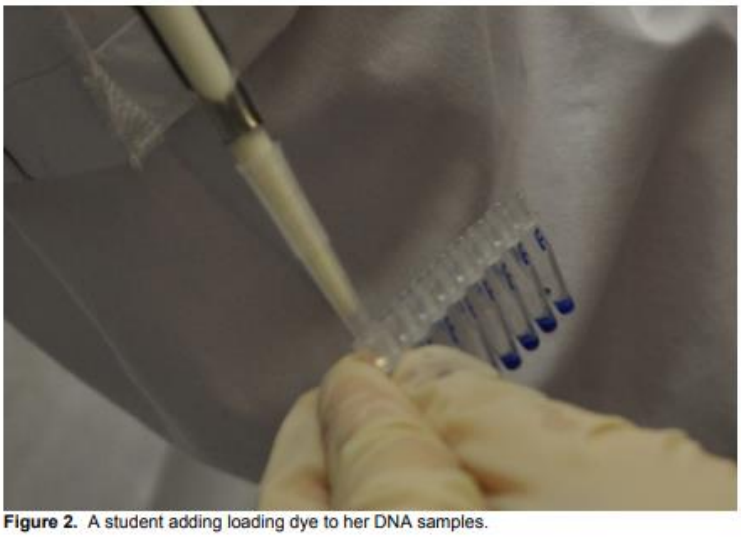


Figure 2. A student adding loading dye to her DNA samples.

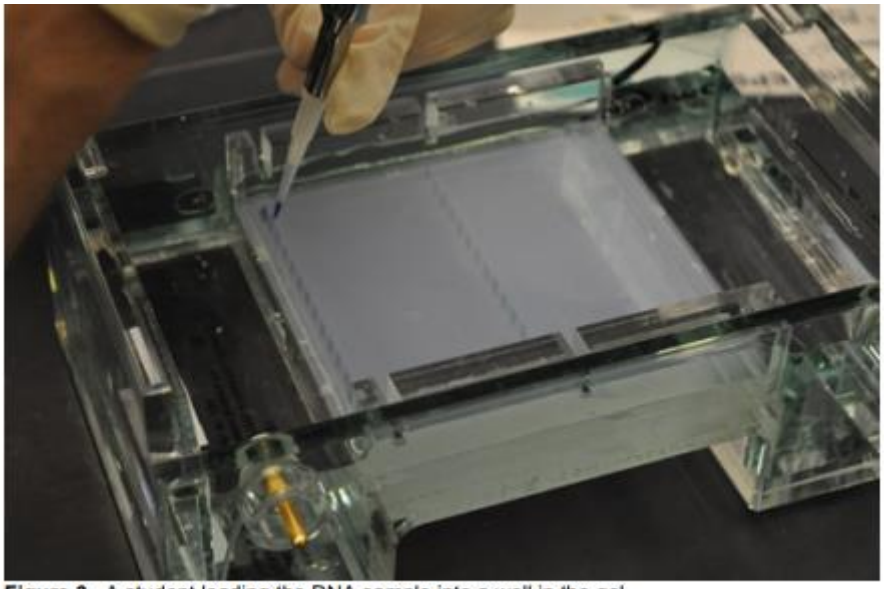


Figure 3. A student loading the DNA sample into a well in the gel.

Figure 4. An example of a gel documentation system.

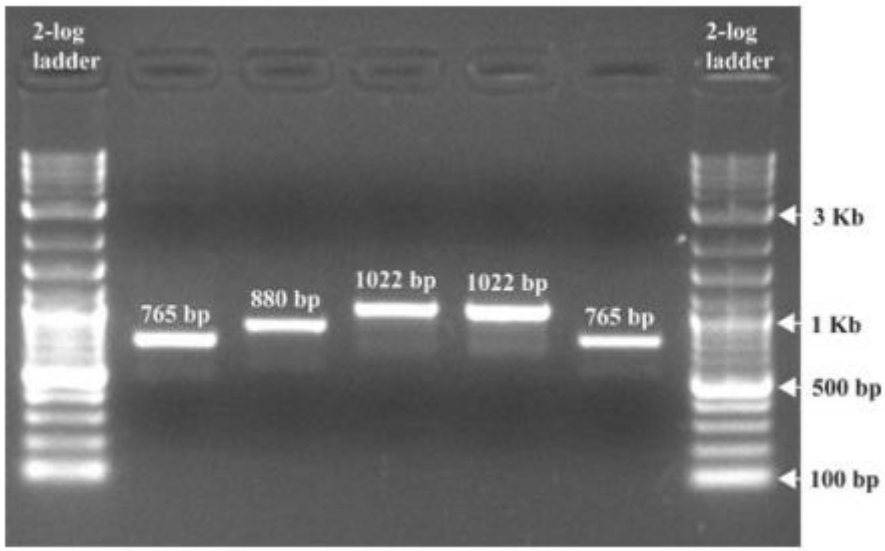


Figure 5. An image of a gel post electrophoresis. EtBr was added to the gel before electrophoresis to a final concentration of 0.5 μg/ml, followed by separation at 100 V for 1 hour. The gel was exposed to uv light and the picture taken with a gel documentation system.

