

Tuesday, 6th October  
2022

## INTRODUCTION TO MEDICAL LABORATORY SCIENCE

### HAEMATOLOGY (MLS 311)

#### Synopsis

- Haematological Stain
- Blood  $\leftarrow$  Cells  
            Liquids
- Romanowsky Stains (Principles of Haematological Stains)
- Blood film preparation and Staining
- Thick and thin smear.
- Components of blood film preparation
- Pipettes
- Chambers  $\leftarrow$  T-WBC - Total white blood cells
- Haemoglobin, PCV & WBC estimation

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## ROMANOSKY STAINS (Haematological Stains)

Romanovsky stains are group of stains that have similar staining qualities used in haematology and cytopathology.

Romanovsky type of stain are used to differentiate cells for microscopic examination, especially blood (cells) and bone marrow films. And to detect parasites. For example, Malaria parasite within the blood.

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### Examples

Examples of Romanovsky stains are:

- (1) Giemsa Stain
- (2) Jenner Stain
- (3) Wright Stain
- (4) Leishman Stain
- (5) Field Stain
- (6) May-Grunwald Stain

## PRINCIPLE OF ROMANOSKY STAIN

The principle of romanosky stain is based on the affinity of a cell or its contents (constituents) to adapt or react at a particular pH in an appropriate pH for example; 6.8.

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### → The Acidic Structure

It takes up the basic dyes which are therefore called "<sup>(Eosin)</sup>Basophilic"

### → The Basic Structures

It takes up the acidic dyes which are therefore called "<sup>(methylene)</sup>Acidophilic".

The Romanosky stain contains Methyl blue (a basic dye), Eosin (acidic dye) and a ~~policrom~~ <sup>policrom</sup> Methyl blue Azore. Using this stain, the acidic component such as nuclear (nucleic) DNA and Cytoplasmic RNA are stained bluish/purple with <sup>(blueish)</sup>

~~policrom~~ methylene blue, iHITE, The basic components such as haemoglobin and Granules are stained orange to pink with Eosin.

# MECHANISM/STAINING DYNAMICS OF ROMANOSKY STAIN

The Value of the Romanovsky Staining lies in the ability to produce a wide range of colour allowing cellular component to be easily differentiated.

This phenomenon is referred to as the Romanovsky effect or more generally as Metachromasia.

In 1891, a Russian Physician called Romanovsky (Medical Doctor) developed a stain using a mixture of Eosin (Eosin-Y) and Aged solution of Methylene blue that formed Colours unattributable to the Staining Component alone, distinctive Shape of purple in the Chromatin of the cell nucleus and within Granules in the Cytoplasm of some of the white blood cells. This became known as the Romanovsky/Giemsa effect. Eosin and pure methylene blue alone or in combination, do not produce the Romanovsky

effect and the active stains effect are now considered to be Azure B and Eosin dyes, including Methylene blue, Azure A, Azure B, Azure C, and Thionine, Methylene violet, Methyl thionine and Thionine.

The exact composition of the polychromed methylene blue depends on the methods used and even batches of the stains.

Although Azure-B and Eosin have been shown to be required components that produce the Romanovsky effect, these stains in their pure forms have not always been used in the formulation of the staining solution.

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Romanovsky type of stains can be made either a combination of pure dye or from a methylene blue that have been subject to oxidative demethylation which results in the breakdown of methylene blue into multiple stains some of which are necessary to produce Romanovsky effect.

Methylene blue that has undergone this oxidative effect is known as Policom Methylene Blue.

The original source of Azure-B (one of the oxidation products of methylene blue) were from policon methylene blue which rose from Policom methylene blue solution which are treated with oxidative agent or allowed to naturally age.

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### BLOOD FILM PREPARATION

In the laboratory, we have two blood films:-

- Thick Smear
- Thin Smear

In haematology, we are concerned with the "Thin Film Smear".

The Value of Blood Film: Examination of the thin blood films important in the examination and management of:

- (i) Anaemia
- (ii) Infection, and other conditions which produces change in the appearance of blood cell.

## DETERMINING SUSCEPTIBILITY

A blood film report can provide rapidly and at a low cost, useful information about a patient's condition. Reliable blood film reporting is only possible when laboratory staff are trained adequately in the recognition of blood cell and follow standard procedure for preparing blood film.

Reporting morphological changes and performing a differential white blood cell count.

## MAKING & STAINING OF BLOOD FILM

This blood film can be made from flowing capillary blood or well-mixed EDTA anti-coagulated blood. It is important to make blood films from EDTA-anti-coagulated blood with little delay (in vitro)

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### TECHNIQUES FOR FILM MAKING

- ① Make a blood spreader; from a slide which has small edges.
  - ② Place a drop of blood on the end of a clean dry slide. Avoid making the slide large.
  - ③ Using a clean smooth edged spreader, draw the spreader backwards to touch the blood, allow the blood to spread and touch the slide. Holding the spreader at an angle of about  $30-45^\circ$ , spread the drop of blood to make a film  $40-50\text{ ml}$  of length.
- NOTE → When the blood is from Anaemic patients, increase the angle of spreading and spread more quickly, BUT when the blood is thick and viscous, reduce the angle.
- ④ Wipe the end off the spreader.
  - ⑤ Immediately dry the film.
  - ⑥ When completely dry, within a few minutes of making a blood film, fix in absolute alcohol.

