# BACHELOR OF MEDICAL MICROBIOLOGY

2nd Semester

Prepared By

**Paramedical & Allied Science Dept.**

BMM

# MIDNAPORE CITY COLLEGE

# BMM 2nd Semester Lab Manual

# Analytical Biochemistry

# Code: BMM 295

# Demonstration of analytical instruments

# (principles and applications)

## Beer-Lambert's Law

The Beer–Lambert law, also known as Beer's law, the Lambert–Beer law, or the Beer–Lambert–Bouguer law relates the attenuation of light to the properties of the material through which the light is travelling. The law is commonly applied to chemical analysis measurements and used in understanding attenuation in physical optics, for photons, neutrons, or rarefied gases. In mathematical physics, this law arises as a solution of the BGK equation.

**Mathematical formulation:**

A common and practical expression of the Beer–Lambert law relates the optical attenuation of a physical material containing a single attenuating species of uniform concentration to the optical path length through the sample and absorptivity of the species. This expression is:

$A = \varepsilon lc$

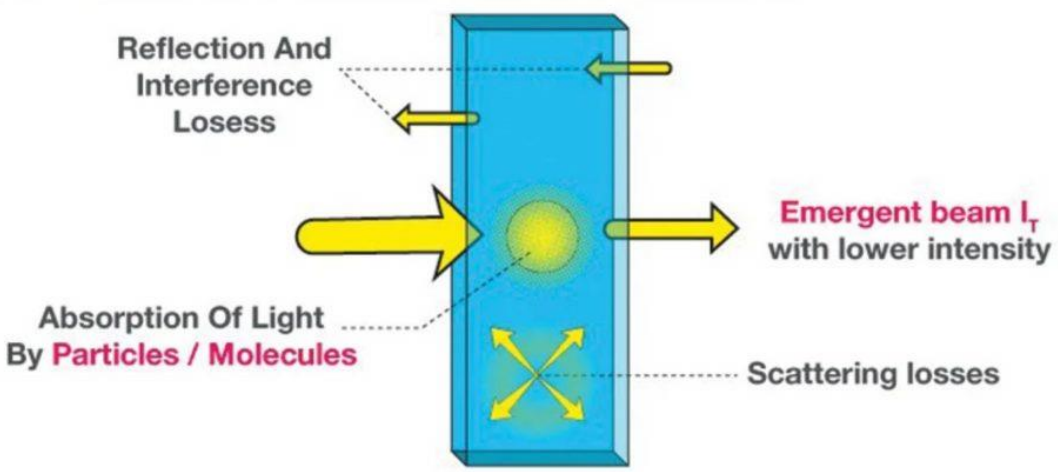
Where

A is the absorbance

ε is the molar attenuation coefficient or absorptivity of the attenuating species

l is the optical path length in cm

c is the concentration of the attenuating species




**Colorimeter:** A colorimeter is an instrument that compares the amount of light getting through a solution with the amount that can get through a sample of pure solvent. A colorimeter contains a photocell which is able to detect the amount of light passing through the solution under investigation.

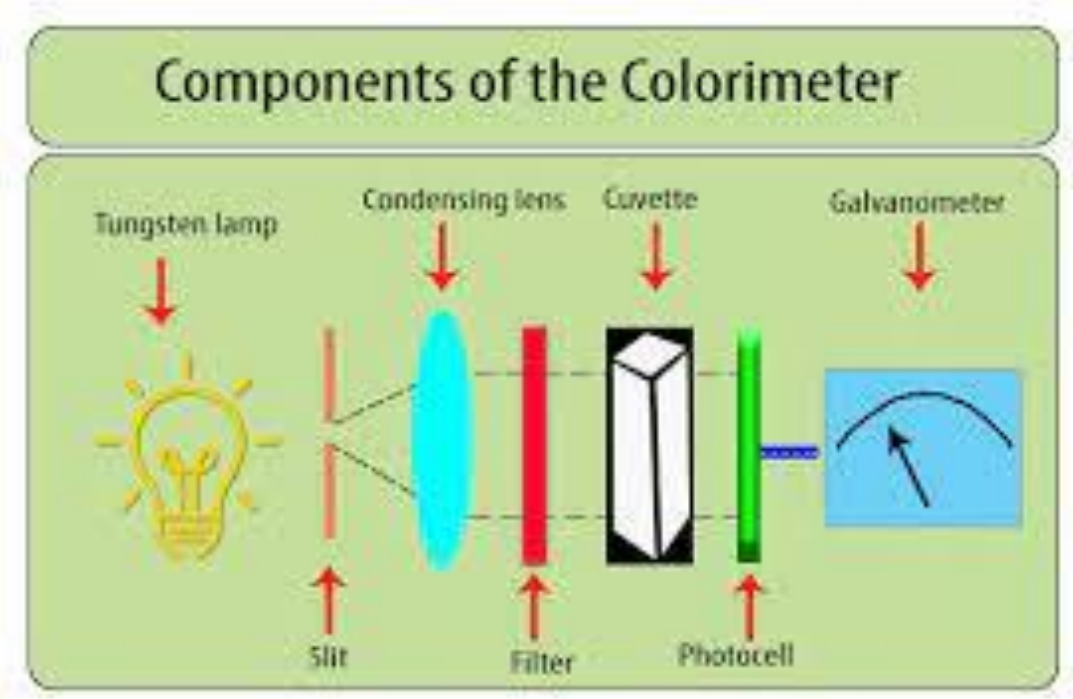
## Principle of colorimeter:

## Beer-lambert's law

- When a monochromatic light passes through a colored solution, that monochromatic light is absorbed by the colored solution, which is depend on –
- ➢ Type of colour
- ➢ Colour density
- ➢ Distance travelled by light.

## Components of colorimeter:

- Light source
- Slit
- Monochromator (Filter)
- Cuvette
- Photocell
- Galvanometer




## Applications of Colorimetry:

- It is used by hospitals as well as laboratories for analysing biochemical samples such as urine, cerebrospinal fluids, plasma, biochemical samples, and serum.
- It is widely used to generate a quantitative estimation of the serum components, proteins, glucose, and various biochemical compounds.
- It is also used in food industries and by manufacturing industries to make textiles and paints. A colorimeter has immense significance in the ever-expanding world of science. Further research is going to enhance its features and functionalities.

## Spectrophotometry

Spectrophotometry is a branch of electromagnetic spectroscopy concerned with the quantitative measurement of the reflection or transmission properties of a material as a
function of wavelength. Spectrophotometry uses photometers, known as spectrophotometers that can measure the intensity of a light beam at different wavelengths. Although spectrophotometry is most commonly applied to ultraviolet, visible, and infrared radiation, modern spectrophotometers can interrogate wide swaths of the electromagnetic spectrum, including x-ray, ultraviolet, visible, infrared, and/or microwave wavelengths.

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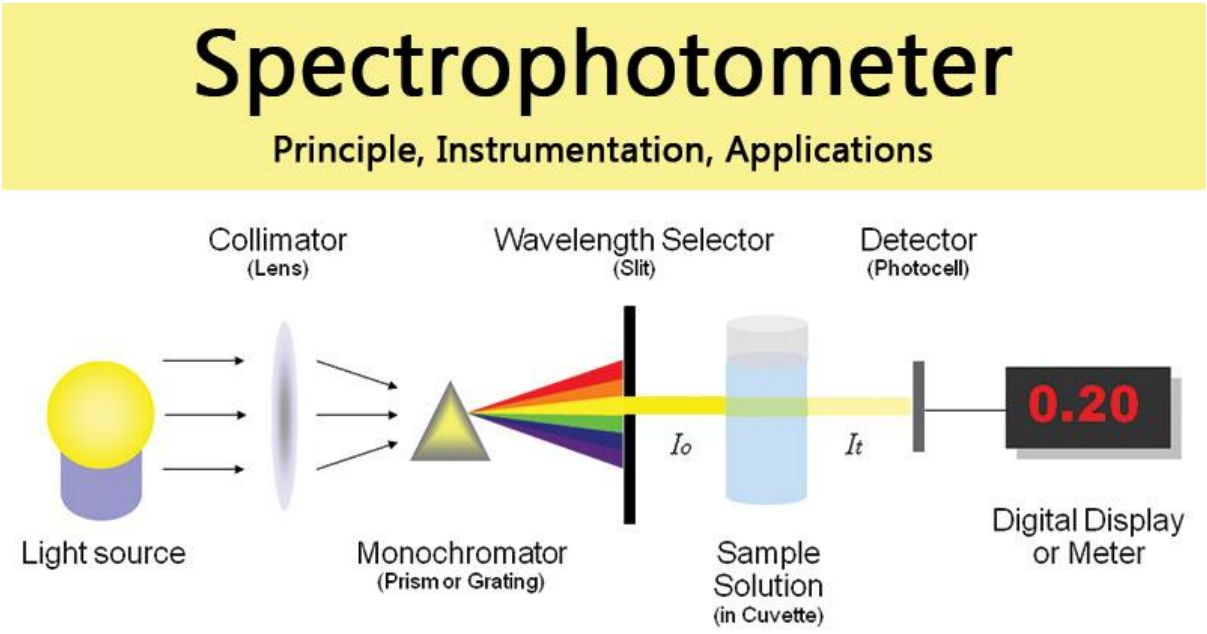
## Principle of Spectrophotometer:

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.
In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths by suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2 nm.

The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

## Instrumentation of Spectrophotometer




## The essential components of spectrophotometer instrumentation include:

## 1. A table and cheap radiant energy source

- Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

**2. A monochromator**, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.

- A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

### Prisms:

A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent.

Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

### Grating:

Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

**3. Transport vessels (cuvettes)**, to hold the sample

- Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as “CUVETTES”.

- Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

### 4. A Photosensitive detector and an associated readout system

- Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.
- Radiation detectors generate electronic signals which are proportional to the transmitter light.
- These signals need to be translated into a form that is easy to interpret.

This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.

### Applications:

- Detection of concentration of substances
- Detection of impurities
- Structure elucidation of organic compounds
- Monitoring dissolved oxygen content in freshwater and marine ecosystems
- Characterization of proteins
- Detection of functional groups

- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds
- The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

# Microscope

## Introduction

The microscope is a valuable instrument. There are many small objects or details of objects which cannot be seen by the unaided human eye. The microscope magnifies the image of such objects thus making them visible to the human eye. Microscopes are used to observe the shape of bacteria, fungi, parasites and host cells in various stained and unstained preparations.

## Types of Microscopy

Microscopes used in clinical practice are light microscopes. They are called light microscopes because they use a beam of light to view specimens.

A compound light microscope is the most common microscope used in microbiology. It consists of two lens systems (combination of lenses) to magnify the image. Each lens has a different magnifying power. A compound light microscope with a single eye-piece is called monocular; one with two eye-pieces is said to be binocular.

Microscopes that use a beam of electrons (instead of a beam of light) and

electromagnets (instead of glass lenses) for focusing are called electron microscopes. These microscopes provide a higher magnification and are used for observing extremely small microorganisms such as viruses.

## Light microscopy

**Brightfield microscopy**

This is the commonly used type of microscope. In brightfield microscopy the field of view is brightly lit so that organisms and other structures are visible against it because of their different densities. It is mainly used with stained preparations. Differential staining may be used depending on the properties of different structures and organisms.

**Darkfield microscopy**

In darkfield microscopy the field of view is dark and the organisms are illuminated. A special condenser is used which causes light to reflect from the specimen at an angle. It is used for observing bacteria such as treponemes (which cause syphilis) and leptospires (which cause leptospirosis).

**Phase-contrast microscopy**

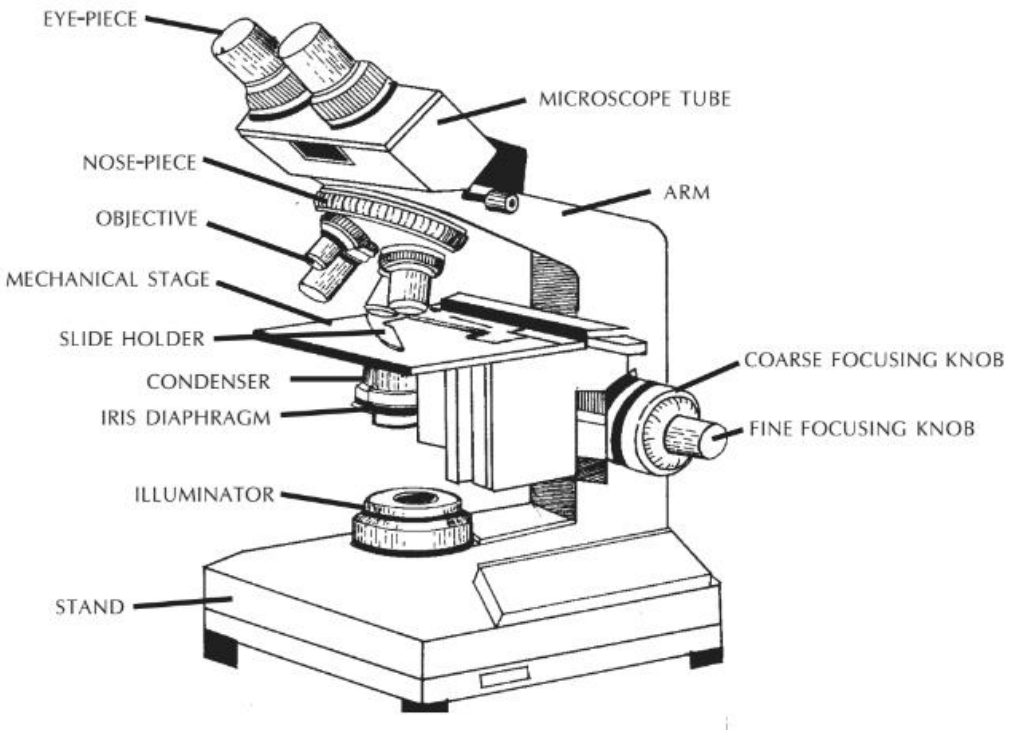
Phase-contrast microscopy allows the examination of live unstained organisms. For phase-contrast microscopy, special condensers and objectives are used. These alter the phase relationships of the light passing through the object and that passing around it.

**Fluorescence microscopy**

In fluorescence microscopy specimens are stained with fluorochromes/fluorochrome complexes. Light of high energy or short wavelengths (from halogen lamps or mercury vapour lamps) is then used to excite molecules within the specimen or dye molecules attached to it. These excited molecules emit light of different wavelengths, often of brilliant colours. Auramine differential staining for acid-fast bacilli is one application of thetechnique; rapid diagnostic kits have been developed using fluorescent antibodies for identifying many pathogens.

**Parts of the Microscope**

The main parts of the microscope are the eye-pieces, microscope tube, nose-piece, objective, mechanical stage, condenser, coarse and fine focusing knobs, and light source.




**Eye-pieces**

- The specimen is viewed through the eye-piece. It has a lens which magnifies the image formed by the objective. The magnifying power of the eye-piece is in the range 5x-20x. A movable pointer may be attached to the inside of the eye-piece.
- In binocular microscopes, the two eye-pieces can be moved closer or farther apart to adjust for the distance between the eyes by pulling pushing motion or by moving a knurled ring.

**Microscope tube**

The microscope tube is attached on top of the arm. It can be of the monocular or binocular type. It supports the eye-piece on the upper end.

**Mechanical tube length**

- Mechanical tube length is the distance between the place where the objective is inserted and the top of the draw-tube into which the eye-pieces fit.
- In modern microscopes it is not tubular; it contains prisms that bend the light coming up, thus providing a comfortable viewing angle. In a binocular tube, the light is also split and sent to both eye-pieces.

**Nose-piece**

The nose-piece is attached under the arm of the microscope tube. The nose-piece houses the objectives and rotates them. The objectives are arranged in sequential order of their magnifying power, from lower to higher. This helps to prevent the immersion oil from getting onto the intermediate objectives.

**Objectives**

The image of the specimen first passes through the objective. Objectives with magnifying powers 4x, 10x, 40x and 100x are commonly used. The magnifying power is marked on the lens and is usually colour-coded for easy identification.

The 100x objective is for oil immersion.

The numerical aperture (NA) is the measure of light-gathering power of a lens. The NA corresponding to the various magnifying powers of the objective is:

| Magnification | Numerical aperture |
|---|---|
| 10x | 0.25 |
| 40x | 0.65 |
| 100x | 1.25 |

A high NA indicates a high resolving power and thus useful magnification.

To provide the best image at high magnification, immersion oil is placed between the slide and the oil immersion objective (100x). Unlike air, immersion oil has the same refractive index as glass. Therefore, it improves the quality of the image. If immersion oil is not used, the image appears blurred or hazy.

**Mechanical stage**

- The mechanical stage holds the slide and allows it to be moved to the left, right, forward or backward by rotating the knobs.
- It is fitted with fine vernier graduations as on a ruler. This helps in relocating a specific field of examination.

**Condenser**

The condenser illuminates the specimen and controls the amount of light and contrast. There are different types of condensers. Some condensers have a rack-and pinion mechanism for up-and-down adjustment.