EXPERIMENT: 8

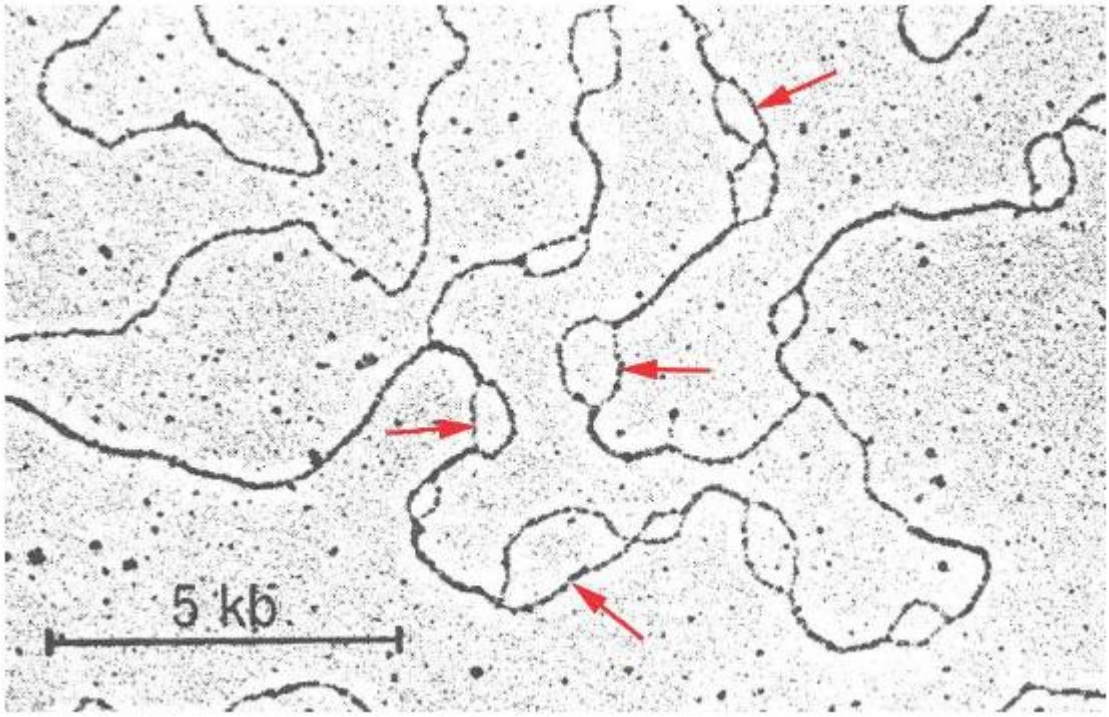


Fig: Electron micrograph showing DNA replication in eukaryotes (*Fritensky, B. and Brien, N*)

An origin of replication (ori) is a site at which DNA replication begins. Origins of replication are often AT rich sequences, which allows them to be more easily pulled apart than GC rich sequences. Without an origin of replication, DNA cannot be replicated and will not be passed on to the next generation.

Eukaryotic chromosomes contain numerous replication origins, allowing for DNA synthesis to proceed in parallel at many sites simultaneously. Each pair of replication forks is referred to as a "replicon". Replicons can be visualized as bubbles of replicated DNA that expand in both directions. Eventually, all replicons merge into a single large bubble, until replication terminates at the telomeres.

The electron micrograph at right shows several replicons, each originating from a different origin (arrows).

Each replicon has two replication forks, moving in opposite directions. Ultimately, replication forks meet, until replication of each template strand is complete.

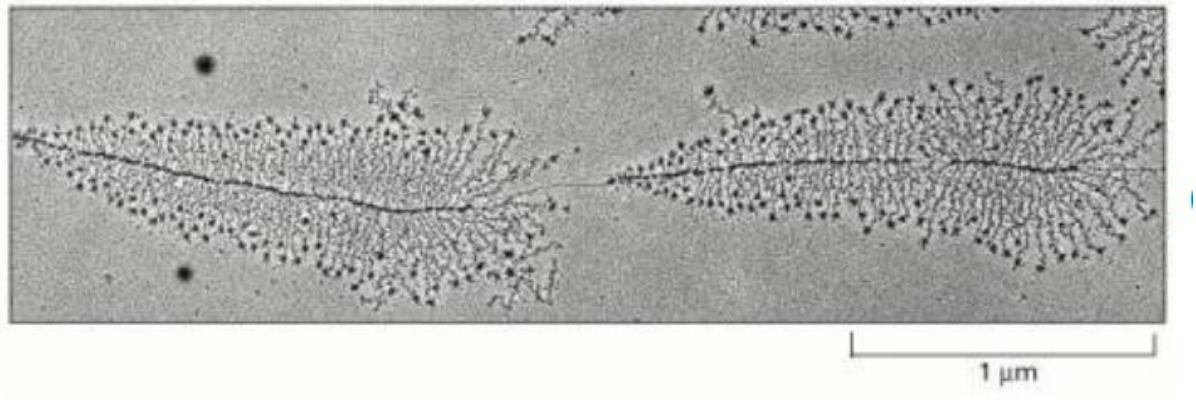


Fig: Electron micrograph of transcription

Transcription of two genes as observed under the electron microscope. Molecules of RNA polymerase are visible as a series of dots along the DNA with the newly synthesized transcripts (fine threads) attached to them. From the lengths of the newly synthesized transcripts, it can be deduced that the RNA polymerase molecules are transcribing from left to right. Adapted from (Miller and Beatty, 1969).