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## ⇒ MATERIALS FOR PREPARING THIN FILM

They are:

- ① Capillary blood or anti-coagulated blood
- (ii) A blood spreader
- (iii) Clean dry slide
- (iv) Alcohol (Absolute)

## FEATURES OF A WELL-MADE FILM

- i) Not too thick or too long
- (ii) Free from lines and holes
- (iii) Has a smooth tail.

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## ⇒ STAINING THIN BLOOD FILM

Thin blood films are usually stained manually using Leishman or Wright stains.

These stains are examples of alcohol containing Romanovsky stain which stains blood cells differently.  
METHODS OF STAINING (Leishman stain)

- ⇒
- ① Cover the blood film with undiluted stain, allow it to stain for about 2 mins to fix the slide.
  - ② Dilute your stain with twice the volume of buffer of PH 6.8

Ensure the water is well-mixed with the stain by blowing or through straw mixing, allow the stain for 8 mins.

(3) Wash off the stain with tap-water. Do not tip off the stain because this will leave fine deposit covering the film. Wash the back of the slide clean and stand in drying bag for the stain to dry.

(4) Examine under microscope  $\times 100$  of objective

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EMULATING STUDENTS

⇒ HEMOGLOBIN ESTIMATION

- Haemoglobin is estimated or measured to:
- 1 Detect Anaemia and its severity.
  - 2 To monitor an anaemic patient's response to treatment
  - 3 Determine Polycythaemia (Blood level above normal patient)
  - 4 To estimate haemoglobin as a medical ahead for blood donors.

Techniques for measuring Haemoglobins

There are two major Techniques

- ① Manual (Visual — Cheap & Less Accurate)
- ② Electronics — Expensive & More Accurate

## Haemoglobin Estimation

- ⇒ Haemoglobin is estimated photometrically or using visual comparative technique.
- ⇒ Haemoglobin is measured in gram per litre (i.e g/l)

### ① Electronic / Photometric Technique

In photometric / electronic method, the absorbance of haemoglobin in a blood sample is measured electronically using a filter colorimeter or direct read-out haemoglobin metre.

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There are several method of measuring the haemoglobin electronically

- ① Haemoglobin Cyanide (Cyanmeth) Method / Cyanmethaemoglobin  
This technique is internationally recognized (it is the most accepted method). It is the Gold - Standard.

- ② Vanzetha Arride methaemoglobin

- ③ Reagentless Method

- ④ Non-invasive Method

- ⑤ Autoanalyser / Haemoglobin Analyzer

- ⑥ Block-gass Analyzer (BGA)

- ⑦ Oxy-haemoglobin Method / technique

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### CYANOMETHAEMOGLOBIN METHOD

This is the Standard method of haemoglobin estimation.

#### ⇒ PRINCIPLE OF CYANOMETHAEMOGLOBIN

Whole blood is diluted in; one-in-two hundred and 1 in 251 (i.e 1 in 251 / 1 in 251). This is called the dilution factor

1 in 201 in a modified Drabkins solution

which contains Potassium Ferricyanide and Potassium Cyanide. The red cells are haemolyzed (broken down) and the haemoglobin is oxidized by potassium ferricyanide to Methaemoglobin.

This is converted by the potassium cyanide to Stable Haemoglobin Cyanide (Cyanmethaemoglobin)

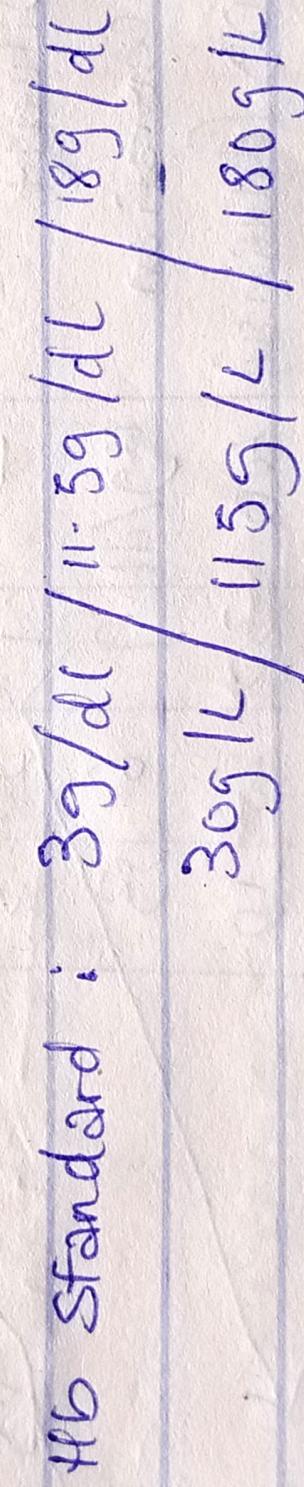
Absorbance of the Cyanmethaemoglobin is read Spectrophotometrically at a wavelength of 540nm or in a filter colorimeter using a yellow green filter. The absorbance obtained is compared with that of a Cyanmethaemoglobin obtained from standard solution.