- The NA of a condenser should be equal to or greater than that of the objective with maximum NA.
- An iris diaphragm is provided below the condenser. This adjusts the NA of the condenser when using objectives having low magnifying power.
- A swing-out type filter holder may be fitted above or under the condenser. In some microscopes the filter holder may not be swing-out type. The filter holder holds detachable filters when required.
- Condenser centring screws, when present, are used to align the condenser with the objective.

- A condenser raising knob may be present (if centring screws are not there), or the distance may be fixed.

**Two-sided mirror**

A mirror is the simplest illuminator. The two-sided mirror provides necessary illumination through reflection of natural or artificial light. It has two surfaces, one plain for artificial light and other concave for natural light. It is supported on two sides by a fork fixed on a mount in a way that permits free rotation.

A mirror is usually fitted on a mount or at the base of the microscope.

**Built-in light sources**

An illuminator is built into the base of the microscope. A halogen bulb provides the best illumination. On top of the illuminator is an in-built filter holder to fit the filter of desired quality.

**Filters**

- Blue filters are used to change the light from ordinary electric bulbs into a more natural white light.
- Neutral density filters are used to reduce brightness without changing the colour of the background.
- Green filters may be useful in some situations.

**Immersion oil**

- Immersion oil must be used with objectives having NA more than 1.0. This increases the resolving power of the objective.
- An immersion oil of medium viscosity and refractive index of 1.5 is adequate. Any synthetic non-drying oil with a refractive index of 1.5 and/or as recommended by the manufacturer should be used.

Cedar wood oil should not be used as it leaves a sticky residue on the objective. If cedar wood oil is used, particular care then needs to be taken to ensure that the objective is thoroughly and promptly cleaned with xylene after each session of use. Petrol can be used in place of xylene for cleaning if xylene is not available.

Liquid paraffin should not be used as it has a low refractive index which produces an inferior image. It is also unsuitable for scanning specimens for long periods, as is required for accurate microscopy.

**Coarse and fine focusing knobs**

The coarse and fine focusing knobs are used to change the distance between the specimen slide and the objective. The coarse focusing knob alters this distance rapidly and is used to bring the specimen into the field of view using an objective having low magnification power. The fine focusing knob changes the distance very slowly and permits better

viewing of the object. One revolution of the fine focusing knob should generally move the mechanical stage by 100μm. The movement should be smooth and free from jerks.

**Halogen lamp**

Halogen lamps are low wattage, high intensity lamps and are the preferred light source. Though costlier, these have the following advantages over tungsten lamps:

❍ emit white light

❍ have higher luminosity (brighter)

❍ have compact filament

❍ have longer life.

**Functioning of the microscope**

There are three main optical pieces in the compound light microscope. All three are essential for a sharp and clear image. These are:

❍ Condenser

❍ Objectives

❍ Eye-pieces.

The condenser illuminates the object by converging a parallel beam of light on it from a built-in or natural source. The objective forms a magnified inverted (upside down) image of the object. The eye-piece magnifies the image formed by the objective. This image is formed below the plane of the slide.

The total magnification of the microscope is the product of the magnifying powers of the objective and the eye-piece.

For example, if the magnifying power of the eye-piece is 10x and that of the objective is 100x, then the total magnification of the compound light microscope is: 10x X 100x = 1000-fold magnification.

## Applications

- Unstained/living biological specimens have little contrast with their surrounding medium. To see them clearly involves:
  - A bright field- closing down diaphragm= decreased NA.
  - B-Dark field illumination, which enhances contrast by reversal, but often fails to reveal internal details.
- The phase Contrast microscopy overcomes these problems by controlled illumination using the full aperture and thus improving the resolution.

- The higher the refractive index of the structure, the darker it will appear against the light background, i.e. with more contrast. (in positive phase contrast)

# pH Meter

## Introduction

- A pH meter is a scientific instrument that measures the hydrogen-ion activity in water-based solutions, indicating its acidity or alkalinity expressed as pH.
- pH is the unit of measure that describes the degree of acidity or alkalinity. It is measured on a scale of 0 to 14.
- The pH value of substance is directly related to the ratio of hydrogen ion [H+] and the hydroxyl ion [OH-]
- The material is acidic if [H+] concentration is greater than [OH-]. The pH value is less than 7.
- The material is neutral if [H+] concentration is equal to [OH-]. The pH value is 7.
- Acids and bases have free [H+] and [OH-] ions respectively.

## Principle

- When the pair of electrodes or a combined electrode (glass electrode and calomel electrode) is dipped in an aqueous solution, a potential is developed across the thin glass of the bulb (of glass electrode).
- The e. m. f. of complete cell (E) formed by the linking of these two electrodes at a given soln temp. is therefore

$$\mathbf{E = E\ ref - E\ glass}$$

- Eref is the potential of the stable calomel electrode which at normal room temp. is +0.250V.
- Eglass is the potential of the glass electrode which depends on the pH of the soln. under test.
- The resultant e.m.f. can be recorded potentiometrically by using vacuum tube amplifier.
- Variations of pH with E may be recorded directly on the potentiometer scale graduated to read pH.

**Electrodes in pH determination**

- For finding the pH of the solution one should use an electrode reversible to H+ ions.
- The glass electrode which is reversible to H+ ions, is the most commonly used as indicator electrode.
- Generally calomel electrode is used as reference electrode.
- The most widely used ion-selective electrode is the glass pH electrode, which utilizes a thin glass membrane that is responsive to changes in H+ activity.

**Important Components of pH Meter**

1. Glass electrode 2. Calomel electrode 3. Electrometer

**1. Glass Electrode:**

- It consists of a very thin bulb about 0.1 mm thick blown on to a hard glass tube of high resistance.
- The bulb contains 0.1 mol/litre HCl connected to a platinum wire via a silver-silver chloride combination.

**2. Calomel electrode:**

- It consists of a glass tube containing saturated KCl connected to a platinum wires through mercury-mercurous chloride paste.

**3. Electrometer:**

- Which is a device capable of measuring very small differences in electrical potentials in a circuit of extremely high resistance.

**Working Mechanism**

- An acidic solution has far more positively charged hydrogen ions than an alkaline one, so it has greater potential to produce an electric current in a certain situation.
- In other words, it is a bit like a battery that can produce a greater voltage.
- A pH meter takes advantage of this and works like a voltmeter: it measures the voltage (electrical potential) produced by the solution.
- When two electrodes (or one probe containing the two electrodes) are dipped into solution, some of the hydrogen ions in the solution move toward the glass electrode and replace some of the metal ions in its special glass coating.
- This creates a tiny voltage across the glass the silver electrode picks up and passes to the voltmeter.
- Reference electrode acts as a baseline or reference for the measurement.
- A voltmeter measures the voltage generated by the solution and displays it as a pH-measurement. An increase in voltage means more hydrogen ions and an increase in acidity, so the meter shows it as a decrease in pH; in the same way, a decrease in

voltage means fewer hydrogen ions, more hydroxide ions, a decrease in acidity, an increase in alkalinity, and an increase in pH.

↑ Voltage = more H+ /less OH - = ↑ acidity = ↓pH

↓ Voltage = less H+ /more OH - = ↓ acidity = ↑pH

**Glass pH electrode**

- The most widely used - For pH measurements (selective ion is H+ ) - Response is fast, stable, and has broad range – pH changes by 1 when [H+ ] changes by a factor of 10 – Potential difference is 0.05196 V when [H+ ] changes by a factor of 10 For a change in pH from 3.00 to 6.00 (3.00 units) Potential difference = 3.00 x 0.05196 V = 0.177

- pH GLASS ELECTRODE - Thin glass membrane (bulb) consists of SiO4 - Most common composition is SiO2, Na2O, and CaO Glass membrane contains - dilute HCl solution - inbuilt reference electrode (Ag wire coated with AgCl).

- Equilibrium establishes across the glass membrane with respect to H+ in inner and outer solutions – This produces the potential, E - Linearity between pH and potential - Calibration plot yields slope = 59 mV/pH units - Electrode is prevented from drying out by storing in aqueous solution when not in use.

**Advantages of glass electrode**

 Used in solutions with pH values ranging from 1 to 12

 Lithium glass electrode can be used to measure upto 14

 Well suited for continuous automatic recording and control of industrial and commercial processes.

 Permits rapid measurements

 Simple to operate

 Used for both oxidizing and reducing solutions

 Does not affect solution under examination

 Can be used in coloured, turbid and colloidal solutions.

**Disadvantages of using glass electrode**

- Due to high resistance ordinary potentiometer cannot be used.
- In solutions of proteins and other colloids that tend to adhere to the sensitive membrane, the glass electrode may yield erroneous results.
- Cannot be used in acid fluoride solutions
- Cannot be used in strongly alkaline solutions
- Cannot be used at high temperatures (about 100°C) for prolonged period

Due to partial dehydration of glass electrode when used in non-aqueous media, it cannot be employed to measure pH in that media.

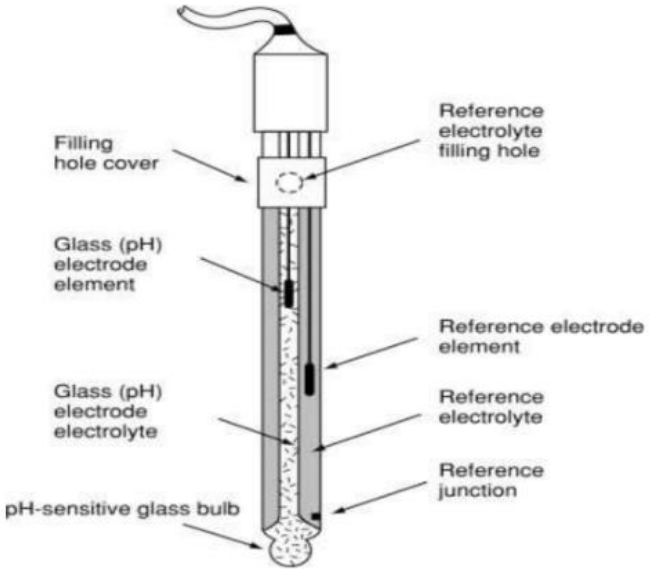
**2 types of pH meters**

1. Potentiometric/slide wire type

2. Direct reading type

## Combination of pH electrode

- Majority of pH electrodes available commercially are combination electrodes that have both glass H+ ion sensitive electrode and additional reference electrode.
- The glass electrode and calomel electrode can be constructed together in a single probe called as combined or combination electrode.
- The combination or pH electrode measures the difference in potentials between the two sides in the glass electrode. To measure the potentials it must be a closed circuit. The circuit is closed through the internal solutions of the electrode and the external solution that is being measured and the pH meter.
- As the electrode is immersed in the test solution the glass bulb senses the hydrogen ions as a millivolts (mV) due to the positive charge of the hydrogen ions.
- The electrolyte or internal solution picks up the mV signal from the glass bulb. That signal is then passed to the internal electrode.
- The Ag/AgCl wire then passes that signal to the electrode cable that leads to the meter.
  The reference electrode containing electrolyte or filling solution generates a constant mV, which is transferred to the Ag/AgCl wire.
- The wire then passes the signal, which can be considered a "control" being measured to the electrode's cable.
- The circuit is closed by a minute amount of internal solution from the reference electrode flowing through a porous membrane made of a ceramic wick. This membrane or junction as it is called is located the electrode body.
- The pH meter measures the difference between the internal electrode and the reference electrode in millivolts DC. This mV reading is then read by the meter and is displayed in pH units.



## Standard Buffer –Reference for pH measurement

- Buffers are solutions that have constant pH values and the ability to resist changes in pH.
- They are used to calibrate the pH meter.
- Before measuring pH we have to calibrate electrode.
- To calibrate electrode we need atleast two solutions of known pH.
- Most commonly used available standard buffers have pH of 4.01, 7.00 and 10.00
- Next step is to put the electrode into pH 7.00 buffer.
- Rinse the electrode with distilled water from a wash bottle into an empty beaker before immersing it into new solution. You should do it every time electrode is moved from one solution to other to minimise contamination.
- Check if the working part of the electrode is completely immersed in the buffer.
- Take care to not hit bottom of the baker with the electrode. Wait for the reading to stabilize (it takes seconds usually, up to a minute sometimes).
- Next steps will depend on the solution you want to measure pH of.
- If you plan to measure pH in acidic solutions, use pH=4.01 buffer.
- If you plan to measure high pH use pH=10.00 buffer.
- If you want to be able to measure pH in the wider range, you may want to proceed with three point calibration and you will need both buffers.
- Remember that high pH buffers tend to absorb atmospheric CO2 thus they should be used as fresh as possible - don't left the bottle open and do the calibration immediately after filling the beaker with the buffer.

**Applications of pH meter:**

- To measure the pH of biological fluids such as blood, urine, gastric acid etc. to ascertain type of biological conditions
- Useful in determination of concentration of substances by pH measurement
- To know pH of buffer solutions
- To maintain the pH of reaction conditions
- To measure the pH of soil, which will be helpful in maximizing the yields and returns from the soil
- To measure the pH of rainwater
- Maintaining perfect and accurate pH levels in several daily activities like keeping the milk from turning sour
- pH meters employed in chemical industries, neutralization of effluent in steel, pulp and paper, pharmaceutical manufacturing , biotechnology and petrochemical industries.
- Helps in analyzing the exact pH value of chemical substances and food grade products, thus ensuring high levels of safety and quality.

# Estimation of DNA

Estimation of DNA is possible by a number of methods based on the physical or chemical property of the nucleic acid. A convenient and easy colorimetric method is available on the basis of quantitative reaction of with diphenylamine reagent.

## Principle

Under extreme acid conditions, DNA is initially depurinated quantitatively followed by the dehydration of sugar to-hydroxylevulinylaldehyde. This aldehyde condenses, in acidic medium, with diphenylamine to produce a deep-blue coloured condensation products with absorption maximum at 595 nm.

## Materials

- DNA Standard (0.5 mg/mL)
- Saline citrate (0.15 M NaCl, 0.015 M Na, Citrate) Solution
- Diphenylamine reagent
- Mix 5 g fresh or recrystallized diphenylamine, 500 mL glacial acetic acid and 13.75 ml conc. H2S04. Stable for six months at 2°C; warm to room temperature and swirl to remix before use.

## Procedure

1. Prepare separate marked tubes containing 1 mL, 2 mL and 3 mL aliquots of the isolated DNA dissolved in standard saline citrate and similar aliquots of a 0.5 mg DNA/mL standard

2. Make all sample tubes, and a separate blank, up to 3 mL with H2O.

3. Add 6 ml of diphenylamine reagent to each tube, and after mixing, heat the tubes in a boiling water bath for 10 minutes. Cool the tubes.

4. Read the absorbance of blue solution at 600 nm against the blank.

5. Construct a standard graph $A_{600}$ (ordinate) versus quantity of DNA and then calculate the concentration of DNA dissolved in the saline citrate solution.

# Estimation of RNA

A number of reactions have been described for the colorimetric estimation of ribose. Colorimetric procedures reactions suitable for pentose determination have been used for measurement of RNA, and include reactions with orcinol, Phloroglucinol, aniline etc. However, the reaction of ribose (Bial's test) in RNA with orcinol have been widely used.

## Principle

The method depends on conversion of the pentose, ribose in the presence of hot acid to furfural which then reacts with orcinol to yield a green colour. The colour formed largely

depends on concentration of HCl, Ferric chloride, orcinol, the time of heating at 100°C etc. up to certain maxima.

## Materials

- Standard RNA, and Sample RNA Solutions
- Orcinol Acid Reagent
- Add 2 mL of a 10% solution (w/v) of ferric chloride. 6H,O to 400 mL of conc. HCl
- 6% Alcohol Orcinol
- Dissolve 6 g orcinol in 100 mL 95% ethanol. Refrigerate in a brown bottle until use.
- Stable for one month.
- Colorimeter

## Procedure

1. Prepare a standard RNA (50 ug RNA/mL) solution in ice-chilled 10 mM Tris-acetate, 1 mM EDTA buffer (pH 7.2) or any other suitable buffer by dissolving RNA completely.

2. Dissolve the isolated RNA in the above buffer soution to an approximate concentration 50 pg/mL.

3. Prepare a series of tubes containing 0.5 mL, 1 mL, 1.5 mL and 3 mL of isolated RNA, 0.5 mL, 1 mL, 1.5 mL and 3 mL of 50 pg standard RNA/mL.

4. Make up each tube to 3 mL with water. In addition set a blank containing 3 ml, of water.

5. Add 6 ml of orcinol acid reagent to each tube.

6 Add 0.4 mL of 6.0% alcoholic oreinol to each tube. Shake the tubes to mix the contents and then heat all tubes in a boiling water-bath for 20 min.

7. Cool the tubes, and read the absorbance at 660 nm against the blank.

8. Draw a standard curve using A660 and the concentration of standard RNA. Calculate the amount in the isolated RNA solution using the graph.