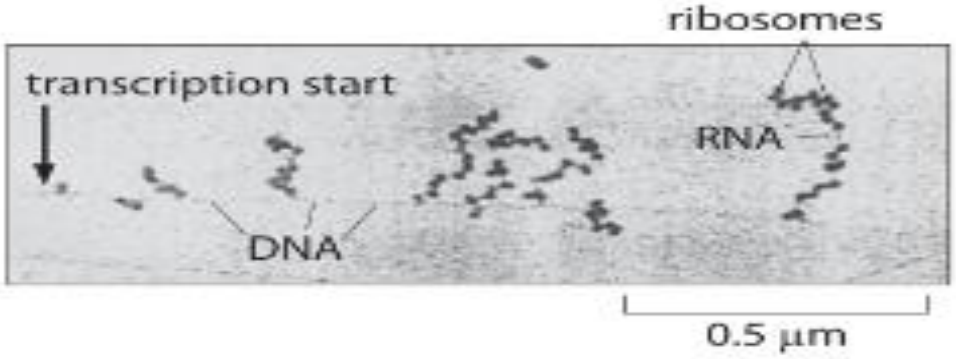


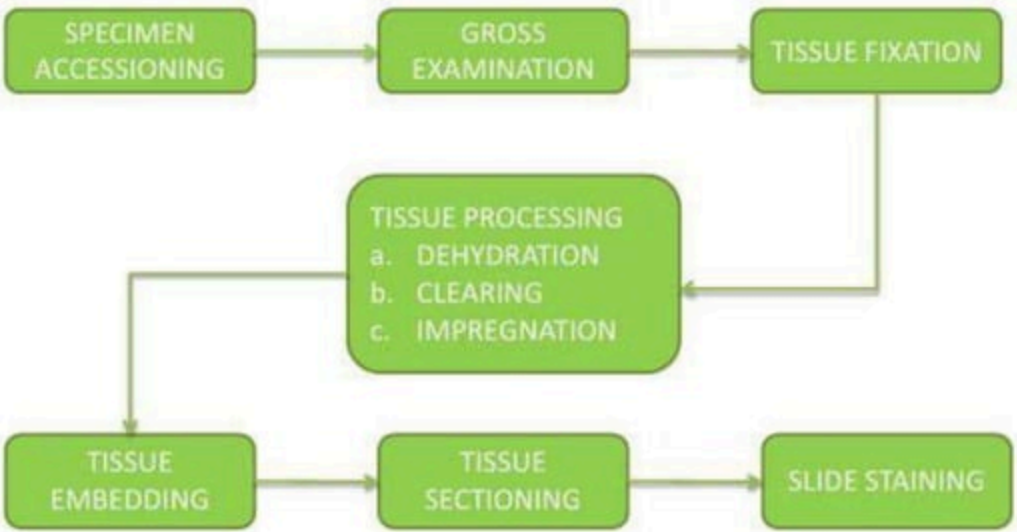
Fig: Electron microscopy image of simultaneous transcription and translation. The image shows bacterial DNA and its associated mRNA transcripts, each of which is occupied by ribosomes. (Adapted from O. L. Miller et al., Science 169:392, 1970.)

EXPERIMENT: 6

DEFINITIONS

- **Histology:** Study of normal tissue at microscopic level
- **Histopathology:** Examination of tissues for presence or absence of changes in their structure due to disease processes.

HISTOLOGY PROCEDURE



Fixation

Method of preserving cells and tissues from decomposition in life-like conditions as far as possible.

The aim of fixation is:

1. To preserve the tissue in as life like manner as possible.
2. To prevent postmortem changes like autolysis and putrefaction.
3. To preserve cells of chemical compounds so that further histochemistry is possible (cell insensitive to hypotonic or hypertonic solutions)
4. To harden the specimen so that easy manipulation of soft tissues is possible
5. Acts as mordant and induces optical contrast

IDEAL FIXATIVE

i. It should be cheap and easily available.
ii. It should be stable and safe to handle.
iii. It should be rapid in action.
iv. It should cause minimal loss of tissue.
v. It should not bind to the reactive groups in tissue which are meant for dyes.
vi. It should give even penetration.
vii. It should retain normal colour of the tissue.
viii. It should not impart its own colour to the tissue.

- Tissue specimen should be placed in a container with fixative immediately after removal to prevent drying and autolysis
- The tissue should be fully immersed in fixative
- Tissue should be kept for adequate time. No refrigeration is needed.
- Large masses fix optimally if incised or sectioned, although this might compromise assessment of margins.

FORMALIN

- Saturated solution of formaldehyde in gas (40% by w/v)
- 10% formalin is used for routine fixation
- Acts by polymerization of cellular proteins forming methylene bridges

- **Merits of formalin:**

➢ Rapidly penetrates the tissues

➢ Normal colour of tissues is retained

➢ Cheap and easily available

➢ Best fixative for neurological tissue

- Demerits of formalin:

➢Causes excessive hardening of tissues.

➢Causes irritation of skin, mucous membranes and conjunctiva.

➢Leads to formation of formalin pigment in tissues having excessive blood at an acidic pH which can be by treatment of section with alcoholic picric acid.

OTHER FIXATIVES

Glutaraldehyde	Electron microscopy Expensive Penetrates tissues slowly
Bouin's fluid (Picric acid)	Renal & testicular needle biopsies Stains tissue yellow, Glycogen Makes tissues harder & brittle Causes lysis of RBCs
Carnoy's fixative (Alcohol)	Cytological smears and endometrial curettings Good fixative for glycogen Dissolves fat
Osmium tetraoxide	CNS tissues Electron microscopy Good fixative for lipids Imparts black colour to tissues

DEHYDRATION

- Process in which water from cells and tissues is removed – so wax can take subsequent space.
- Passing series ascending grades of alcohol – 70%, 80%, 95% and absolute alcohol
- Alternative of ethyl alcohol – methyl alcohol, isopropyl alcohol or acetone

CLEARING

- process in which alcohol from tissues and cells is removed and replaced by a fluid which is miscible with wax making tissue transparent
- Xylene – commonly used clearing agent
- Other – toluene, benzene(carcinogenic), chloroform, cedar wood (expensive and very viscous)

IMPREGNATION

- Process in which empty spaces in the tissue and cells after removal of water are taken up by paraffin wax
- Hardening of tissue – helps in section cutting
- Done by molten paraffin wax of melting point 54-62°C

TISSUE PROCESSOR

- **12 chambered automated device**
- **10 stations are steel/glass jars and two are thermostatically controlled wax bath**

➢ For *fixation* in formalin: 1 jar.

➢ For *dehydration* in ascending grades of alcohol: 6 jars, one each of 70%, 80%, 90% and 3 for 100%.

➢ For *clearing* in xylene: 3 jars.

➢ For *impregnation* in molten paraffin wax: 2 wax baths.

- Tissues move automatically from one jar to next after scheduled time, usually 1.5 hours in each jar

- **Closed(Vacuum) Tissue Processor:**

➢ Tissues cassettes are placed in a single container

➢ Processing fluids are moved in and out sequentially according to electronically programmed cycle

➢ No hazard of contamination by toxic fumes unlike in open system

EMBEDDING AND BLOCKING

- Embedding is done by molten wax
- Conventionally prepared using metallic L(Leuckhart's) moulds. Plastic moulds are available
- The moulds are placed over smooth surfaced glass tile
- Molten wax is pored into the cavity and allowed to solidify
- If L-moulds are used they are removed while plastic moulds remain with the wax block carrying the tissue piece
- Can be performed in device – embedding center
- After embedding the tissue sections are available in block for microtomy

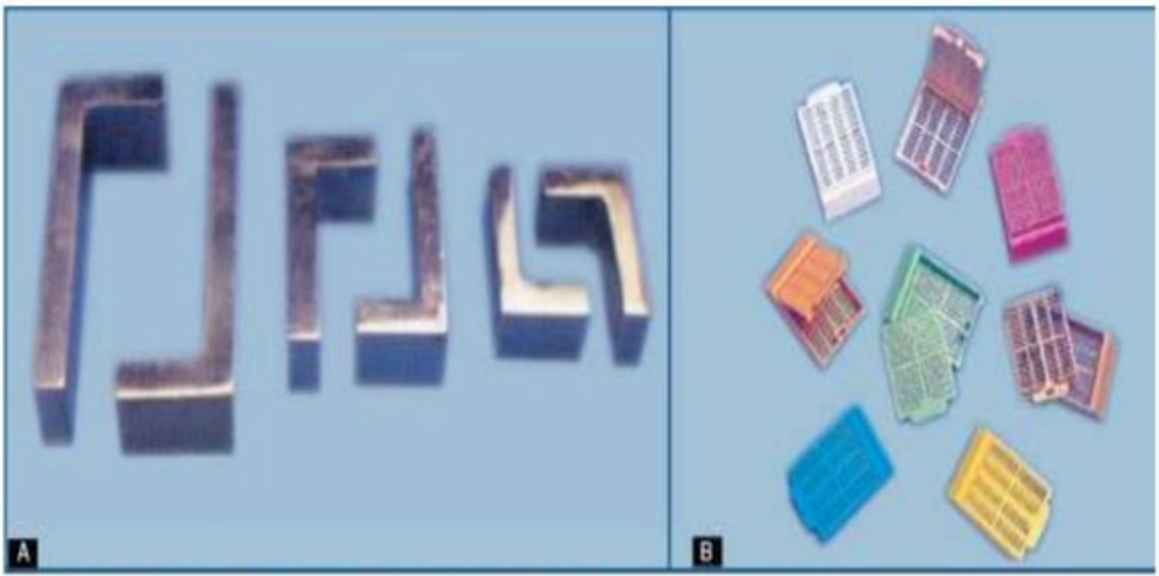


FIGURE 2.3 ♦ A, L (Leuckhart's) metal moulds. B, Plastic block moulds in different colours.

MICROTOMY (SECTION CUTTING)

- Microtome – equipment for cutting section
- 5 types
  - Rotary (Commonly Used)
  - Sliding
  - Freezing
  - Rocking
  - Base-sledge

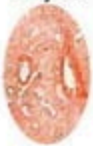
- Cut section by adjusting thickness 3-4 µm and picked from knife by help of forceps and camel hair brush
- Water bath at 40-45°C then place on clean slide at 56°C for 20-30 mins → fishing
- Coating adhesives for section like egg albumin and gelatin can be used before hematoxylin and eosin stain

STAINING

- Sections are deparaffinised by placing in jar of xylene for 10-15 minutes
- Sections are then rehydrated by passing through series of descending grades of alcohol and finally to water
- Then, place slide in haematoxylin for 8-10 minutes followed by ringing with water and differentiation (excess dye removed by putting in 1% acid alcohol for 10 seconds)

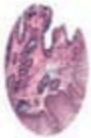
- Blueing is done by putting section in sodium bicarbonate and magnesium sulphate or saturated solution of lithium carbonate for 2-10 minutes
- Counterstain with 1% aqueous eosin for 0.5-1 minute and dip in tap water
- Sections are then dehydrated passing through a series of ascending grades of alcohol and finally cleared in xylene, 2-3 dips in each solution
- Mount in DPX (Dextrene Polystyrene Xylene)