# Protein Estimation by Lowry's Method

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolysing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry et al. is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

## Principle

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

## Materials

- 2% Sodium Carbonate in 0.1 N Sodium Hydroxide (Reagent A)
- 0.5% Copper Sulphate (CuSO,.5H,0) in 1% potassium sodium tartrate (Reagent B)
- Alkaline copper solution: Mix 50 mL of A and 1 mL of B prior to use (Reagent C)
- Folin-ciocalteau reagent (reagent D): Reflux gently for 10 hours a mixture consisting of 100 g sodium tungstate (Na, Wo04,2H,O), 25 g sodium molybdate (Na,Mo04,2H,0), 700 mL water, 50 mL of 85% phosphoric acid, and 100 mL of concentrated hydrochloric acid in a 1.5 L flask. Add 150 g lithium sulphate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 L and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1 N NaOH to a phenolphthalein end-point)
- Protein solution (Stock standard)
  Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled
  water and make up to 50 mL in a standard flask.

- Working standard
  Dilute 10 mL of the stock solution to 50 mL with distilled water in a standard flask
  mL of this solution contains 200 μg protein.

## Procedure

**Extraction of Protein from Sample**

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 mL of the buffer. Centrifuge and use the supernatant for protein estimation.

**Estimation of Protein**

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 0.1 mL and 0.2 mL of the sample extract in two other test tubes.

3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of water serves as the blank.
4. Add 5 mL of reagent C to each tube including the blank. Mix well and allow to stand for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temp. in the dark for 30 min. Blue colour is developed.
6. Take the readings at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

### Calculation

Express the amount of protein mg/g or 100 g sample.

# Protein Purification

## Introduction

There are many thousand kinds of proteins with different properties and functions in a cell. To study a protein, one has to obtain the highly purified intact form of the protein. A major protein is not so difficult to be purified while a minor one may need many purification steps and high skills on the techniques. This chapter describes the principles underlying techniques for separation and purification of proteins extracted from cells and tissues. The strategy involves extraction and purification.

## Extraction of protein

Generally both extraction and purification processes should be done under a cold condition, mostly at 0-4oC, except for some proteins. An ice box or another cooling system is normally needed for sample cooling. The extraction procedure varies according to the type of sample and physicochemical properties of the protein. The first step is to disrupt cells or tissues. The more gentle procedure is used the more intact protein is obtained. Most of animal cells and tissues are soft and easy to break, so gentle methods can be applied. For bacteria, fungi and most plant cells which have tough cell envelope, more vigorous procedures are required. Extraction buffer or solvent is also important. Buffer with suitable concentration, ionic strength, and pH is used for extraction of water-soluble protein. In some case, mild detergent or other appropriate dissociating substance is added to the extraction buffer. Protease inhibitors are sometimes necessary to prevent destruction of the extracted proteins by proteases. From the fact that the protein extract obtained from subcellular component has contaminants less than that obtained from whole cell and will be easier to be purified. So separation of subcellular components may be done before the protein extraction. Disruption of cells should be adjusted to get intact sub-cellular components. The required cell component is obtained by centrifugation under appropriate condition. The required cell component is obtained by centrifugation under appropriate condition.

- **Extraction of water-soluble protein from animal cells and tissues**

The proteins which are components of fragile unicellular tissues such as animal blood cells can be extracted by using hypotonic buffer solution. If the sample contains different types of cells, separation of the cell types before the extraction will make the purification easier. This osmosis-based method is also used for animal cells grown in culture media. In some case a mild surfactant is included in the extraction buffer. Sonication or freeze/thaw cycle or a mild mechanical agitation may be used to help disruption of the cells and dissociation of the cellular components. For multicellular soft tissue, a hand homogenizer or an electrical one with optimal size is a good choice. The tiny pestle using with a micro-centrifuge tube is also commercially available for the sample with a small volume. Equipments with stronger breaking power such as a blade blender are needed for extraction of proteins from tougher tissue such as animal muscle. Cell disruption by nitrogen decompression is another method for animal cells and some plant cells. In this method, large quantities of nitrogen are dissolved in the cells under high pressure in a vessel. When the pressure is suddenly released, the dissolved nitrogen becomes bubbles. The expanding bubbles cause rupture of the cell. Intact organelles are also obtained by this method.

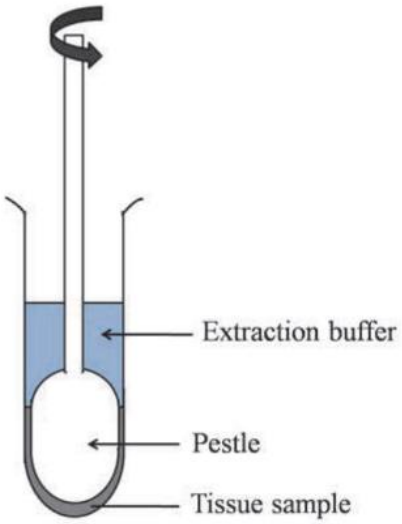



Fig. 1. Homogenization of tissue by a hand homogenizer.

- **Extraction of water-soluble protein from unicellular organisms**
  This group of organisms includes bacteria, yeast, fungi and some algae. Their cell envelopes are tougher than those of animal cells. The stronger methods are needed for disruption of the cells. A simple method is to shake the suspension of cells, as well as spores, with small glass beads or the other kind of beads. If the collision with the beads is done in a blender, the method is called a bead mill. Sonication is another way

to break the cells. The solution used to suspend the cells can be only an appropriate buffer, or with the addition of some enzyme and/or mild detergent. The examples of the enzymes are lysozyme, cellulase and chitinase. Nonionic and zwitterionic detergents, especially Triton X-100 and CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), are commonly used since they are mild and have little effects on protein denaturation. To disrupt the unicellular organisms with very strong envelope, a high-pressure press apparatus may be needed such as French press, Microfluidizer processors. In these systems, the cell suspension in a suitable chamber is pressurized (up to 30,000 psi) by using a hydraulic pump. Shearing force is generated when the pressurized suspension is squeezed pass a very narrow outlet into the atmospheric pressure (Walker, 2005).

- **Extraction of water-soluble protein from plant tissue**

Grinding with or without sands, in the presence of an appropriate buffer, by using a pestle and mortar actually works well with plant sample. If the plant tissue is too strong to be ground, rapid freeze with liquid nitrogen will make the plant more fragile. The frozen plant tissue is ground before shaking in the buffer (Fido et al., 2004). Some plant cells can be disrupted by the mean of nitrogen decompression.

**Extraction of lipid-soluble protein**

Most of lipid-soluble proteins are membrane proteins. These proteins may be called proteolipids which are extracted from the samples by using organic solvents such as a mixture of chloroform and methanol (Velours et al., 1984). An aqueous solution containing mild detergents such as Triton X-100 and CHAPS is an alternative way to dissolve the proteins from cells or organelles. Strong detergents can be used but they may cause permanent denaturation of the proteins.

**Extraction of aggregated protein**

High expression of recombinant proteins in bacterial host cell often form insoluble aggregates which is known as inclusion bodies. This form of proteins is very difficult to be solubilized. To extract any protein from bacterial inclusion bodies, strong denaturants such as 6M guanidine-HCl or 6 to 8M urea are included in the solubilizing solution. This solution efficiently extracts the aggregated protein. However, the extracted protein is also denatured and sometimes cannot be renatured (Rudolph & Lilie, 1996). The other procedure is to use a mixture of mild detergents such as a combination of Triton X-100, CHAPS and sarkosyl. This method is less efficiency but more native forms of the protein can be recovered (Tao et al., 2010).

# Purification of protein

To purify any protein, various separation techniques are used depending on physical and chemical properties of the protein. The purification process can be concluded as follows.

Step 1. Crude extract of protein

↓

Step 2. Detection of the desired protein- Detection by size, activity or property

↓

Step 3. Separation into fractions- i) Separation based on solubility, ii) Separation based on molecular size and density

↓

Step 4. Detection of the desired protein- Detection by size, activity or property

↓

Step 5. Separation for the purified protein – i) Chromatographic techniques

ii) Other separation techniques

↓

Step 6. Detection of the desired protein - Detection by size, activity or property

↓

Step 7. Determination of the purity – Electrophoresis

- **Separation based on solubility**

  Solubility of hydrophilic protein depends on its charge and hydrogen bonding with water molecule. Net charge of a protein is zero at its isoelectric pH (pI); so the protein molecule is easy to aggregate and then precipitate. The method is called isoelectric precipitation. If the pI is not known, high concentration of some salt can precipitate protein by destroying hydrogen bond between protein and water molecule. The method is called salting out or salt precipitation. Ammonium sulphate is normally used since it has high ionic strength. Proteins in crude extract can be separated into a number of fractions by gradually addition of the salt. The precipitated proteins are re dissolved and used for further steps. Combination of the two methods is used for precipitation of some protein.

- **Separation based on size and density**

The techniques include centrifugation, dialysis and molecular filtration. A high speed centrifugation (10,000-20,000g) is normally used to remove cell debris and large organelles from crude protein extract if the desired protein is a water-soluble molecule. The supernatant is then used for further purification steps. To know the location of the desired protein in cell, the cell disruption should be a soft procedure to get intact organelles and the other subcellular structures. The subcellular structures including organelles are then fractionated by using differential centrifugation and/or density gradient centrifugation. In differential centrifugation, the subcellular particles are separated according to their sizes into fractions by the stepwise increase of the centrifugal force. Density gradient centrifugation is a procedure to separate particles including protein aggregates by their densities. A liquid density gradient may be pre-formed before use for the separation. Various materials can be used to make the gradients such as sucrose, Ficoll. In addition the liquid density gradient can be formed during the separation. By this way, the sample is layer on top of a suitable concentration of cesium chloride. The strong centrifugation force of ultracentrifuge causes migration of cesium chloride into the bottom of the tube. The liquid density gradient is then formed by the gradient concentration of cesium chloride (Ohlendieck, 2005). The density gradient is also spontaneously generated during centrifugation by using Percoll (polymer-coated silica particle).

Dialysis is a widely used method. The method is simple, but time consuming because the separation depends on diffusion. The sample is placed inside a dialysis bag prepared from a tube made of semipermeable membrane. Rely on commercially available dialysis tube, only small molecules whose sizes less than 10 kDa is removed from the sample to the surrounding medium. So this technique is normally used to remove salts from the solution of protein. Dialysis is also used to concentrate protein solution. Water molecules are removed from the inside of dialysis bag by using a hydrophilic polymer such as polyethylene glycol.

Molecular filtration (or ultrafiltration) is similar to dialysis. A membrane with specific pore size is used to fractionate proteins. By using pressure or centrifugation force, only the molecules smaller than the pore pass through the membrane (Fig 2). There are various sizes of the membrane pore; the largest one can cut off the large molecule whose mass is almost 100 kDa. So proteins in the sample can be separated into various ranges of molecular masses by using the membranes with different pore sizes. The protein solution is also concentrated by this technique.

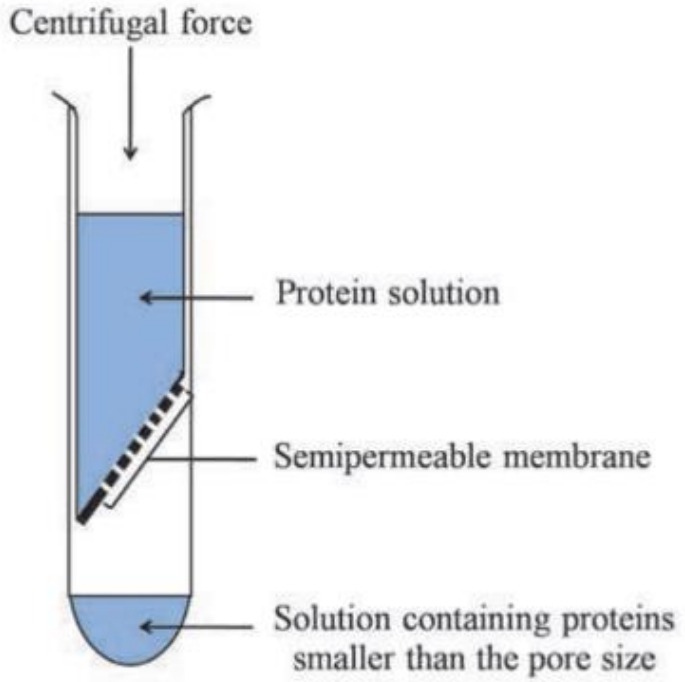



Fig. 2. Separation of proteins by using molecular filtration.

## Chromatographic technique

Chromatography is the powerful method for detection and purification of biological substances. The principle of chromatographic separation is distribution or partition of separated molecules between two immiscible phases called mobile phase and stationary phase. Chromatographic methods are classified by different criteria including physical shape of stationary phase, nature of mobile phase and/or stationary phase, mechanism of separation or the other properties of the chromatographic systems. So the methods are called depending on their popularity. For examples, paper chromatography is called according to the material used as the stationary phase; thin layer chromatography (TLC) and column chromatography are named by the physical shapes of the stationary phases. Gas-liquid chromatography has

gas mobile phase and liquid stationary phase. By using mechanisms of separation, chromatography is classified as adsorption chromatography, partition chromatography, size-exclusion chromatography, ion-exchange chromatography and affinity chromatography. Column chromatography is the most popular method for purification of proteins.

The conventional column chromatography is performed under low pressure. The mobile phase flows through the stationary phase in the column by the gravity or by low-pressure pump(s). So it can be called low pressure liquid chromatography (LPLC). High performance liquid chromatography (HPLC) is an advanced version of column chromatography. High pressure pumps, high-quality materials used as stationary phase and detectors with high sensitivity are used to improve the separation and to reduce the operating time. HPLC is now very popular for detection and purification of biological molecules. The other chromatographic methods are fast protein liquid chromatography (FPLC), capillary chromatography, reversed-phase liquid chromatography etc. FPLC is similar to HPLC but its operating pressure is lower than that of HPLC. LPLC, HPLC and FPLC are widely used for purification of proteins. In these methods, proteins are normally separated according to their size, charge or binding affinity. The separated proteins are eluted from the column as peaks which can be seen by various means such as absorption spectrometry, spectrofluorimetry (Wilson, 2005).

**Size-exclusion chromatography**

The other names of this method are gel filtration chromatography and gel permeation chromatography. This chromatographic technique also separates proteins by molecular sizes. Size-exclusion chromatography is the best to conserve native structure and function of the purified protein since wide varieties of buffers can be selected to obtain the suitable condition for the protein. The stationary phase of size-exclusion chromatography normally contains porous hydrophilic gel beads (Fig. 3). The gel beads used for LPLC are made of agarose, dextran, polyacrylamide, and chemical derivatives of these substances. The beads used for HPLC and FPLC are made of stronger materials such as cross-linked dextran and polystyrene. The cross-linked polystyrene can be used for separation of hydrophobic substances in the presence of organic solvents. The principle of the technique is the diffusion of molecules into the porous cavities of the beads. The molecules larger than the pores cannot enter inside the beads whereas the smaller ones can do. Since the pores have many sizes, the molecules including proteins are separated according to their molecular masses.The larger molecules pass the column faster than the smaller molecules. Size-exclusion chromatography can also be used for estimation of molecular mass of protein. Aset of suitable proteins with known molecular masses are separated by the same condition as the purified protein. The fraction numbers or elution volumes are then plotted against log molecular masses of the standard proteins. The standard curve is used to estimate molecular mass of the purified protein. To obtain the good result, one have to choose the right beads since different types of beads are suitable for different molecular masses of the proteins.

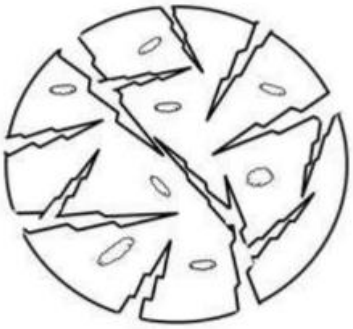


Fig. 3. A model demonstrates the pores in the hydrophilic gel bead used in size-exclusion chromatography.

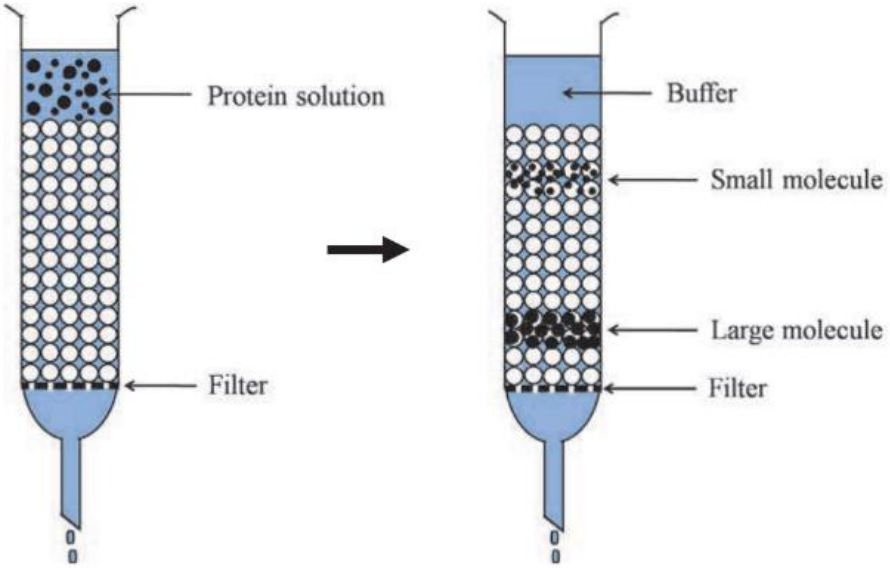



Fig. 4. Separation of two proteins by using size-exclusion chromatography. The protein mixture is loaded on the top of the gel. Then the large molecules pass the column faster than the small molecules.