Amyloid



Congo Red
Cat # 24614

Carbohydrates



Alcian Blue/ PAS
Cat # 25086

PAS
Cat # 24200

Rapid Mucin
Cat # 24208

Neuronal Tissue

Cresyl Violet
Cat # 21083

Bielschowsky
Cat # 25994

Luxol® Fast Blue
Cat # 24611

Triglycerides & Lipids

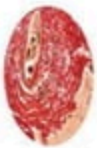
Oil Red O
Cat # 25962

Reticulin Fibers

Reticulin
Cat # 25094

Jones PAS-M
Cat # 25091

Connective Muscle Tissue



Picrosirius Red
Cat # 24901

Verhoeff Van Gieson
Cat # 25089

Gomori's Trichrome
Cat # 24205

Masson's Trichrome
Cat # 25088

Rapid PTAH
Cat # 25715

Pigment, Minerals & Granules

Prussian Blue Iron
Cat # 24199

Fontana Masson
Cat # 25104

Von Kossa Method
Cat # 24633

Villanueva Osteochrome Bone
Cat # 16280

Microorganisms

AFB Kinyuon
Cat # 25765

AFB Ziehl-Neelson
Cat # 24669

Auramine O
Cat # 24665

Differential Quik
Cat # 24606

Fungi-Fluor®
Cat # 17442

Grocott Methenamine Silver
Cat # 25482

TB Fluorostain
Cat # 22422

Warthin-Starry
Cat # 25093

Gram's Stain
Cat # 24668

EXPERIMENT: 7

ELISA

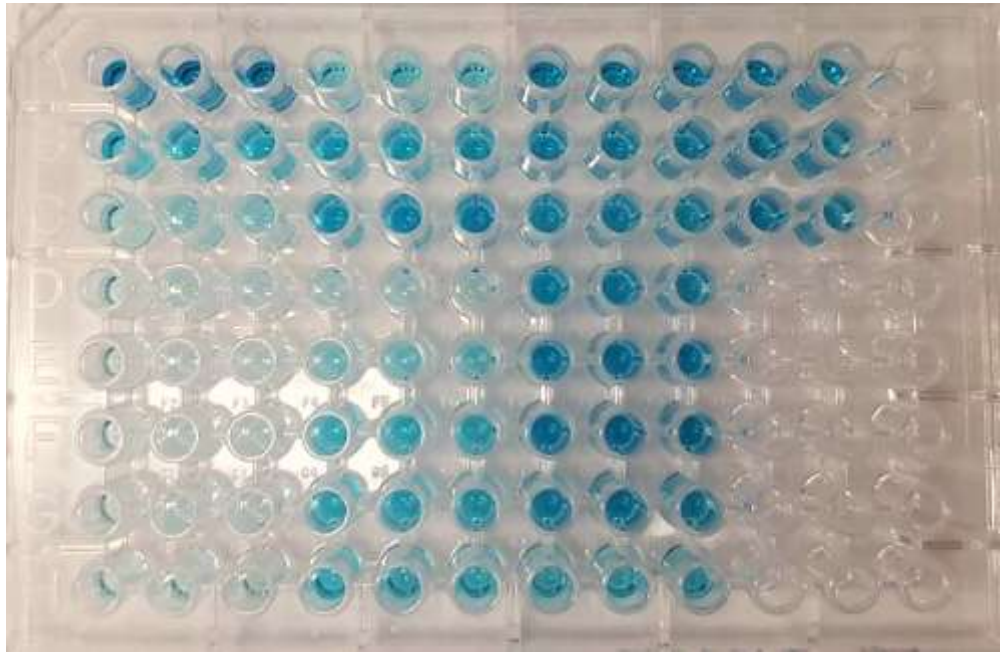
Enzyme Linked Immunosorbant Assay

- **ELISA is a plate based assay technique which is used for detecting and quantifying substances such as peptides, proteins, antibodies and hormones.**

- **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 alkaline phosphatase, horse radish peroxidase and beta galactosidase.**

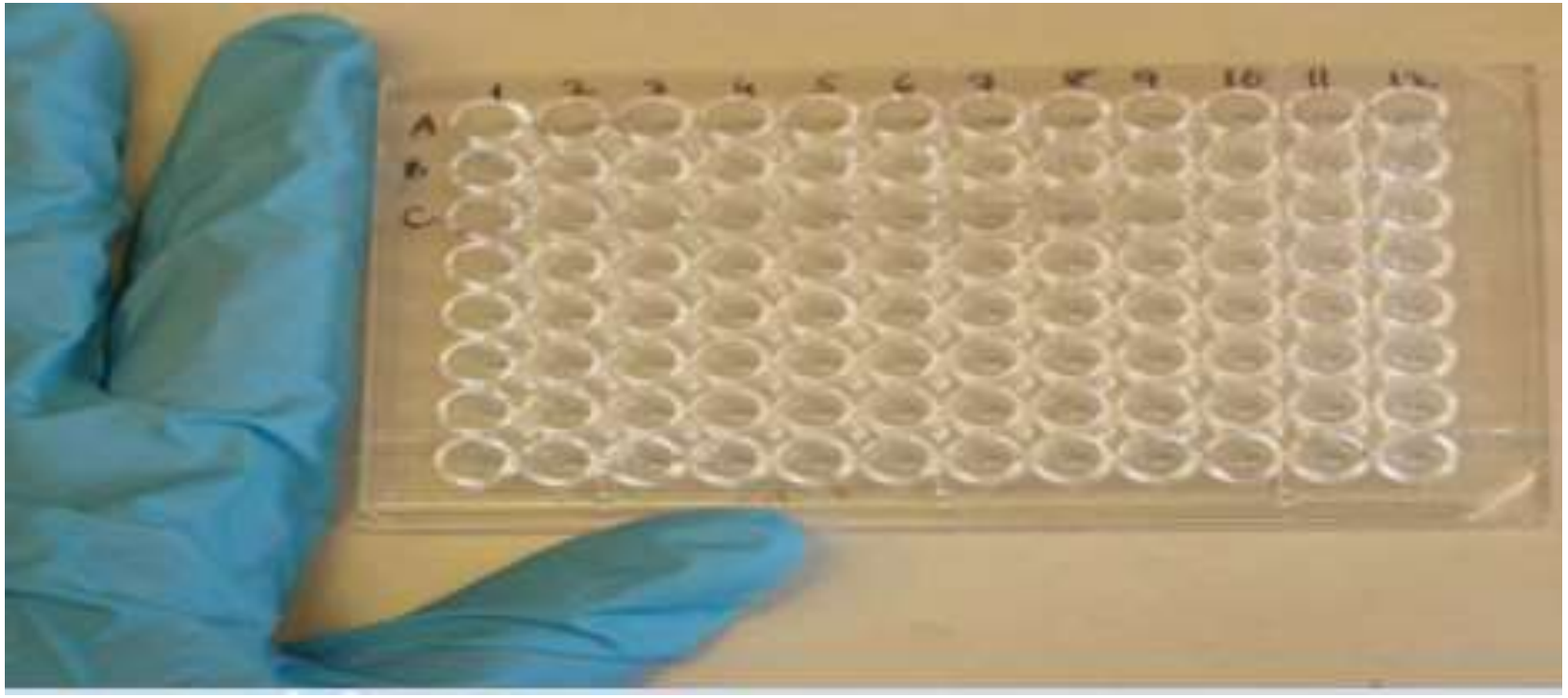
- **Specific substrate such as ortho-phenyldiamine dihydrochloride (for peroxidase), paranitrophenyl phosphate (for alkaline phosphatase) are used which are hydrolysed by above enzymes to give colored end product.**



Basic terms

Solid phase:

- **Usually a microtitre plate well having 8*12 well format**



Adsorption:

The process of adding an antigen/antibody, diluted in buffer , so it attaches to the solid phase on incubation.