**Ion-exchange chromatography**

This chromatographic technique conducts the separation according to magnitude of net electric charge of the proteins. There are two types: anion-exchange chromatography and cation-exchange chromatography. The material packed in the column is called ion exchanger which also have two types, anion and cation exchangers. Anion exchangers possess positively charged groups while cation exchangers have negatively charged groups.

Anion-exchange chromatography consists of anion exchanger and used for separation of proteins containing net negative charges. In contrast cation-exchange chromatography consists of cation exchanger and used for separation of proteins containing net positive charges. There are various varieties of ion exchangers depending on the matrix materials and ionic groups. Cellulose, agarose, dextran and polystyrene are used for LPLC while polystyrene, polyethers and silica are strong enough for HPLC. The matrix is chemically

modified to contain ionic groups which are weakly acidic, strong acidic, weakly basic, or strong basic. The matrix with weakly acidic group (cation exchanger) as well as that with weakly basic group (anion exchanger) is suitable for separation and purification of most proteins. Choice of the exchanger depends on the pH range in which the protein is stable and the pI value of the exchanger. Generally the pH of the system should make opposite charges between the stationary phase and the protein (Fig. 5). Elution of proteins from the column can be isocratic or gradient system. The pH and ionic strength of elution buffer is important for getting well separation of proteins.

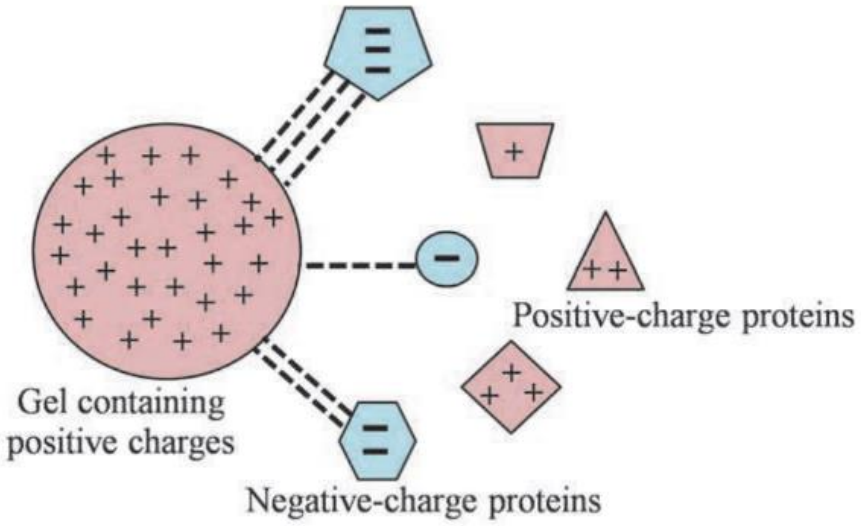



Fig. 5. Principle of ion-exchange chromatography. The anion-exchanger binds to negative-charge proteins but positive-charge proteins. The binding strength varies according to the charges of the proteins.

**Affinity chromatography**

This is an efficient technique but the material used as stationary phase is costly. The separation of molecules is based on binding affinity between macromolecules and macromolecules, or between macromolecules and low molecular mass ligands. The binding is specific for a certain molecule or a group of molecules. For examples, monoclonal antibody binds to its antigen only while avidin can bind to any biotin-coupled proteins. In this technique, one of the partner molecules is immobilized on a matrix by using a chemicalreaction. The matrix can be polyacrylamide, polystyrene, cross-linked dextrans and agarose etc. There are a number of chemical substances which react to different functional groups of the immobilized molecule. The chemical reaction must not destroy the binding activity. So it is necessary to use the right reaction for the immobilization. However, there are a lot of commercially available affinity matrices for different purposes such as protein A Sepharose for purification of immunoglobulins, mannan-agarose for purification of mannose-binding lectins. After washing off the impurities, the matrix-bound protein is eluted by change of the pH of elution buffer, or by a competing substance. Affinity chromatography is always operated in a simple way by packing the matrix in a small column. Sophisticated system is not necessary for this technique.

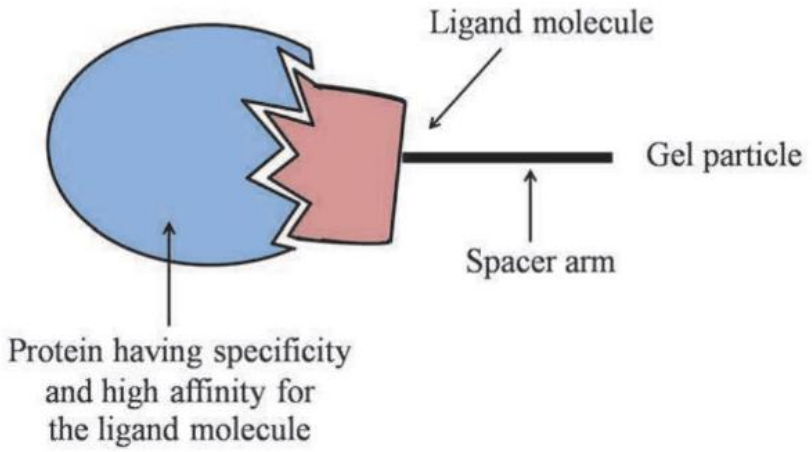



Fig. 6. Principle of affinity chromatography. The ligand is immobilized on a matrix and used for purification of its partner molecule.

## Hydrophobic interaction chromatography

This chromatographic method separates proteins on the basis of hydrophobicity, the same as reversed-phase liquid chromatography (RPLC). The stationary phases of both methods are hydrophobic ligands attached to a matrix. However, hydrophobicity and number of the ligands in hydrophobic interaction chromatography (HIC) is less than those in RPLC. So HIC may be referred to as a milder form of RPLC. RPLC is mainly used for separation of peptides and low molecular mass proteins that are stable in aqueous-organic solvents. HIC is suitable for purification of proteins since it uses less extreme condition for elution of the adsorbed proteins. The hydrophobic ligands of HIC are mainly alkyl (ethyl to octyl) or phenyl or polyamide groups; the matrices are cross-linked agarose or silica. For the general procedure of HIC, the sample is applied onto the column equilibrated with a mobile phase of relatively high salt concentration. The adsorbed proteins are then eluted by a solvent of decreasing salt concentration. HIC is suitable for separation of proteins after salt precipitation and/or ion-exchange chromatography since the proteins already dissolves in the solution of high salt. Although HIC gives only moderate resolution, it opens new possibilities for purifying a number of biomolecules such as receptor proteins, membrane proteins (Wilson, 2005).

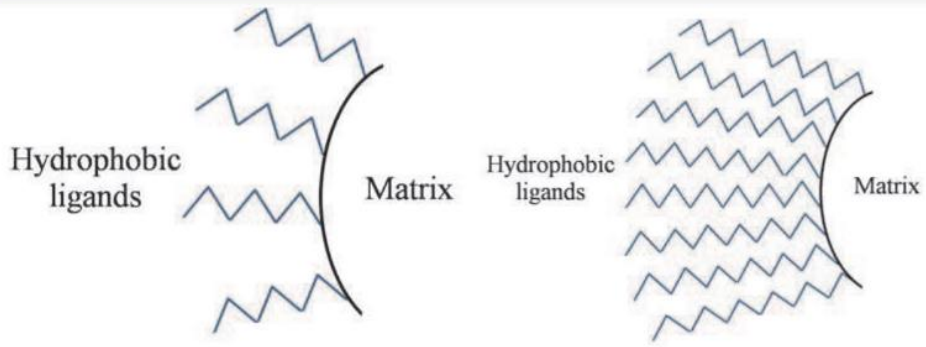



Fig. 8. Comparing the structures of materials used as stationary phases in hydrophobic interaction chromatography (A) and reversed-phase liquid chromatography (B).

## Other separation techniques

There are some minor methods used for purification of protein such as electro elution of the protein separated by SDS-PAGE. The stained protein band is cut out of the gel. The gel slices with a suitable buffer are packed in a dialysis bag. The bag is placed in the buffer between two electrodes. The protein is then electrically eluted from the gel slices. One can make a simple electroelution cell or can take any commercially available apparatus. SDS is removed from the protein by dialysis in the presence of a nonionic detergent. Only a small amount of protein is obtained by electroelution and it is possibly denatured.

### Detection of the desired protein

Actually, purification process includes many steps and is laborious. Therefore it is necessary to be certain that the desired protein is present in the crude protein extract and all purification steps. For the protein with known molecular mass or size, the most versatile method is gel electrophoresis, especially SDS-polyacrylamide gel electrophoreis. Determination of enzymatic activities or other properties such as absorption spectra are also widely used. In some case the sophisticated technique, LC-MS/MS, is performed for identification of the protein after separation by electrophoresis.

### Electrophoresis

It is the most widely used technique for separation, detection and determination of the purity of protein. It is sometimes used for protein purification. The technique is based on migration of charged proteins in an electric field. Electrophoresis of proteins is generally conducted in a gel medium, mostly polyacrylamide. The widely used polyacrylamide gel electrophoreses (PAGE) are sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF).

### Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE is normally employed for estimation of purity and molecular mass of protein. This versatile method is reproducible and low-cost. For the principle, sodium dodecyl sulfate (SDS) in the system binds to most proteins in amount approximately proportional to the molecular mass of the proteins. So each protein has similar charge-to-mass ratio and migrates proportional to its molecular mass. The widely used SDS-PAGE is the Laemmli protocol, as

well as its modified protocols, which consists of the discontinuous buffer system. In this buffer system, there are two parts of polyacrylamide gel, stacking gel and separating gel. There are differences in composition, concentration and pH among electrode buffer, stacking buffer and separating buffer (Fig. 10). The usefulness of the buffer stem is stacking effect which occurs in the stacking gel. The effect increases concentration of proteins in the sample which helps well

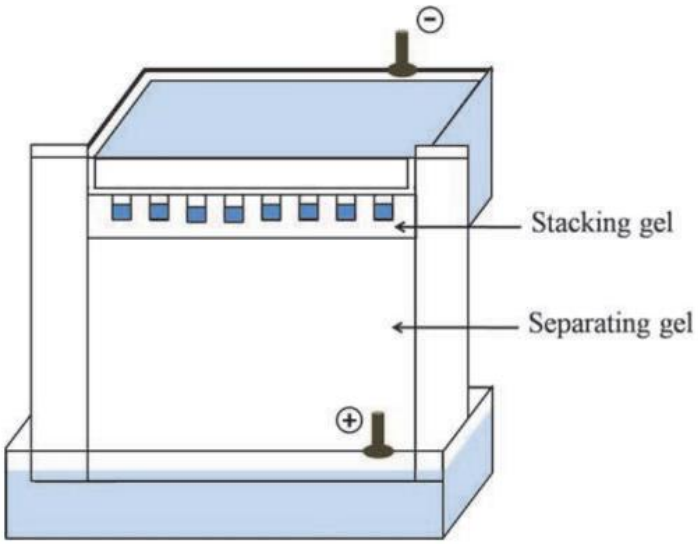



Fig. 10. Polyacrylamide gel electrophoresis. The electrophoresis set composes of the upper and lower chambers which are filled with an electrode buffer. The gel is polymerized in the space between two glass plates and then connected between the two chambers. Protein samples are load in wells at the top of the gel. Protein molecules will migrate into the gel under an electric field. For SDS-PAGE, the proteins migrate from cathode to anode.

separation of the proteins in the separating gel. SDS-PAGE can be used to estimate molecular mass of the protein by using a calibration curve between relative mobility and log molecular mass of standard protein. There are the other protocols of SDS-PAGE including urea-SDS-PAGE, Tricin-SDS-PAGE etc. The other types of polyacrylamide gel electrophoresis are also available such as acid-urea-PAGE, native-PAGE. Although SDS-PAGE is commonly used, some proteins may need specific PAGE for the detection. The proteins separated on polyacrylamide gel can be visualized by many types of staining. The staining with dye especially Coomassie Brilliant Blue R250 is most widely used since it is easy, low cost and effective. Staining with some other dyes and silver staining are also available for specific purposes. Examples are Sudan Black B for staining of proteolipids, Thymol for staining of glycoproteins (Hames, 1981; Holtzhauer, 2006).

## Isoelectric focusing

IEF is used for determination of isoelectric pH (pI) of protein. The technique is also useful for separation, detection and determination of the purity of protein. Proteins are separated according to their pI in a medium containing pH gradient. For analytical slap gel, the medium

commonly used is polyacrylamide gel or agarose gel. The pH gradient is established by a mixture of low molecular mass organic acids and bases which is called ampholytes (Berg et al., 2002). The proteins in the mixture migrate in an electrical field depending on their charges and then standstill in the gel at the pH equal to their pI. The separated proteins can be visualized by an activity staining or a dye staining. The activity stain depends on the protein property. There is a problem concerning with the dye staining of IEF gel. The ampholytes can be stained with some dyes especially Coomassie Brilliant Blue R250. So it must be removed by appropriate solutions before staining.

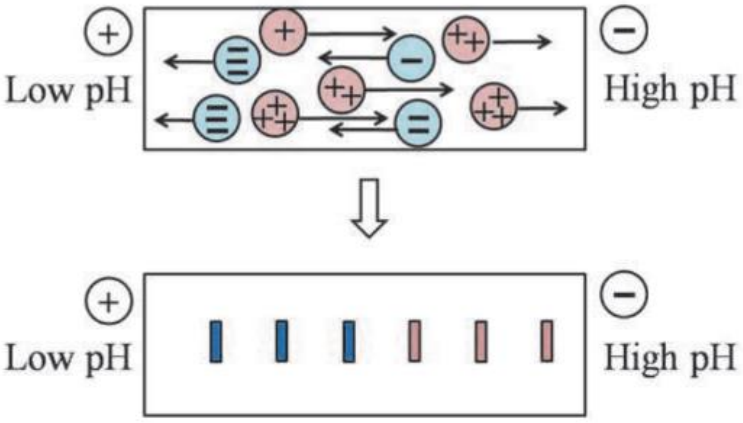



Fig. 11. Principle of isoelectric focusing. A pH gradient is established between two electrodes by using an electric field, a mixture of ampholytes and suitable electrode buffers. Each protein migrates in the gel and then stop moving at the pH equal to its pI.

For some work, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is performed to obtain a very high resolution. The technique is a sequential separation of proteins. The protein mixture is separated by using IEF in the first dimension and then SDS-PAGE in the second dimension. High skill is needed to perform the conventional procedure. Fortunately, the commercially available apparatus are designed to make it much easier and more efficient. Since the cost of the technique is rather high, it is done only when necessary.

After dye staining or silver staining, the protein separated by SDS-PAGE or 2D-PAGE may be identified by using Liquid Chromatography-Mass spectrometry (LC-MS). The protein band is cut from the gel and then in-gel digestion with some protease which is mostly trypsin. The peptide fragments are separated by HPLC and injected to a series of Mass Spectrometers. This mean of mass spectrometric analysis is called tandem mass spectrometry. The MS spectra of the peptides are used to search in the data bases through the internet. The amino acid sequences of some peptides are obtained as well as the protein(s) which has them as parts of molecule (Berg et al., 2002).

**Enzyme activity or the other property of protein**

Apart from electrophoresis, there are some other methods for detection of the desired protein during purification process. Various specific techniques are used depending on the property of the protein. Many proteins can be stained in gel by using their enzyme activity. Since the native conformation is important for the activity, IEF and native-PAGE are compatible with activity stains. SDS-PAGE is not suitable for activity staining of protein, excepted that the

proteins can be re-natured by soaking the gel with non-ionic detergent. The enzyme activity is also determined in solution. Its activity is measured by absorption spectrometry and the activity unit can be determined. Specific activity calculated from enzyme activity and the amount of protein in milligram is normally used for determination of the purity fold during the purification process. Some enzyme activity may be detected by other techniques such as paper chromatography (Arunchaipong et al., 2009).