Washing:

The flooding or emptying the wells with a buffered solution to separate bound from unbound reagents in ELISA

Antigen:

Any molecule that elicits the production of antibodies when introduced into body.

Antibodies:

Proteins produced in response to antigenic stimuli.

Enzyme conjugate:

An enzyme that is attached irreversibly to an antibody.

Chromogen:

A chemical alters color as a result of an enzyme interaction with substrate

Stopping:

The process of stopping the action of the enzyme on substrate

Reading:

Spectrophotometric measurement of color developed in ELISA

Principle

- ELISAs are typically performed in 96-well polystyrene plates.
- The serum is incubated in a well, and each well contains a different serum.
- A positive control serum and a negative control serum would be included among the 96 samples being tested. Antibodies or antigens present in serum are captured by corresponding antigen or antibody coated on to the solid surface.
- After some time, the plate is washed to remove serum and unbound antibodies or antigens with a series of wash buffer.
- To detect the bound antibodies or antigens, a secondary antibodies that are attached to an enzyme such as peroxidase or alkaline phosphatase are added to each well.
-
- After an incubation period, the unbound secondary antibodies are washed off. When a suitable substrate is added, the enzyme reacts with it to produce a color.
- This color produced is measurable as a function or quantity of antigens or antibodies present in the given sample. The intensity of color/ optical density is measured at 450nm.
- The intensity of the color gives an indication of the amount of antigen or antibody.

Types of ELISA

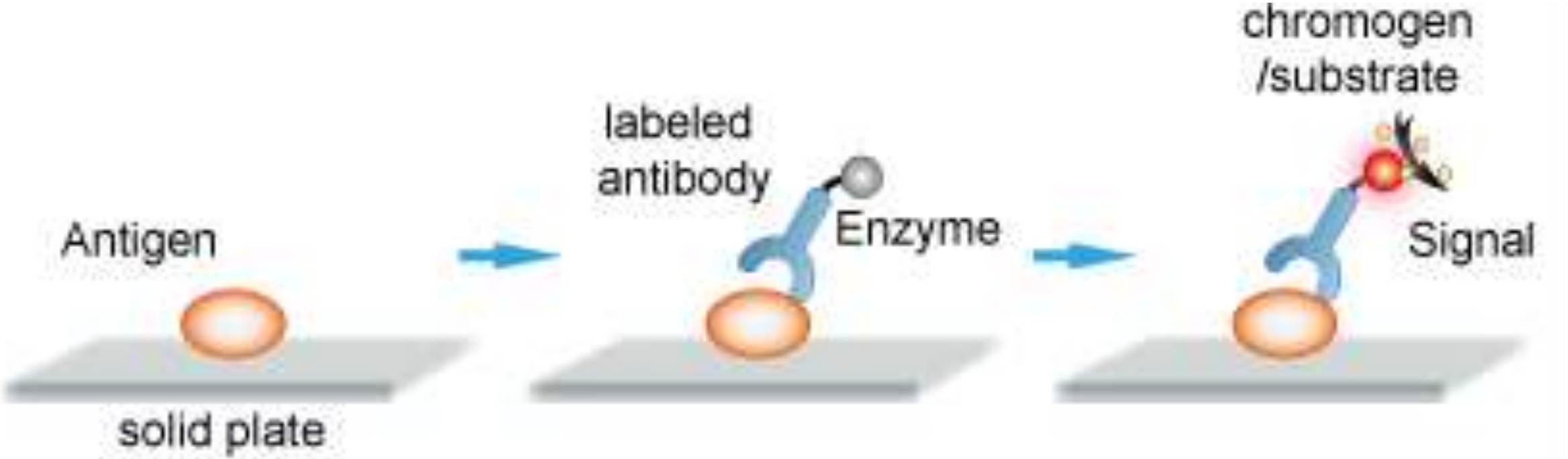
1. Direct ELISA

2. Indirect ELISA

3. Sandwich ELISA

4. Competitive ELISA

1. Direct ELISA

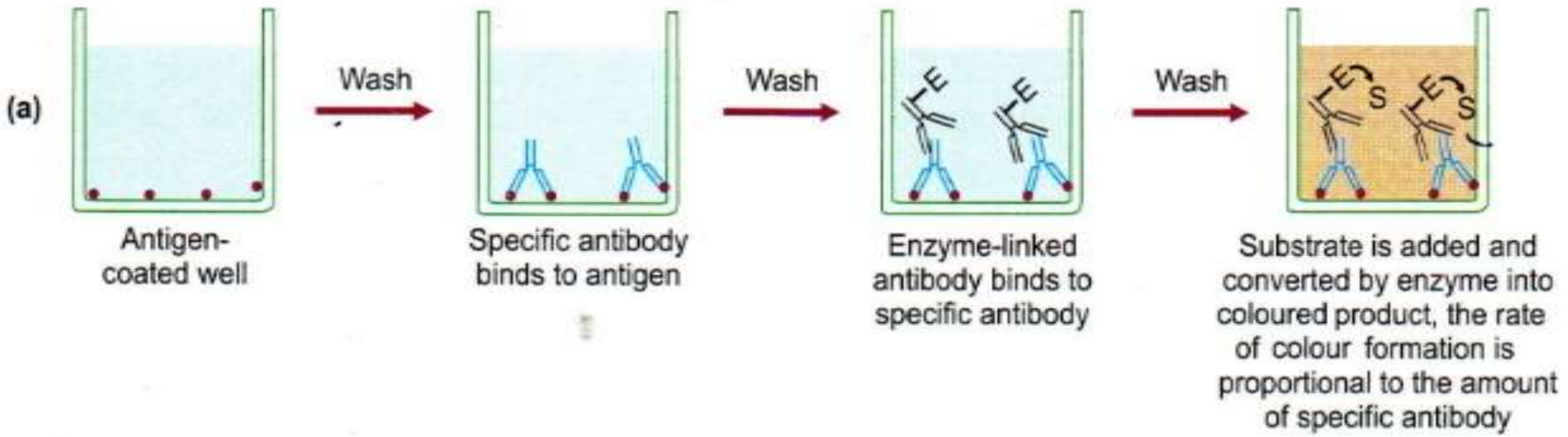


1. Antigen is coated onto wells by passive adsroption
2. Antibody conjugated with enzyme is added and incubated with antigen.and incubation.
3. Substrate / chromophore is added and colour develops.

2. Indirect ELISA

- **Antibody can be detected or quantitatively determined by indirect ELISA.**
- In this technique, antigen is coated on the microtiter well. Serum or some other sample containing primary antibody is added to the microtiter well and allowed to react with the coated antigen.
- Any free primary antibody is washed away and the bound antibody to the antigen is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody.
- Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added.
- Enzyme hydrolyzes the substrate to form colored products.
- The amount of colored end product is measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.

INDIRECT ELISA

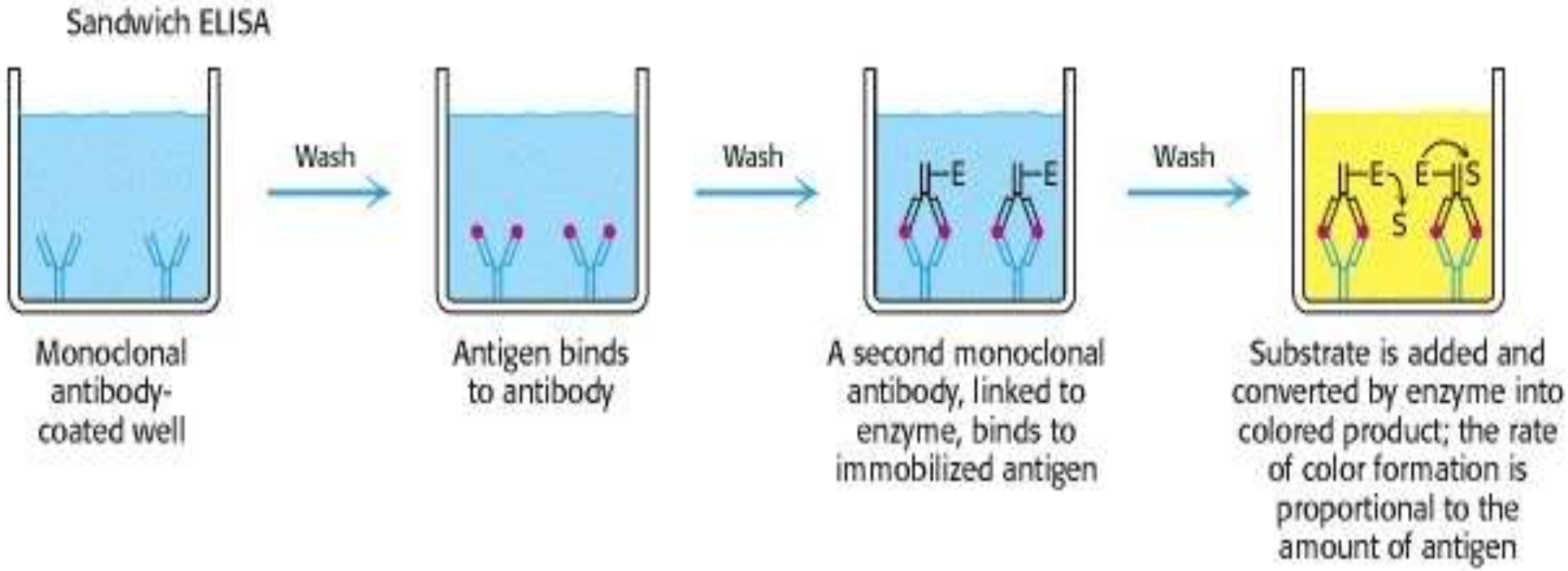


Procedure of Indirect ELISA

1.Coat the micro titer plate wells with antigen.
2.Block all unbound sites to prevent false positive results.
3.Add sample containing antibody (e.g. rabbit monoclonal antibody) to the wells and incubate the plate at 37°c.
4.Wash the plate, so that unbound antibody is removed.
5.Add secondary antibody conjugated to an enzyme (e.g. anti- mouse IgG).
6.Wash the plate, so that unbound enzyme-linked antibodies are removed.
7.Add substrate which is converted by the enzyme to produce a colored product.
8.Reaction of a substrate with the enzyme to produce a colored product.