Many proteins are not enzymes but they may have other activities or properties. Some activities or properties are used for detection of the proteins. For examples, Anti-microbial activities can be detected by microbiological methods. Glycoproteins are pursued during purification process by using lectins. In this case, the binding activity has to be done on a membrane surface since the protein molecules cannot freely move in the gel matrix and the specific binding will be interfered. The membrane method is called Western blot analysis. In this method the proteins are separated using SDS-PAGE. The separated proteins are then electrically transferred onto a membrane sheet made of nitrocellulose or polyvinylidene fluoride (PVDF). The protein bands are adsorbed on the membrane surface. The membrane is then used for binding with a lectin-linked enzyme. Only glycoprotein band(s) binds to the lectin-linked enzyme. The band(s) can be visualized by addition of a suitable substrate. Western blot analysis is also suitable for detection of protein by specific antibody.

# Molecular Weight Determination by SDS-PAGE

## Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a reliable method for determining the molecular weight (MW) of an unknown protein. The first step in MW determination of a protein is to separate the protein sample on the same gel with a set of MW standards. Next, a graph of log MW vs. relative migration distance (Rf) is plotted, based on the values obtained for the bands in the MW standard. The MW of the unknown protein band is then calculated by interpolation using this graph. The key to determining MW accurately is selecting separation conditions that will produce a linear relationship between log MW and migration within the likely MW range of the unknown protein.

## SDS-PAGE Conditions for MW Determination

To ensure accurate MW determination, both the protein standards and the unknown protein must be electrophoresed on the same gel under identical separation conditions. It is also important to generate multiple data points (at least three gels) so that the estimated MW has statistical significance. The sample buffer used to solubilize the proteins should contain reducing agents (dithiothreitol or β-mercaptoethanol) to break disulfide bonds, which minimizes the effect of secondary structure on migration. In addition, a strong ionic detergent such as SDS is a required component of the sample buffer. SDS provides two functions: It denatures secondary, tertiary, and quaternary structures by binding to hydrophobic protein regions, and its binding confers a net negative charge on the proteins, which also results in a constant charge-to-mass ratio. The proteins are then separated through a gel in an electrical field according to their mass. However, other factors may also influence protein separation.

## Analysis of Electrophoresed Proteins

An example of the approach is shown in Figure 1 using Green Fluorescent Protein (GFP) as a hypothetical example of an unknown protein. A dilution series of an E. coli lysate spiked with GFP and Precision Plus Protein TM unstained standards was electrophoresed in different lanes of a Criterion TM 4–20% SDS-PAGE gel, then stained with Bio-SafeTM Coomassie stain and destained in distilled water to visualize the protein bands. The gel was then analyzed to obtain the Rf values for each band. The Rf is defined as the migration distance of the protein through the gel divided by the migration distance of the dye front. The distance should be measured from the top of the resolving gel to the band of interest, as illustrated on the gel.

A plot of log MW versus Rf was generated from the bands in the gel shown in Figure 1 to determine the MW of the unknown protein. The simplest method for this is to base the MW determination on a standard curve. If the curve is nearly linear, it can be described by the formula y = mx + b, where y is the log MW, m is the slope, x is the Rf, and b is the y-intercept, as shown in Figure 2.

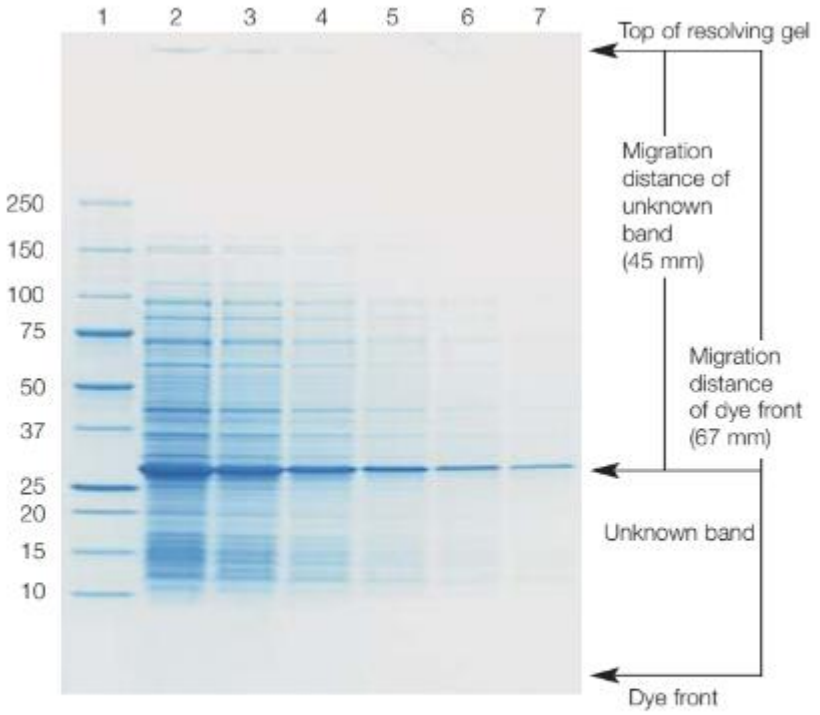



**Fig. 1. Example showing approach for MW determination of an unknown protein.** Lane 1, 10 µl of Precision Plus Protein unstained standards; lanes 2–7, a dilution series of an E. coli lysate containing a hypothetically unknown protein (GFP). Proteins were separated by SDS-PAGE in a Criterion 4–20% Tris-HCl gel and stained with Bio-Safe Coomassie stain. Gel is shown actual size. MW standards are in kD.

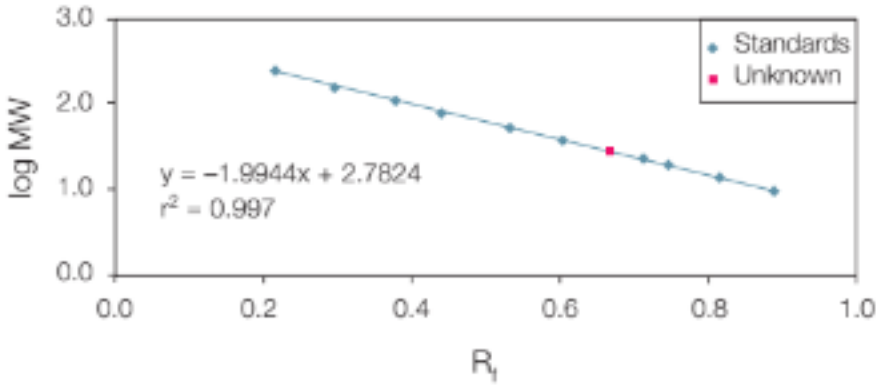



**Fig. 2. Determining the MW of an unknown protein by SDS-PAGE.**
A standard curve of the log MW versus $R_f$ was generated using the Precision Plus Protein standards from Figure 1. The strong linear relationship ($r^2 > 0.99$) between the proteins' MW and migration distance demonstrates exceptional reliability in predicting MW.

## Procedure

1. Run the standards and unknown on an SDS-PAGE gel.

2. Process the gel with the desired stain and then destain to visualize the protein bands.

3. Determine the Rf graphically or using Quantity One software (or equivalent).

4. Use a graphing program to plot the Rf versus log MW. From the program, generate the straight line equation y = mx + b, and solve for y to determine the MW of the unknown protein.

## Determining Rf Graphically

- Use a ruler to measure the migration distance from the top of the resolving gel to each standard band and the dye front.
- Calculate the Rf value of each band using the following equation:

**Rf** = migration distance of the protein/migration distance of the dye front

**Determining Rf With Quantity One Software**

The Rf and r2 values are determined automatically by the software.

**Limitations**

MW determination by SDS-PAGE is a dependable method. However, an unknown protein's MW should always be obtained by mass spectrometry if a more precise MW determination is needed. Mass spectrometry has a higher degree of accuracy because each amino acid of a protein is analyzed. Protein-to-protein variation can be minimized by denaturing samples, reducing proteins, normalizing the charge-to-mass ratio, and electrophoresing under set conditions. However, factors such as protein structure, posttranslational modifications, and amino acid composition are variables that are difficult or impossible to minimize and can affect the electrophoretic migration.

## Study of enzyme by native gel electrophoresis (Zymogram)

Zymography is an electrophoretic technique for the detection of hydrolytic enzymes, based on the substrate repertoire of the enzyme. Three types of zymography are used; *in gel* zymography, *in situ* zymography and *in vivo* zymography For instance, gelatin embedded in a polyacrylamide gel will be digested by active gelatinases run through the gel. After Coomassie staining, areas of degradation are visible as clear bands against a darkly stained background.

Modern usage of the term zymography has been adapted to define the study and cataloging of fermented products, such as beer or wine, often by specific brewers or winemakers or within an identified category of fermentation such as with a particular strain of yeast or species of bacteria. Zymography also refers to a collection of related, fermented products, considered as a body of work. For example, all of the beers produced by a particular brewery could collectively be referred to as its zymography.

### Gel zymography

Samples are prepared in a standard, non-reducing loading buffer for SDS-PAGE. No reducing agent or boiling are necessary since these would interfere with refolding of the enzyme. A suitable substrate (e.g. gelatin or casein for protease detection) is embedded in the resolving gel during preparation of the acrylamide gel. Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100, followed by incubation in an appropriate digestion buffer, for an optimized length of time at 37 °C. The zymogram is subsequently stained (commonly with Amido Black or Coomassie brilliant blue), and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

### Variations on the standard protocol

The standard protocol may require modifications depending on the sample enzyme; for instance, *D. melanogaster* digestive glycosidases generally survive reducing conditions (i.e. the presence of 2-mercaptoethanol or DTT), and to an extent, heating. Indeed, the separations following heating to 50 °C tend to exhibit a substantial increase in band resolution, without appreciable loss of activity.

A common protocol used in the past for zymography of α-amylase activity was the so-called starch film protocol of W.W. Doane. Here a native PAGE gel was run to separate the proteins in a homogenate. Subsequently, a thin gel with starch dissolved (or more properly, suspended) in it was overlaid for a period of time on top of the original gel. The starch was then stained with Lugol's iodine.

Gel zymography is often used for the detection and analysis of enzymes produced by microorganisms. This has led to variations on the standard protocol e.g. mixed-substrate zymography.

Reverse zymography copolymerizes both the substrate and the enzyme with the acrylamide, and is useful for the demonstration of enzyme inhibitor activity. Following staining, areas of inhibition are visualized as dark bands against a clear (or lightly stained) background.

In imprint technique, the enzyme is separated by native gel electrophoresis and the gel is laid on top of a substrate treated agarose.

Zymography can also be applied to other types of enzymes, including xylanases, lipases and chitinases.

# Enzyme Activity and Assays

Enzyme activity refers to the general catalytic properties of an enzyme, and enzyme assays are standardized procedures for measuring the amounts of specific enzymes in a sample.

**Factors that Affect Enzymatic Analysis**

Enzyme activity is measured in vitro under conditions that often do not closely resemble those in vivo. The objective of measuring enzyme activity is normally to determine the amount of enzyme present under defined conditions, so that activity can be compared between one sample and another, and between one laboratory and another. The conditions chosen are usually at the optimum pH, 'saturating' substrate concentrations, and at a temperature that is convenient to control. In many cases the activity is measured in the opposite direction to that of the enzyme's natural function. Nevertheless, with a complete study of the parameters that affect enzyme activity it should be possible to extrapolate to the activity expected to be occurring in vivo.

The factors that affect the activity of an enzyme include substrate concentrations(s), pH, ionic strength and nature of salts present, and temperature. Activity is measured as the initial rate of substrate utilization when no products are present (a situation that rarely occurs in vivo). There are many compounds that may act as inhibitors which repress the activity, so they should not be present. The subject of enzyme inhibitors is a complex one which will not be dealt with here. However, it is worth noting that the converse, namely the involvement of

nonsubstrate activators, must be attended to with many enzymes, since they can be totally inactive without an activator present.

**The effect of substrate concentration**

The traditional enzyme has a hyperbolic response to substrate concentration, according to the Michaelis–Menten equation:

$$V=Vmax[S]/Km+[S]$$

Where [S] is the substrate concentration, v is the rate measured, Vmax is the maximum theoretical rate at infinite substrate concentration, and Km is the Michaelis constant. Applying this formula we find that the rate v is one-half of Vmax when [S] = Km, and 91% of Vmax when [S] = 10×Km. Km. The substrate concentration that is used in enzyme assays is chosen according to parameters such as the Km, the solubility of the substrate, whether high concentrations may inhibit, and the cost of the substrate. If values much less than 2×Km are used, it becomes more critical to know exactly what the concentration is (some preparations of unusual substrates may be impure, or the exact amount present may not be known). This is because the rate measured varies with substrate concentration more rapidly as the substrate concentration decreases, as can be seen in Figure 1. In most cases an enzyme assay has already been established, and the substrate concentration, buffers and other parameters used previously should be used again. There are many enzymes which do not obey the simple Michaelis–Menten formula. The most extreme deviations are with those enzymes known as ‘allosteric’, in which a sigmoid shape of response is found (Figure 1). With many allosteric enzymes an activator is required, and this can convert the sigmoid shape to a hyperbolic curve. Each allosteric enzyme has its own specific characteristics, so we cannot generalize about their behaviour.

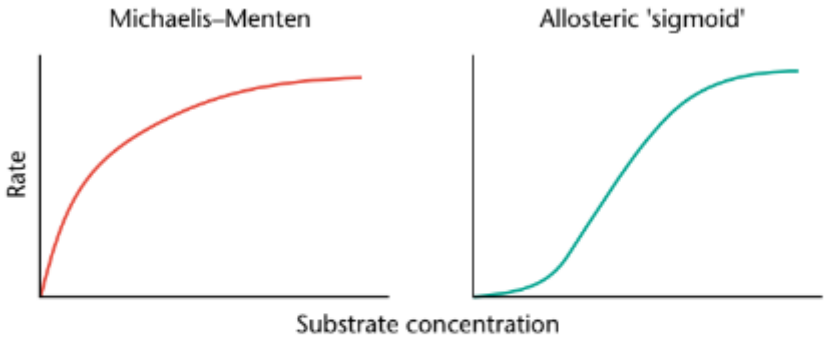


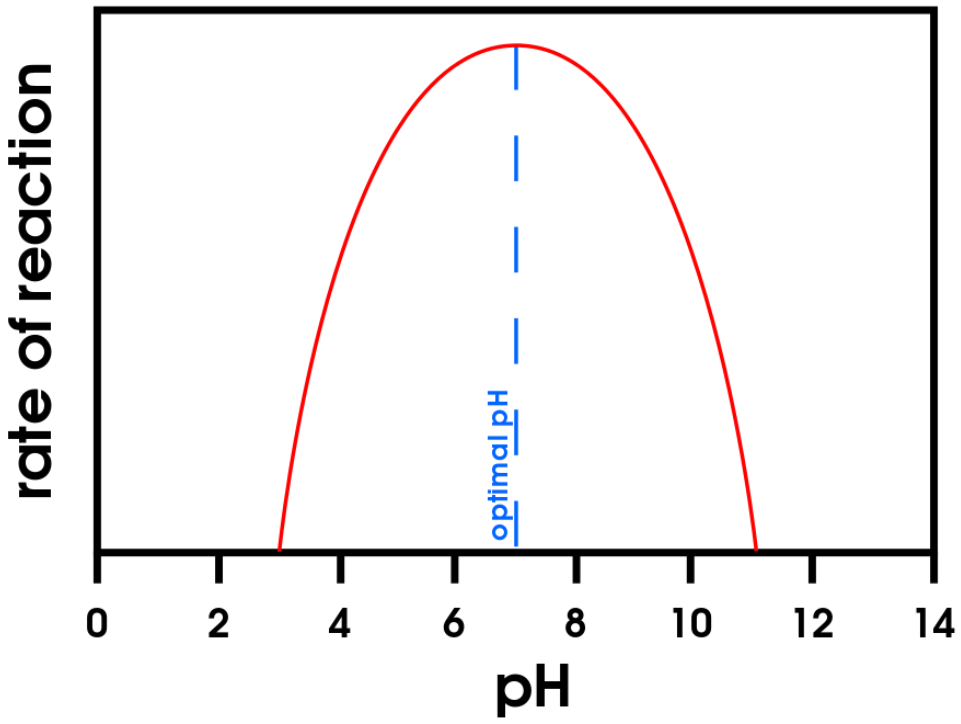

**Figure 1** Comparison of a conventional Michaelis–Menten enzyme with an allosteric enzyme: the rate variation with substrate concentration.

**The effect of pH on enzyme activity**

Enzymes are active only within a limited range of pH. But the limits may be wide, e.g. pH 5 to 10, or narrow, e.g. over 1 pH unit. Within the range there will be an optimum at which the maximum activity (the highest value for Vmax) is attained: this could be a short range in itself. The activity can also be affected by the nature of the buffer used. There could be a discontinuity in activity over the pH range tested because of the use of different buffers. Alternative buffers for a given pH should be tested.