3. Sandwich ELISA

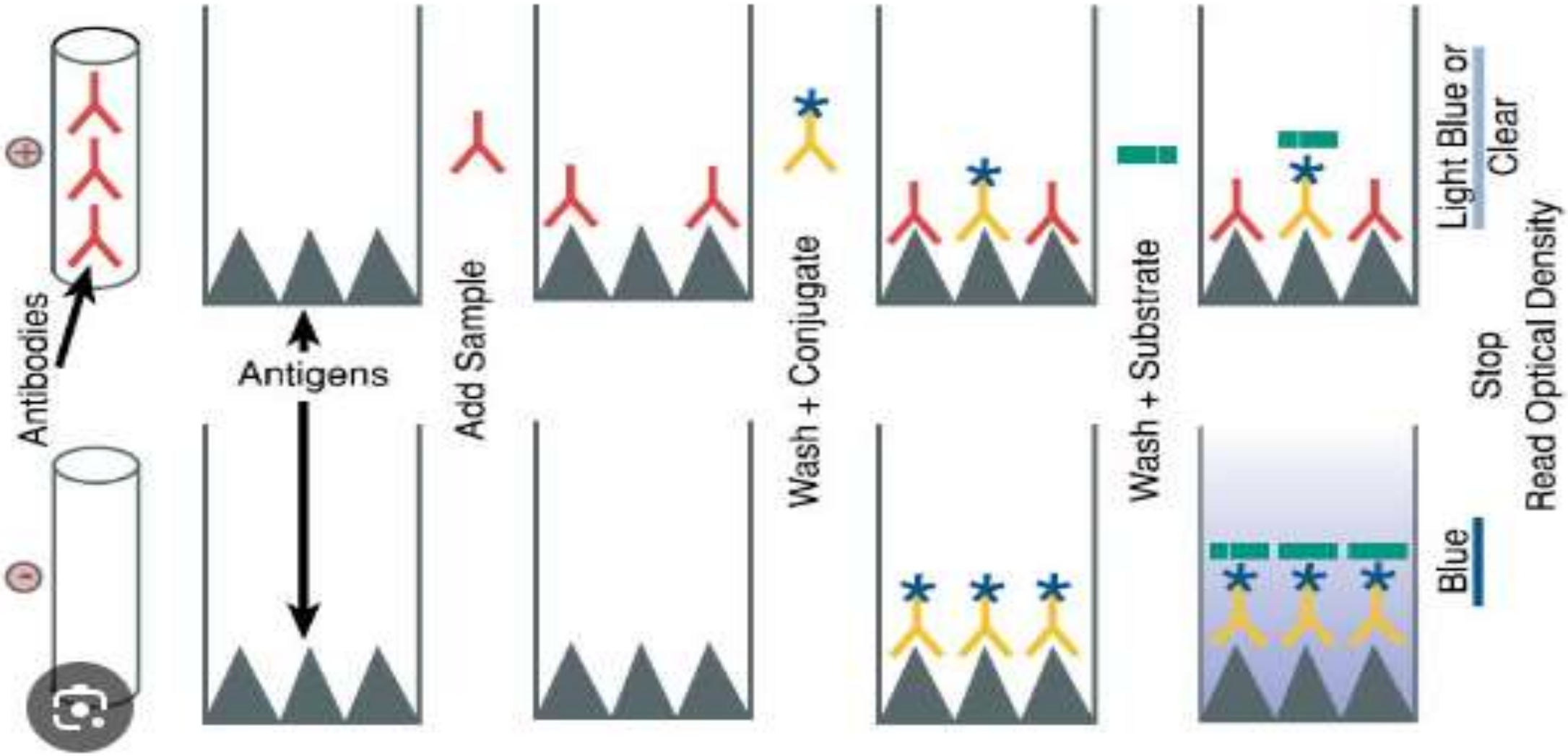
- Antigen can be detected by sandwich ELISA.
- In this technique, antibody is coated on the microtiter well.
- A sample containing antigen is added to the well and allowed to react with the antibody attached to the well, forming antigen-antibody complex.
- After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen.
- Then after unbound secondary antibody is removed by washing.
- Finally substrate is added to the plate which is hydrolyzed by enzyme to form colored products.

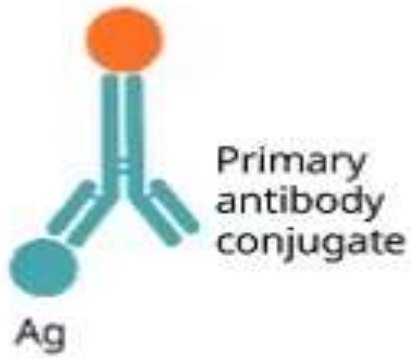


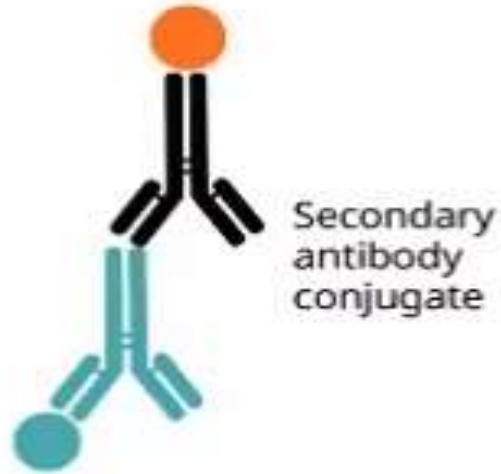
Procedure of sandwich ELISA

1.Prepare a surface to which a known quantity of antibody is bound.
2.Add the antigen-containing sample to the plate and incubate the plate at 37°c.
3.Wash the plate, so that unbound antigen is removed.
4.Add the enzyme-linked antibodies which are also specific to the antigen and then incubate at 37°c.
5.Wash the plate, so that unbound enzyme-linked antibodies are removed.
6.Add substrate which is converted by the enzyme to produce a colored product.
7.Reaction of a substrate with the enzyme to produce a colored product.

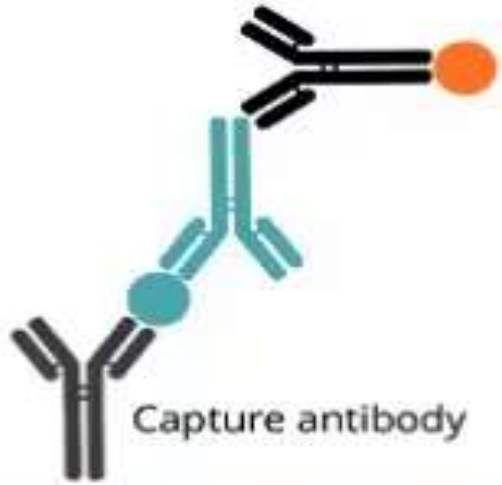
4. Competitive ELISA

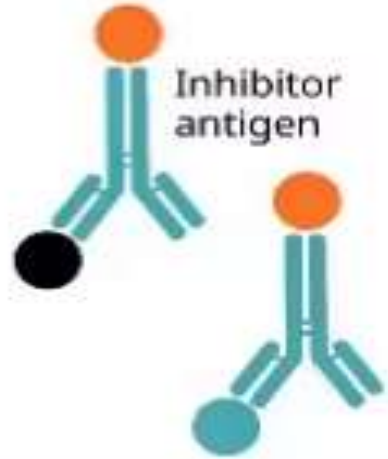






Direct ELISA | **Indirect ELISA**





Sandwich ELISA | **Competitive ELISA**