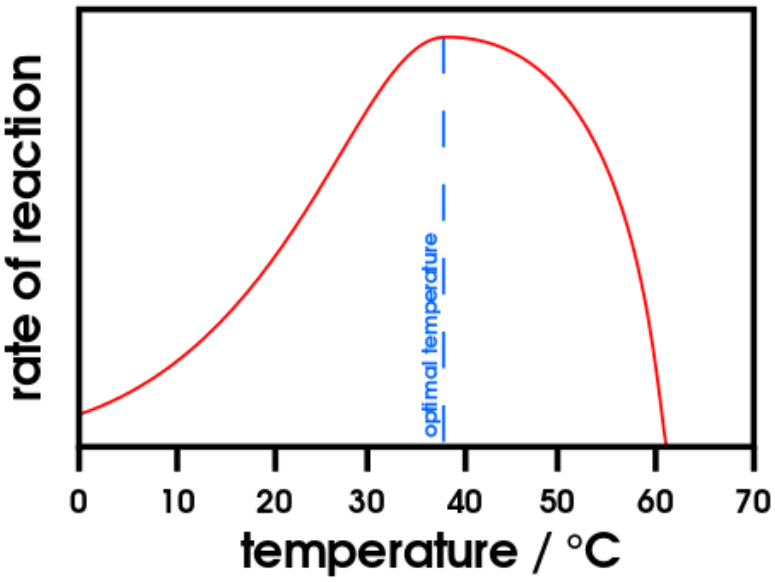
The effect of pH is generally tested at high substrate concentration. However, if tested at low concentration, it is possible that the value of v may change with pH because of an effect not on Vmax, but on Km. For standard enzyme assays, a value of pH is chosen that is close to the optimum, unless some other component of the assay mixture cannot operate at that pH.

It should be noted that the optimum pH is reaction direction-specific. In more cases than not, the optima in the two directions will be different, especially if there is uptake/evolution of a proton in the reaction. Some examples of this are with nicotinamide cofactor (NAD(P))-specific dehydrogenases, in which the optimum pH with NAD(P) 1 as oxidizing cofactor is generally in the range 9 to 10, whereas the optimum for reduction of substrate by NAD(P)H is around pH 6 to 7.



**Effect of temperature**

Temperature affects enzyme activity in much the same way as it affects other chemical reactions. Rates increase by between 4 and 8% per degree C, although at high temperatures denaturation of the enzyme protein decreases product formation. Thus it is important when carrying out an enzyme assay to ensure that the temperature remains constant, and also that you know exactly what it is. For comparison with other results that may have been reported at other temperatures, the exact temperature coefficient should be known; if not, a figure of 6% per degree is a useful approximation.




**Effect of ionic strength, salts**

This is a complex subject as each enzyme responds in a unique way to ionic strength I (salt content). Some enzymes are maximally active at the lowest I, while others require substantial levels of salt for significant activity. Most are inhibited at high (>0.5mol/L) salt. For most enzymes there is a variation of activity with I, so the value should be fixed and recorded (it is implicit in the total composition of the assay buffer). A natural intracellular ionic strength is typically in the range of 0.15 to 0.2 mol/L

**Initial Rates and Steady State Turnover**

Definition of the initial rate of an enzymatic reaction. An enzyme assay is set up with appropriate buffers and with the substrates present, but no products. The enzyme is added, and products begin to be formed. The initial rate of reaction occurs at this moment (after a short lag, which may be only milliseconds). In theory the reaction rate declines thereafter, owing to product accumulation. This decline is often not observable because the thermodynamics of the reaction being catalysed greatly favours product formation. However, if the equilibrium of thereaction substantially favours the starting reactants, then a significant slowing of product formation may be observed early, owing to reverse reaction. It is the aim of a successful enzyme assay to measure the product formation before it has accumulated

sufficiently to affect the initial rate. Coupled reactions help by removing the product as it forms.

**Steady state turnover**

This term refers to the situation in which there is a steady and unchanging flow of substrate through to product. 'Initial steady state' is the term sometimes used to describe the initial rate as above. More useful, though, is the use of the term steady state in a metabolic pathway, in which the net flow through an enzyme is determined by both the concentration of its substrate, which is constantly being replenished by the previous enzyme, and of its product, which is steadily being removed by the next enzyme, all enzymes having the same flux, or steady state net forward rate.

**Measurement of Enzyme Activity**

**Stopped assays**

To measure the activity of an enzyme one must measure how much product is formed over a given time or, in some cases, how much substrate has been used up, which should be the same thing. Thus ideally a method for measuring either product or substrate in the presence of the other is required. There are many different approaches; this section will deal with what are known as 'stopped assays'. Stopped assays involve stopping the reaction after a fixed time, then measuring how much product has been formed. Any method is possible, from chemical, enzymatic to bioassay, and generally the simplest is chosen provided it is reliable. In many cases a selective method can distinguish between substrate and product, so that no separation step is required. For example, phosphate release from a phosphate ester can be measured by the standard phosphomolybdate procedure. Otherwise separation of unused substrate from product may be needed. This is essential with radiochemical assays, in which the measurement is of radioactivity, not a specific test for the product itself. Separation methods include chromatographic (thin-layer chromatography, TLC; high-performance liquid chromatography, HPLC), solubility and partition procedures.

Methods for stopping the reaction include those which denature the enzyme, such as strong acid, alkali or detergent; heat; or treatments with irreversible inhibitors such as heavy metal ions. In some cases the enzyme can be stopped by addition of a complexing agent such as ethylenediaminetetraacetic acid (EDTA), which removes metal ions essential for activity; even chilling on ice may be sufficient. It is important that stopped assays are checked at least once with varying times of incubation, to ensure that the rate is linear through the period selected for the standard method.

**Continuous assays**

The alternative to a stopped assay is a continuous one in which the progress of the reaction is followed as it occurs. Continuous assays are much more convenient in that the result is seen immediately, and any deviation the initial rate shows from linearity can be observed. On the other hand not all enzymes have an assay method that can be observed continuously. The simplest continuous assay is one in which the action of the enzyme itself can be followed by changes in absorbance (e.g. NAD(P)H at 340 nm with dehydrogenases), fluorescence, viscosity, pH, or one of several other possible physical parameters. In many examples of hydrolase assays, an artificial substrate which releases a coloured or fluorescent product is

used. But most enzymes do not produce any change in a readily detectable physical parameter by their activity. This can be overcome using a coupled continuous method. In this process, the product is acted on further (usually by other enzymes that are added to the mixture), until an ultimate product is formed which can be observed physically. A great advantage of coupled assays is that the product is removed, so helping to keep the measured rate constant over a long period by avoiding product inhibition and reversal of reaction.

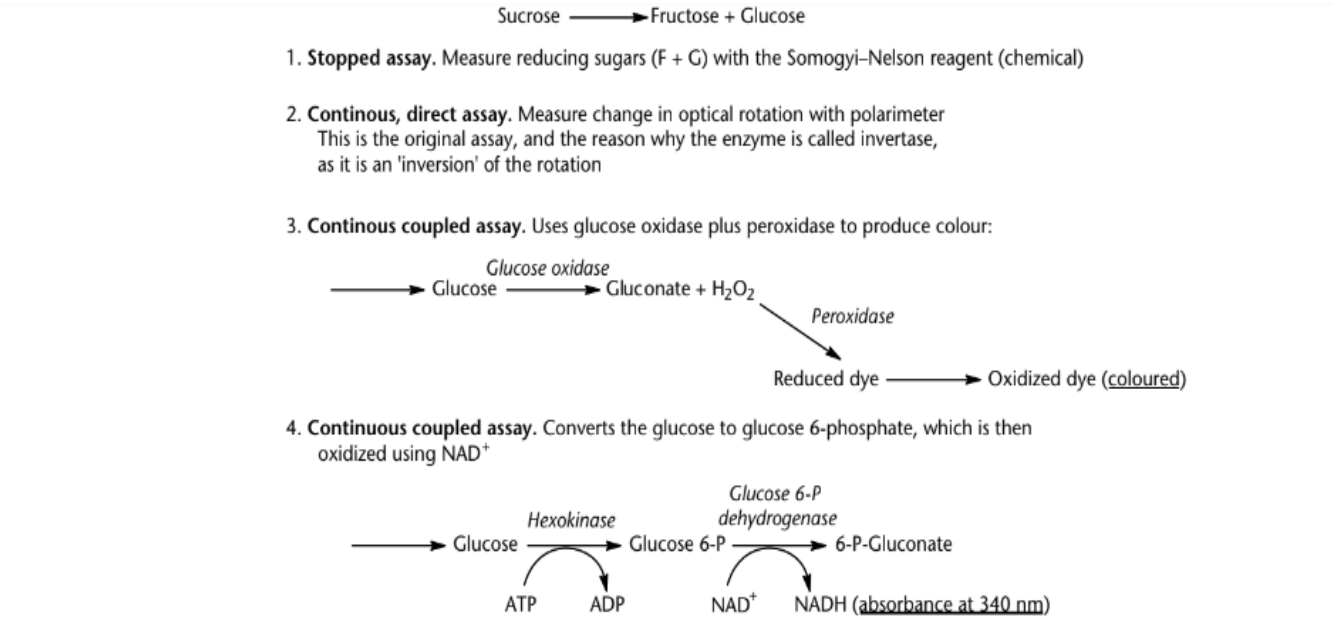



**Figure 2** Four different ways of assaying the enzyme invertase.

# B.SC MEDICAL MICROBIOLOGY 2ND SEMESTER LAB MANUAL, PAPER- BMM 296

## 1. Estimation of blood glucose

Atlas Medical CE

**Glucose (Mono Reagent)**
**(GOD/POD method)**

IVD *For in vitro diagnostic use only*

Store at 2-8°C

**INTENDED USE**

For the determination of glucose in serum or plasma.

**INTRODUCTION**

Glucose is the major carbohydrate present in blood. Its oxidation in cells is the source of energy for the body.
Increased levels of glucose are found in diabetes mellitus, hyperparathyroidism, pancreatitis, and renal failure.
Decreased levels are found in insulinoma, hypothyroidism, hypopituitarism, and extensive liver disease.

**PRINCIPLE OF THE METHOD**

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconate. The formed hydrogen peroxide ($H_2O_2$) is detected by a chromogenic oxygen acceptor, phenol, 4-Aminophenazone (4-AP) in the presence of peroxidase (POD):

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + (4\text{-AP}) \xrightarrow{POD} \text{Red Quinone dye} + H_2O$$

The intensity of the color formed is proportional to glucose concentration in serum.

**REAGENTS COMPOSITION**

| | | |
|---|---|---|
| R | GOD | 15ku/L |
| | POD | 1.0ku/L |
| | Phenol | 0.3mmol/L |
| | 4-AP | 2.6mmol/L |
| | BufferpH7.55 | 92mmol/L |
| | Stabilizers and activators | |
| GLUCOSE STD | Glucose aqueous primary standard 100mg/dl | |

**EQUIPMENTS NEEDED BUT NOT PROVIDED**

- Spectrophotometer or colorimeter measuring at 505nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

**PREPARATION**

- Reagent and standard provided are ready to use.

**STORAGE AND STABILITY**

- All components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminants during their use.
- Do not use reagents beyond the expiration date.
- Signs of reagent deterioration:
  - Presence of particles and turbidity.
  - Blank absorbance against water is more than 0.2.

**COLLECTING AND HANDLING OF SPECIMENS**

Use serum, or plasma free of hemolysis.
When blood is drawn and permitted to clot and to stand uncentrifuged at room temperature, the average decrease in serum glucose is 7%/1h (5-10mg/dl).
In separated, unhemolyzed serum, glucose concentration is generally stable up to 8h at 25°C or 72h at 4°C, if kept free of bacterial contamination.

**ASSAY PARAMETER**

| | | | |
|---|---|---|---|
| **Reaction** | End point | **Interval** | ---- |
| **Wavelength** | 505nm | **Sample Vol.** | 0.01ml |
| **Blank** | Reagent blank | **Reagent Vol.** | 1.0ml |
| **Incub. Temp.** | 37°C/15-25°C | **Standard** | 100mg/dl |
| **Incub. Time** | 5min/10min | **Factor** | ----- |
| **Reac. Slope** | increasing | **linearity** | Up to 600mg/dl |
| **Units** | mg/dl | | |

**ASSAY PROCEDURE**

1. Wavelength.....505nm (500-510)
2. Cuvette.....1cm.light path
3. Temperature.....37°C/15-25°C.
4. Adjust the instrument to zero with distilled water.
5. Pipette into clean dry test tubes labeled as Blank (B), Standard(S), and Sample:

| Addition Sequence | Blank | Standard | Sample |
|---|---|---|---|
| Glucose mono reagent | 1.0ml | 1.0ml | 1.0ml |
| Glucose standard | - | 0.01ml | - |
| Sample | - | - | 0.01ml |

6. Mix well and incubate at 37°C for 5 min or at 15-25°C. (25°C) for 10 min.
7. Measure the absorbance of the standard and test sample against blank.
8. After incubation the color is stable between 15-30min.

**CALCULATIONS**

Glucose (mg/dl) = (A) Sample x100(Standard Conc.)
(A) STD

**Conversion factor**: mg/dL x 0.0555= mmol/L.

**QUALITY CONTROL**

- To ensure adequate quality control, it is recommended that each run includes assayed normal and abnormal controls.
- If control values are found outside the defined range, check the instrument calibration, and reagent for problems.
- Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

**REFERENCE VALUES**

Serum or plasma:
60-110 mg/dL = 3.33-6.11 mmol /L
These values are for guidance purposes; each laboratory should establish its own reference range, according to its own geographic area.

**PERFORMANCE CHARACTERISTICS**

***Measuring range (Linearity):***
The assay is linear between 10mg/dl and 600 mg/dl.
If the results obtained were greater than 600mg/dl, dilute the sample to half with Nacl 9g/L and multiply the result by 2.

**Sensitivity:**
1 mg/dl = 0.0032 (A)

***Accuracy:***
Results obtained using the reagent compared well with other commercial reagents.

***Precision:***

| | Intra-assay(n=20) | | Inter-assay(n=20) | |
|---|---|---|---|---|
| Mean(mg/dl) | 91.73 | 228.51 | 97.68 | 244.17 |
| STD | 3.51 | 10.64 | 3.42 | 10.69 |
| C.V% | 3.83 | 4.66 | 3.50 | 4.38 |

The results of the performance characteristics depend on the analyzer used.

GOD-POD METHOD

## Principle

The Glucose Tolerance test is used for the diagnosis of Diabetes Mellitus and related disease conditions. The oral glucose tolerance test requires a fasting glucose blood draw followed by a 75 gram dose of glucola, then a 2 hour post load glucose blood draw. An abnormally high fasting glucose value with a delayed return to normal indicates decreased tolerance to glucose and supports a diagnosis of either impaired tolerance or a provisional diagnosis of diabetes.

## Patient Preparation

The following conditions should be met before performing an oral glucose tolerance test.

1. Discontinue, when possible, all non-essential medications known to affect glucose metabolism. Medications include: apmetamine, arginine, beta-adrenergic blockers, diuretics, epinephrine, glucocorticoids, glucose administered intravenously, insulin, lithium, oral contraceptives, oral hypoglycemic agents, phenothiazines, phenytoin, and salicylates.

2. Patient must be fasting for at least 8 hours, but no more than 14 hours before the test (water is acceptable).

3. Individual should remain at rest, and avoid medications, smoking, caffeine, and alcohol before and during the test. Alcohol should not be consumed a minimum of 3 days prior to the test.

4. The individual should not be ill and should have had normal physical activity and carbohydrate intake greater than 150 grams/day for at least 3 days before the test.

5. Should not be done during recovery from acute illness, emotional stress, surgery, trauma, pregnancy, inactivity due to chronic illness; therefore, is of limited value in hospitalized patients. A two week recovery time is recommended before tolerance testing.

## Reagents

Glucose Tolerance Beverage, 75 grams

## Procedure

1. Patient must be fasting for at least 8 hours, but no more than 14 hours before the test. Water is acceptable during the fast.

2. Collect a fasting blood glucose specimen from the patient.

3. Give adult patients 75 grams of glucose (1 full bottle of glucose tolerance beverage). The glucose beverage should be consumed within 5 minutes. The patient should remain at rest and avoid medications, smoking, caffeine and alcohol for the duration of the test. Water is allowed. For children, the dose of glucola must be adjusted for the weight of the child. Children: 1.75g/kg up to 75g of Glucola. (Weight in lbs/2.2)1.75 = grams of Glucola to be given not to exceed 75 grams.

4. Obtain blood glucose specimen 2 hours after the patient finishes drinking the glucose tolerance beverage. Continue sample collection at 3, 4, 5 or 6 hours if other than the standard 2-hour tolerance is requested. ** This procedure is not to be used for gestational patients.

## Reference Ranges

All tolerance tests are reported along with the following comment: Reference ranges based on the American Diabetes Association Clinical practice recommendations: Glucose load: 75 gram glucose drink Fasting glucose: Normal: fasting glucose - <100mg/dL, impaired fasting glucose –

100-125mg/dL, Diabetes: ≥126 mg/dL, 2 hour post load of glucose: Normal- <140mg/dL,

# CREATININE KIT

(Alkaline Picrate method)

For the determination of Creatinine in serum and urine.
(For Invitro Diagnostic Use Only)

**Summary**

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

**Principle**

Picric acid in an alkaline medium reacts with creatinine to form a orange coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample.

**Creatinine + Alkaline Picrate - Orange Coloured Complex**

**Reference values**

| | Serum | Urine, 24hrs. collection |
|---|---|---|
| Males | 0.6-1 .2mg% | 1.1- 3 .0gm |
| Females | 0.5-1 .1mg% | 1.0- 1 .8gm |

It is recommended that each laboratory establish its own normal range representing its patient population

| Contents | 15 Tests | 35 Tests | 70 Tests |
|---|---|---|---|
| L1 : Picric Acid Reagent | 60 ml | 140 ml | 2 x 140ml |
| L2 : Buffer Reagent | 5ml | 12 ml | 25 ml |
| S : Creatinine Standard (2 mg/di) | 5ml | 5ml | 10ml |

**Storage / stability**

All reagents are stable at R.T. till the expiry mentioned on the labels.

**Reagent Preparation**

Reagents are ready to use. Do not pipette with mouth.

**Sample material**

Serum or Urine.
Creatinine is stable in serum for 1 day at 2-8°C
Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/ deionised water before the assay.

**Procedure**

| | |
|---|---|
| Wavelength/ filter | 520nm (Hg546 nm)/ Green |
| Temperature | R.T. |
| Light path | 1cm |

**Deproteinization of specimen:**

Pipette into a clean dry test tube

| | |
|---|---|
| Picric acid reagent (L1) | 2.0ml |
| Sample | 0.2ml |

Mix well and centrifuge at 2500 - 3000 rpm for 10 min. to obtain a clear supernatant

**Colour development :**

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T):

| Addition Sequence | B (ml) | S (ml) | T (ml) |
|---|---|---|---|
| Supernatant | | | 1.1 |
| Picric Acid Reagent (L1) | 1.0 | 1.0 | |
| Distilled water | 0.1 | | |
| Creatinine Standard (S) | | 0.1 | |
| Buffer Reagent (L2) | 0.1 | 0.1 | 0.1 |

Mix well and keep the test tubes at R.T. for exactly 20 minutes. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank.

**Calculations**

$$\text{Creatinine in mg\%} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 2.0$$

$$\text{Urine Creatinine in gm/Lit.} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 1.0$$

Urine Creatinine gm/24Hrs. = Urine Creatinine in gm/L x *Vol* of urine in 24 Hrs.

**Linearity**

The procedure is linear upto 8 mg/di of creatinine.
If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

**Note**

Maintain the reaction time of 20 min. as closely as possible since a longer incubation causes an increase in the values due to the reaction of pseudochromogens.
The determination is not specific and may be affected by the presence of large quantities of reducing substances in the sample.
The reaction is temperature sensitive and all the tubes should be maintained at a uniform temperatur.e

# TRIGLYCERIDES KIT

## (GPO / PAP method)

For the determination of Triglycerides in serum or plasma.

(For Invitro Diagnostic Use Only)

### Summary

Triglycerides are a form of fatty acid esters. They are produced in the liver by binding glycerol and other fatty acids. They are transported by VLDL and LDL and act as a storage source for energy. Increased levels are found in hyperlipidemias, diabetes, nephrotic syndrome, hypothyroidism. Increased levels are risk factor for arteriosclerotic coronaiy disease and peripheral vascular disease. Decreased levels are found in malnutrition and hyperthyroidism.

### Principle

Lipoprotein lipase hydrolyses triglycerides to glycerol and free faky acids' The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxidefurther reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directlyproportional totheamountof triglycerides presentinthesample.

$$\text{Triglycerides} \xrightarrow{\text{LipoproleinLipase}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GlycefiolKinase}} \text{Glycerol 3 Phophate} + \text{ADP}$$

$$\text{Glycerol 3 Phophate} + O_2 \xrightarrow{\text{Glycero/3PO}} \text{Dihydroxyacetonephos.} + H_2O_2$$

$$H_2O_2 + \text{4Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2O$$

### Normal reference values

Serum/Plasma (Suspicious) : 150 mg/dl and above
(Elevated) : 200 mg/dl and above

It is recommended that each laboratory establish its own normal range representing its patient population.

| Contents | is=i | 7smi | 2x75ml 2x150ml |
|---|---|---|---|
| L1 : Enzyme Reagent 1 | 20 ml | 60 ml | 2 x 60 i lx 120 ml |
| L2 : Enzyme Reagent 2 | 5 ml | 15 ml | 2 x 15 l2k 30 ml |
| 5 : Triglycerides Standard (200 mg/dl) | 5 ml | 5 ml | 5 6fril |

### Storage / stability

Contents are stable at 2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent : Pour the contents of 1 bottle of L2 (Enzyme Reagent 2) into 1 bottle of L1 (Enzyme Reagent 1).This working reagent is stable for at least 8 weeks when stored at 2-8' C. Upon storage the working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent 1) & 1 part of L2 ( Enzyme Reagent 2 ). Alternatively0.8 ml ofL1 and 0.2 ml ofL2 may also be used instead of 1 ml of the working reagent directly during the assay.

### Sample material

Serum, plasma. Triglycerides is reported to be stable in the sample for Sdayswhenstoredat 2-8°C.

### Procedure

| | |
|---|---|
| Wavelength/filter | 505nm(Hg 546 nm)/Green |
| Temperature | 37 C/R.T. |
| Light path | 1 cm |

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

| Additiion Sequence | B (ml) | S (ml) | (ml) |
|---|---|---|---|
| Working reagent | 1.0 | 1.0 | 1.0 |
| Distilled water | 0.01 | | |
| Triglycerides Standard(S) | | 0.01 | |
| Sample | | | 001 |

Mix well and incubate at 37 C for 5 min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 60 Min.

### Calculations

$$\text{Triglycerides in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 200$$

### Linearity

This procedure is linear upto 1000 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay.

### Note

Fasting samples of 12 to 14 hrs. are preferred. Fatty meals and alcohol may cause elevated results. Patient should not drink alcohol for 24 hrs. before the test

# UREA KIT

(GLDH Kinetic method)

For the determination of Urea in serum or plasma.
(For Invitro Diagnostic Use Only)

**Summary**

Urea is the end product of protein metabolism. It is synthesised in the liver from the ammonia produoed by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liverfailure and pregnancy.

Principle

Urease hydrolyzes urea to ammonia and CO,. The ammonia formed further combines with a Ketoglutarate and NADH to form Glutamate and NAD.The rate of oxidation of NADH to NAD is measured as a decrease in absorbance in a fixed time which is proportional to the urea concentration in the sample.

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\text{-Ketoglutarate} + 2NADH \xrightarrow{GLDH} 2\text{L-glutamate} + 2NAD^+ + 2H_2O$$

Normal reference values

| | |
|---|---|
| Serum/Plasma | 14 - 40 mg/dl |
| Urine | Upto 20 g/l |

It is recommended that each laboratory establish its own normal range representing its patient population.

| Contents | 75 ml | 2 x 75 ml | 2 x 150 ml |
|---|---|---|---|
| L1 : Enzyme Reagent | 60 ml | 2 x 60 ml | 2 x 120ml |
| L2 : Starter Reagent | 15 ml | 2 x 15 ml | 2 x 30ml |
| S : Urea Standard (40 mg/dl) | 5 ml | 5 ml | 5 ml |

Storage / **stability**

Contents are stable at2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.
Workingreagent: For sample startassays a singlereagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottleofL1 (Enzyme Reagent). Thisworking reagent isstableforatleast 10dayswhen stored at2-8'C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively0.8mlofL1 and 0.2 ml ofL2 may also be used instead of 1 ml of the working reagent directly during the assay.

Allow the Working Reagent to stand for 30 min. before use.

**Sample material**

Serum, plasma, Urine. Dilute urine 1+ 49 with distilled water before the assay(results x 50). Urea is reported to be stable in the serum for 5 days when stored at2-8'C.

Procedure

| | |
|---|---|
| Wavelength/filter | 340 nm |
| Temperature | 37°C/30'CI25'C |
| Light path | 1 cm |

Substrate StartAssay:

Pipette into a clean dry test tube labelled Standard (S) or Test(T):

| AdditionSequence | \Sjl\jfl 37'C/30'CI25'C |
|---|---|
| Enzyme Reagent (L1) | 0.8 ml |
| Urea Standard/Serum/Diluted urine | 0.01 ml |
| Incubate at the assay temperaturefor 5 minute | |

Mix well and read the initial absorbanceA,forthe Standard and Test after exactly 30 seconds. Read another absorbance A, of the Standard and Testexactly 60 seconds later. Calculate the change in absorbance A A for both the Standard and Test.

Sample StartAssay :
Pipette into a clean dry test tube labelled Standard (S) or Test(T):

| Addition Sequence | (S)/(T) 37°C/30'CI25°C |
|---|---|
| Working reagent | 1.0 ml |
| Bringtoassaytemperatureand add Urea Standard/Serum/ Dilutedurine | 0.01ml |

Mix well and read the initial absorbanceA,for the Standard and Test after exactly 30 seconds. Read another absorbanceA, of the Standard and Testexactly 60 seoonds later. Calculate the change in absorbance AA for both the Standard and Test.

| | | | |
|---|---|---|---|
| ForStadad | dAS | @s | A,s |
| ForTest | bAT | QT | A,T |

Calculations

$$\text{Urea in mg/dl} = \frac{AAT}{AAS} \times 40$$

# URIC ACID KIT

## (Uricase/PAP method)

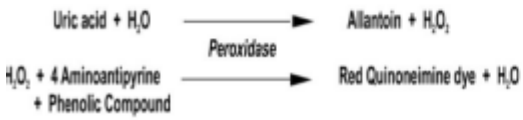
For the determination of Uric Acid in serum or plasma.
(For Invitro Diagnostic Use Only)

### Summary

Uric acid is the end product of purine metabolism. Uric acid s excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation. Increased levels are found in Gout, arthritis, impaired renal functions,and starvation. Decreased levels are found in Wilson'sdisease, Fanconis syndrome and yellow atrophy of the liver.

### Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenoliccompound and4 aminoantipyrinebythecatalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.




### Normal reference values

| | | |
|---|---|---|
| Serum/Plasma | (Males) | 3.4 - 7.0 mg/dl |
| | (Females) | 2.5 - 6.0 mg/dl |

It is rec0mmended that each laboratory establish its own normal range representing its patient population.

| Contents | 25 ml | 75 ml | 2 x 75 ml | 2 x 150 ml |
|---|---|---|---|---|
| L1 : Buffer Reagent | 20 ml | 60 ml | 2 x 60 ml | 2 x 120 ml |
| L2 : Enzyme Reagent | 5 ml | 15 ml | 2 x 15 mg | x 30 ml |
| S : Uric Acid Standard (8 mg/dl) | 5 ml | 5 ml | 5 ml | 5 ml |

### Storage / stability

Contents are stable at2-8'C till the expiry mentioned on the labels.

### Reagent Preparation

Reagents are ready to use.
Working reagent : Pour the contents of 1 bottle of L2 (Enzyme Reagent) into 1 bottle of L1 (Buger Reagent). This working reagent is stableforatleast4weeks when stored at 2-8'C. Upon storage the working reagent may develop a slight pink colour however this does not a8ect the performanceofthe reagent.
Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Buffer Reagent) and 1 part of L2 (Enzyme Reagent). Alternatively0.8mlofL1 and 0.2 ml ofL2 may also be used instead of 1 ml of the working reagent directly during the assay.

### Sample material

Serum, plasma. Uric Acid is reported to be stable in the samplefor3-5 days when stored at 2-8'C.

### Procedure

| | |
|---|---|
| Wavelength /filter | 520 nm(Hg 546 nm)/YellowGreen |
| Temperature | 37'C/R.T. |
| Lightpath | 1 cm |

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

| Addition **Sequence** | B (ml) | 5 (ml) | T (ml) |
|---|---|---|---|
| Workingreagent | 1.0 | 1.0 | 1.0 |
| Distilled water | 0.02 | | |
| UricAcid Standard (S) | | 0.02 | |
| Sample | | | 0.02 |

Mix well and incubate at37'C for 5 min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 30 Min.

### Calculations

$$\text{Uric Acid in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 8$$

### Linearity

This procedure is linear upto 20 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Protein Estimation:

Proteins are constituents of muscle, enzymes, harmones and several other key functional and structural entities in the body. They are involved in the maintenance of the normal distribution of water between blood and the tissues. Consisting mainly of albumin and globulin in the fractions vary independently and widely in diseases. Increased levels are found mainly in dehydration. Decreased levels are found mainly in malnutrition, impaired synthesis, protein losses as in hemorrhage or excessive protein catabolism. PRINCIPLE: Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

CONTENTS: Reagent 1 : Biuret Reagent Reagent 2 : Protein Standard 6 g/dl MATERIALS REQUIRED BUT NOT PROVIDED:- Clean & Dry Glassware. - Laboratory Glass Pipettes or Micropipettes & Tips. - Colorimeter or Bio-Chemistry Analyzer.

SAMPLES: Serum, Heparinized/EDTA Plasma. Proteins are reported to be stable in the sample for 6 days at 2-8°C

PREPARATION OF REAGENT & STABILITY : All reagents are stable till the expiry date mentioned on the label at room temperature. Standard vial once opened should be stored at 2 -8°C, it is stable till the expiry date mentioned on the vial. All reagents are in ready to use form.

GENERAL SYSTEM PARAMETERS: Reaction type : End point Wave length : 546 nm (530 - 570 nm) Temperature : Room temperature Incubation : 5 minutes Reagent volume : 1.0 ml Sample volume : 10 µl Standard concentration : 6 gm/dl. Zero setting : Reagent blank Light path : 1 cm

PROCEDURE: Pipette into clean dry test tube labeled as Blank (B), Standard (S) and Test (T).

| Addition sequence | B | S | T |
|---|---|---|---|
| Biuret Reagent | 1ml | 1ml | 1ml |
| Standard | | 10µl | |
| Sample | | | 10µl |

Mix well, Incubate for 5 minutes at Room temperature. Measure the absorbance of the standard Abs. S and sample Abs. T against the reagent blank , within 60 minutes.

CALCULATION : Total Protein Conc. (gm/dl) = Abs. T/ Abs. S x 6 NORMAL VALUE : Serum : 6.0 - 8.0 gm/dl It is recommended that each laboratory establish its own normal range. LINEARITY : This procedure is linear upto 10 gm/dl. Samples above this concentration should be diluted with normal saline and results should be multiplied by dilution factor.

QUALITY CONTROL : For accuracy it is necessary to run known controls with every assay. LIMITATION & PRECAUTIONS : 1. Storage condition mentioned on the kit must be adhered. 2. Do not in any case freeze or expose reagent to high temperature as it may effect the performance of the kit. 3. Before the assay bring all the reagents to room temperature. 4. Avoid contamination of the reagents during the assay process. 5. Use clean glassware free from dust or debris

Quantia® HbA1c

**Auto Kit**

**Turbidimetric immunoassay for quantitative determination of HbA1c in human blood suitable for automated analyzers**

**SUMMARY**

Glycated hemoglobin (GHb) also commonly known as glycosylated hemoglobin, glycohemoglobin, HbA1, HbA1c or A1c is a term used to describe a series of stable minor hemoglobin components formed slowly and non-enzymatically from hemoglobin and glucose. The glycation of hemoglobin can occur at various sites present on the polypeptide chains of the hemoglobin molecule with different carbohydrate (sugar) molecules. The glycohemoglobin is subdivided into subfractions depending on each of the glycation sites and reaction partners involved in glycation. More recently HbA1c is defined as Hb that is irreversibly glycated at one or both N-terminal valines of the β-chain. The remaining GHbs have glucose, glucose-6-phosphate, fructose-1, 6-diphosphate, or pyruvic acid bound to one of the 44 additional sites occurring at ε-amino group of lysine residues or at the $NH_2$ terminal of the α-chain. Formation of HbA1c is irreversible and the blood levels depend on both the life span of the red blood cell (average 120 days) and the blood glucose concentration. The rate of formation of HbA1c is directly proportional to the ambient glucose concentration. The amount of HbA1c therefore represents the integrated values of glucose over the preceding six to eight weeks and provides an additional means of assessing glycemic control. The results of HbA1c are not influenced by recent meals, physical activity or emotional stress.

Maintaining glycemic levels as close to diabetic range as possible has been demonstrated to have a powerful beneficial impact on diabetes-specific complications, including retinopathy, nephropathy and neuropathy in the setting of type 1 diabetes; in type 2 diabetes, more intensive treatment strategies have likewise been demonstrated to reduce complications. Intensive glycemic management resulting in lower HbA1c levels has also been shown to have a beneficial effect on cardiovascular disease complications in type 1 diabetes.

The measurement of HbA1c in human blood is therefore considered the most important marker for long-term assessment of glycemic state in patients with diabetes, and goals for therapy are set at specific HbA1c target values.

The two seminal studies, The Diabetes Control and Complications trial (DCCT) and the United Kingdom Prospective Diabetes study (UKPDS) proved the usefulness of HbA1c measurement in predicting the risk of developing microvascular complications and, as a consequence, have led to the widespread recommendations of its increased use.

**Quantia HbA1c is a turbidimetric immunoassay for the direct determination of HbA1c in human blood without the need to estimate total hemoglobin.**

**REAGENTS**

1. **Quantia HbA1c** Latex Reagent (R1): ready to use uniform suspension of latex particles.
2. **Quantia HbA1c** Antibody Reagent (R2): ready to use solution of mouse anti human HbA1c monoclonal antibody.
3. **Quantia HbA1c** Antibody Reagent (R3): ready to use solution of goat anti mouse human IgG antibody.
4. **Calibrators: Quantia HbA1c** calibrator set contains three lyophilised calibrators with different HbA1c concentrations (Level I, Level II, Level III). The **Quantia HbA1c** calibrator utilizes lysed human blood that is prepared from healthy non-diabetic blood donors. The elevated levels of HbA1c have been produced by controlled glycation of normal HbA1c level red cells. The % HbA1c concentration "S" for each calibrator is printed on the respective vial labels and at the end of the package insert.

The assigned % HbA1c values are lot specific and traceable to a NGSP (National Glycohaemoglobin Standardisation Program) certified method that has documented traceability to Diabetes Control and Complications Trial (DCCT) eference method. In turn the assigned values are traceable to the International Federation of Clinical Chemists (IFCC) reference method through the master equation:

NGSPA1c = 0.915 (IFCCA1c) + 2.15
IFCCA1c = (NGSPA1c-2.15) / 0.915

5. **Quantia HbA1c** Hemolysing Reagent: ready to use solution

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its performance.

**REAGENT STORAGE AND STABILITY**

1. Store the reagents at 2-8°C. DO NOT FREEZE.
2. The shelf life of the latex reagent (R1), antibody reagent (R2), antibody reagent (R3) and Hemolysing reagent is as per the expiry date mentioned on the respective vial labels.
3. Working reagent must be prepared for application on automated analyzers with two reagent systems. The working reagent

must be prepared by mixing 1 part of R3 reagent to 2 parts of R2 reagent. The working reagent must be used as R2 reagent in these analyzer. The working reagent is stable for 7 days when stored at 2-8°C provided it is not contaminated.

4. Once reconstituted the reagent can be used for 7 days when stored tightly capped at 2-8°C provided it is not contaminated. The reagent should not be frozen or stored uncapped.

**Onboard stability (at 18-20°C):**
In appropriate bottles the Quantia HbA1c activation buffer (R1) is stable for 8 weeks if kept closed when not in use. The working reagent is stable for 5 days if the reagent bottle is kept closed when not in use.

**PRINCIPLE**
**Quantia HbA1c** is a turbidimetric immunoassay for direct determination of HbA1c and is based on the principle of agglutination reaction. The test specimen after treatment with Hemolysing solution is allowed to react with latex reagent (R1). Total Hb and HbA1c bind with same affinity to latex particles. The amount of binding is proportional to the relative concentration of both substances in blood. The reaction mixture is then allowed to react with mouse anti human HbA1c monoclonal antibody (R2) wherin the mouse anti human HbA1c antibody bind to the HbA1c on the latex. Goat anti mouse human IgG (R3) is then allowed to interact with the above reaction mixture which interacts with the HbA1c-mouse anti human HbA1c complex resulting in agglutination reaction that is measured at 630 nm. The increase in turbidity corresponds to the concentration of HbA1c in the test specimen.

**NOTE**
1. In vitro diagnostic reagent for professional use only. Not for medicinal use.
2. All reagents derived from human source have been tested for HbsAg and HIV antibodies and are found to be non-reactive. How ever handle the material as if infectious.
3. Reagents contain 0.09% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.
4. The reagents can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagents be verified with **Quantia HbA1c** controls (Cat. No.: 108130002) periodically.
5. Gently mix the **Quantia HbA1c** latex reagent well before use to achieve optimum test performance.
6. Gently mix each of the Quantia reagents to attain homogeneity prior to use for obtaining better performance.
7. As the reagents within lots have been matched, reagents from different lots must not be interchanged.
8. Do not use damaged or leaking reagents.
9. The **Quantia HbA1c Auto Kit** assay is standardised with **Quantia HbA1c** calibrators having values traceable to NGSP (National Glycohaemoglobin Standardisation Programme) certified method that has documented traceability to DCCT reference method.
10. The **Quantia HbA1c Auto Kit** reagents are not adaptable to Nephelometer.

**SPECIMEN COLLECTION AND PREPARATION**
No special preparation of the patient is required prior to specimen collection by approved techniques. No special additives or preservatives other than anticoagulants are required. Collect venous blood in EDTA using aseptic techniques.

**Specimen preparation:**
1. Mix the specimen (sample or reconstituted calibrator or reconstituted control) thoroughly to obtain uniform distribution of erythrocytes. Avoid bubble formation.
2. Take 500µl **Quantia HbA1c** Hemolysing solution in a test tube.
3. Add 10µl of homogenised specimen (sample/reconstituted calibrator/reconstituted control). Mix well and allow to stand for **15 minutes** or until complete lysis is apparent. This hemolysed specimen is referred as **Lysate**.

**Specimen stability:**
- Whole blood : 1 week at 2-8°C
- Lysate : 10 hours at 15-25°C
- Lysate : 10 days at 2-8°C

**ADDITIONAL MATERIAL REQUIRED**
Spectrophotometer with 630 nm wavelength filter and cuvette mode, well calibrated micropipettes, disposable tips, particulate free distilled water, test tube rack, incubator/waterbath set at 37°C, optically clean disposable cuvettes such as Quantiamate semi micro cuvettes/glass cuvettes, **Quantia HbA1c** lyophilised control (Cat. No. 108160002).

**TEST PROCEDURE**
Applications suitable for Hitachi 902, Olympus AU 400, can be made available on request.

**General Application parameter set up**
A defined application for the **Quantia HbA1c Auto kit** must be installed in accordance with the general instrument settings given below. For instructions refer the respective instrument manual.

# SGOT (ASAT) KIT

(Reitman & Frankel's method)

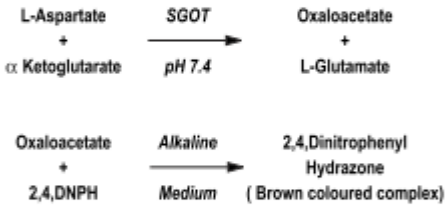
For the determination of SGOT (ASAT) activity in serum.
(For Invitro Diagnostic Use Only)

**Summary**

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood stream. Elevated levels are found in myocardial infarction, Cardiac operations, Hepatitis, Cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in pregnancy, Beri Beri and diabetic ketoacidosis.

**Principle**

SGOT converts L-Aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2,4,Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGOT (ASAT) is read off this calibration curve.




**Normal reference values**

Serum : 8 - 40 Units/ml

It is recommended that each laboratory establish its own normal range representing its patient population.

| Contents | 40 Assays | 80 Assays |
|---|---|---|
| **L1 :** Substrate Reagent | 25 ml | 50 ml |
| **L2 :** DNPH Reagent | 25 ml | 50 ml |
| **L3 :** NaOH Reagent (4N) | 25 ml | 50 ml |
| **S** : Pyruvate Standard (2mM) | 5 ml | 5 ml |

**Storage / stability**

Contents are stable at 2-8°C till the expiry mentioned on the labels. Sodium Hydroxide can be stored at R.T. till the expiry mentioned.

**Reagent Preparation**

All reagents are ready to use except NaOH Reagent (4N) which has to be diluted 1:10 with distilled/deionised water.
**Working NaOH reagent** : Dilute the Sodium Hydroxide to 250 ml or for every 1.0 ml of NaOH Reagent (4N) add 9.0 ml of distilled water. The Working Sodium Hydroxide reagent is stable at R.T. till the expiry mentioned, in a **plastic bottle**.

**Sample material**

Serum. Free from hemolysis. SGOT (ASAT) is reported to be stable in serum for 3 days at 2-8°C.

**Procedure**

Wavelength / filter : 505 nm (Hg 546 nm) / Green
Temperature : 37°C & R.T.
Light path : 1 cm

**Plotting of the Calibration curve :**

Pipette into five clean dry test tubes labelled as 1, 2, 3, 4, & 5

| Addition sequence | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Enzyme Activity (U/ml) | 0 | 24 | 61 | 114 | 190 |
| | (ml) | (ml) | (ml) | (ml) | (ml) |
| Substrate Reagent (L1) | 0.50 | 0.45 | 0.40 | 0.35 | 0.30 |
| Pyruvate Standard (S) | - | 0.05 | 0.10 | 0.15 | 0.20 |
| Distilled Water | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| DNPH Reagent (L2) | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Mix well and allow to stand at R.T. for 20 minutes. | | | | | |
| Working NaOH Reagent (L3) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |

Mix well and allow to stand at R.T. for 10 min. Measure the absorbances of the tubes 2 - 5 against tube (Blank). Plot a graph of the absorbances of tubes 2 - 5 on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis.

**Assay :**

Pipette into clean dry test tubes labelled as Blank (B) & Test (T) :

| Addition Sequence | (B) (ml) | (T) (ml) |
|---|---|---|
| Substrate Reagent (L1) | 0.50 | 0.50 |
| Incubate at 37°C for 3 minutes | | |
| Sample | - | 0.10 |
| Mix well and incubate at 37°C for 60 minutes | | |
| DNPH Reagent (L2) | 0.50 | 0.50 |
| Mix well and allow to stand at R.T. for 20 minutes. | | |
| Distilled Water | 0.10 | - |
| Working NaOH Reagent (L3) | 5.00 | 5.00 |

Mix well and allow to stand at R.T. for 10 min. Measure the absorbances of the Test (T) against Blank ( Blank) and read the activity of the test from the calibration curve plotted earlier

# CalKine SGPT (ALAT) Kit

(Mod. IFCC Method)

For the determination of SGPT (ALAT) activity in serum.

(For Invitro Diagnostic Use Only)

**Summary**

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzyme is also seen in myocardial infarction.

**Principle**

SGPT (ALAT) catalyzes the transfer of amino group between L-Alanine and α Ketoglutarate to form Pyruvate and Glutamate. The Pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGPT (ALAT) activity in the sample.

$$\text{L- Alanine} + \alpha\text{ Ketoglutarate} \xrightarrow{SGPT} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{LDH} \text{Lactate} + NAD^{+}$$

**Normal reference values**

Serum (males) : upto 40 U/L at 37°C
(females) : upto 31 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

| Contents | 4 x 25 ml | 2 x 150 ml |
|---|---|---|
| **L1 :** Enzyme Reagent | 4 x 20 ml | 2 x 120 ml |
| **L2 :** Starter Reagent | 20 ml | 60 ml |
| **C :** Bovine Serum Control | 1 ml | 1 ml |

**Storage / stability**

Contents are stable at 2-8°C till the expiry mentioned on the labels.

**Reagent Preparation**

Reagents are ready to use.

**Working reagent :** For sample start assays a single reagent is required. A working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 parts of L2 (Starter Reagent). This working reagent is stable for at least 3 weeks when stored at 2-8° C. Alternatively 0.8 ml of L1 and 0.2 ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

**Control :** Reconstitute with 1 ml of good quality D.W. Leave for 5 min to hydrate and mix by swirling, to avoid frothing. The reconstituted control is stable for at least 3 days when stored tightly stoppered at 2-8°C without contamination.

**Sample material**

Serum. Free from hemolysis. SGPT (ALAT) is reported to be stable in serum for 3 days at 2-8°C.

**Procedure**

Wavelength / filter : 340 nm
Temperature : 37°C / 30°C / 25°C
Light path : 1 cm

**Substrate Start Assay :**

Pipette into a clean dry test tube labelled as Test (T) :

| Addition Sequence | (T) 25°C / 30°C | (T) 37°C |
|---|---|---|
| Enzyme Reagent (L1) | 0.8 ml | 0.8 ml |
| Sample or Control (C) | 0.2 ml | 0.1 ml |
| Incubate at the assay temperature for 1 minute and add | | |
| Starter Reagent (L2) | 0.2 ml | 0.2 ml |

Mix well and read the initial absorbance $A_0$ & repeat the absorbance reading after every 1, 2 & 3 minutes. Calculate the mean absorbance change per minute (Δ A/ min.)

**Sample Start Assay :**

Pipette into a clean dry test tube labelled as Test (T) :

| Addition Sequence | (T) 25°C / 30°C | (T) 37°C |
|---|---|---|
| Working Reagent | 1.0 ml | 1.0 ml |
| Incubate at the assay temperature for 1 minute and add | | |
| Sample or Control (C) | 0.2 ml | 0.1 ml |

Mix well and read the initial absorbance $A_0$ after 1minute & repeat the absorbance reading after every 1, 2 & 3 minutes. Calculate the mean absorbance change per minute (ΔA/ min.)

**Calculations**

**Substrate / Sample start**

SGPT (ALAT) Activity in U/L 25°C / 30°C = ΔA / min. x 952
SGPT (ALAT) Activity in U/L 37°C = ΔA / min. x 1746

**TEMPERATURE CONVERSION FACTORS**

| Assay Temperature | Desired Reporting Temperature 25°C | 30°C | 37°C |
|---|---|---|---|
| **25°C** | 1.00 | 1.32 | 1.82 |
| **30°C** | 0.76 | 1.00 | 1.38 |
| **37°C** | 0.55 | 0.72 | 1.00 |

**Linearity**

The procedure is linear upto 500 U/L at 37°C. If the absorbance change (ΔA/ min.) exceeds 0.250, use only the

# ALKALINE PHOSPHATASE KIT (DEA)

(pNPP Kinetic Method)

For the determination of Alkaline Phosphatase activity in serum.

(For Invitro Diagnostic Use Only)

**Summary**

Alkaline Phosphatase (ALP) is an enzyme of the Hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in the liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone disease. Moderate increases are seen in Hodgkins disease and congestive heart failure.

**Principle**

ALP at an alkaline pH hydrolyses p-Nitrophenylphosphate to form p-Nitrophenol and Phosphate. The rate of formation of p-Nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

$$\text{p-Nitrophenylphosphate} \xrightarrow{ALP} \text{p-Nitrophenol + Phosphate}$$

**Normal reference values**

Serum (Adults) : 80 - 290 U/L at 37°C
(Children) : 245 - 770 U/L at 37°C

It is recommended that each laboratory establish its own normal range representing its patient population.

| Contents | 10 x 3 ml | 5 x 15 ml | 20 x 15 ml |
|---|---|---|---|
| **L1 :** Buffer Reagent | 35 ml | 80 ml | 2 x 150 ml |
| **T1 :** Substrate Reagent | 10 Nos. | 5 Nos. | 2 x 10 Nos. |

**Storage / stability**

Contents are stable at 2-8°C till the expiry mentioned on the labels.

**Reagent Preparation**

**Working reagent :** Dissolve 1 substrate tablet in 3.2 ml (10 x 3 ml pack) or 15 ml (5 x 15 ml / 20 x 15 ml pack) of buffer reagent.

This working reagent is stable for at least 15 days when stored at 2-8°C.

The substrate is light and temperature sensitive. Take adequate care, especially after reconstitution.

**Sample material**

Serum. Free from hemolysis. ALP is reported to be stable in serum for 3 days at 2-8°C.

**Procedure**

Wavelength / filter : 405 nm
Temperature : 37°C / 30°C / 25°C
Light path : 1 cm

Pipette into a clean dry test tube labelled as Test ( T ) :

| Addition Sequence | ( T ) ( ml ) |
|---|---|
| Working Reagent | 1.0 |
| Incubate at the assay temperature for 1 minute and add | |
| Sample | 0.02 |

Mix well and read the initial absorbance $A_0$ after 1 minute & repeat the absorbance reading after every 1, 2, & 3 minutes. Calculate the mean absorbance change per minute ($\Delta A$ / min.)

**Calculations**

ALP Activity in U/L = $\Delta A$ / min. x 2754

**TEMPERATURE CONVERSION FACTORS**

| Assay Temperature | Desired Reporting Temperature 25°C | 30°C | 37°C |
|---|---|---|---|
| **25°C** | 1.00 | 1.22 | 1.64 |
| **30°C** | 0.82 | 1.00 | 1.33 |
| **37°C** | 0.61 | 0.75 | 1.00 |

**Linearity**

The procedure is linear upto 700 U/L at 37°C. If the absorbance change ($\Delta A$ / min.) exceeds 0.250, use only the value of the first two minutes to calculate the result, or dilute the sample 1 + 9 with normal saline (NaCl 0.9%) and repeat the assay (Results x 10).

**Note**

Samples having a very high activity show a very high initial absorbance. If this is suspected then dilute the sample and repeat the assay.

**References**

Bowers, G.N., McCommb, R.B., (1972) Clin. Chem. 18 : 97
Recommendations of the German Society for Clinical Chemistry, (1972)
Z. Clin. Chem. Bio. 10 : 182