Haemoglobin values are obtained from tables prepared from a Calibration graph (curve)

## ⇒ Components of Drabkins Solution

- (1) Potassium Ferricyanide - 200 mg
  - (2) Potassium Cyanide - 50 mg
  - (3) Potassium Hydrogen phosphate - 140 mg
  - (4) Non-ionic Detergent (Nonidet) - 1 ml
- This fluid must be stored in a light opaque container (Brown-Glass bottle). Drabkins solution is a clear fluid and must not be used if it loses its colour or becomes turbid.

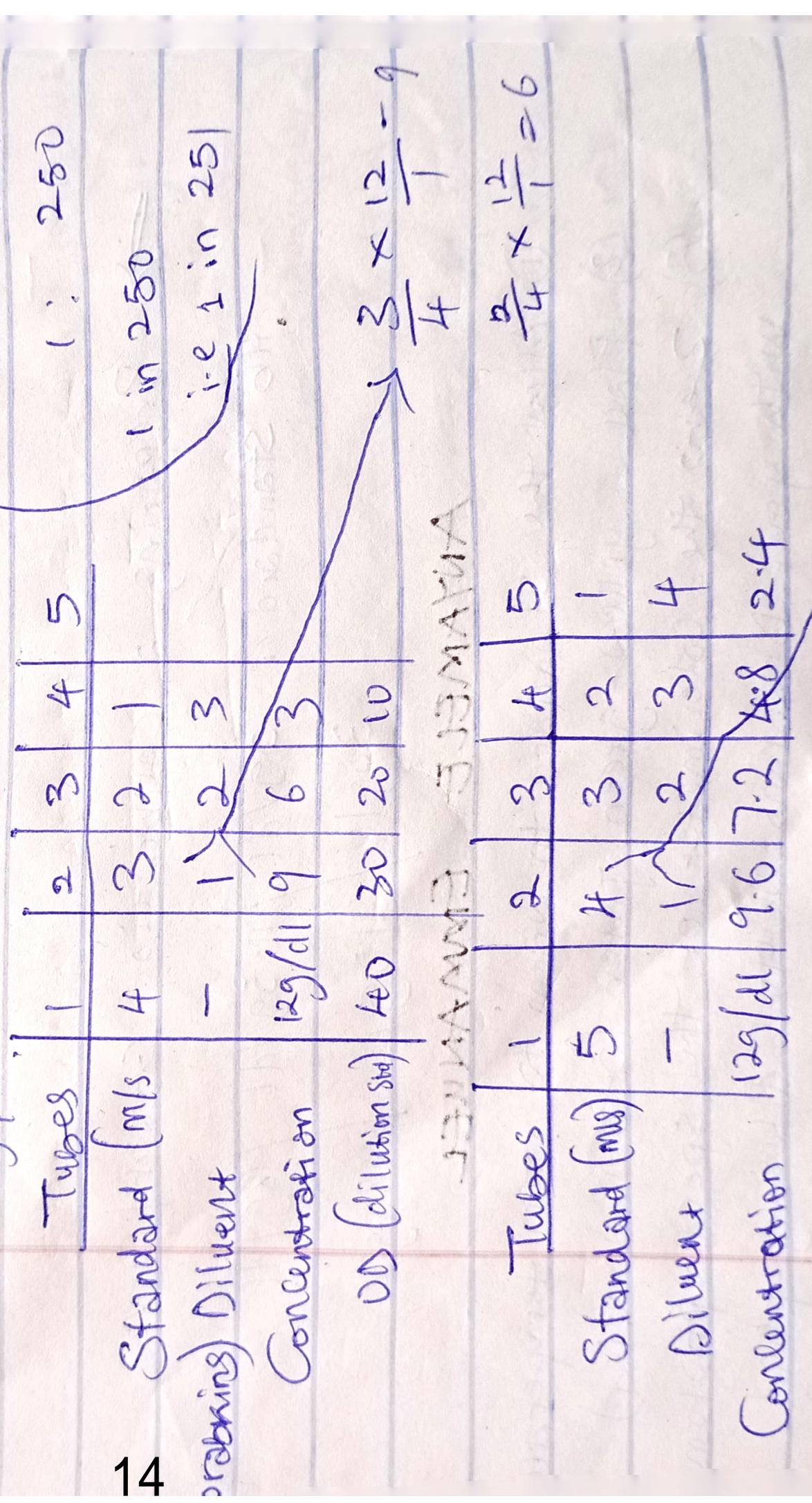
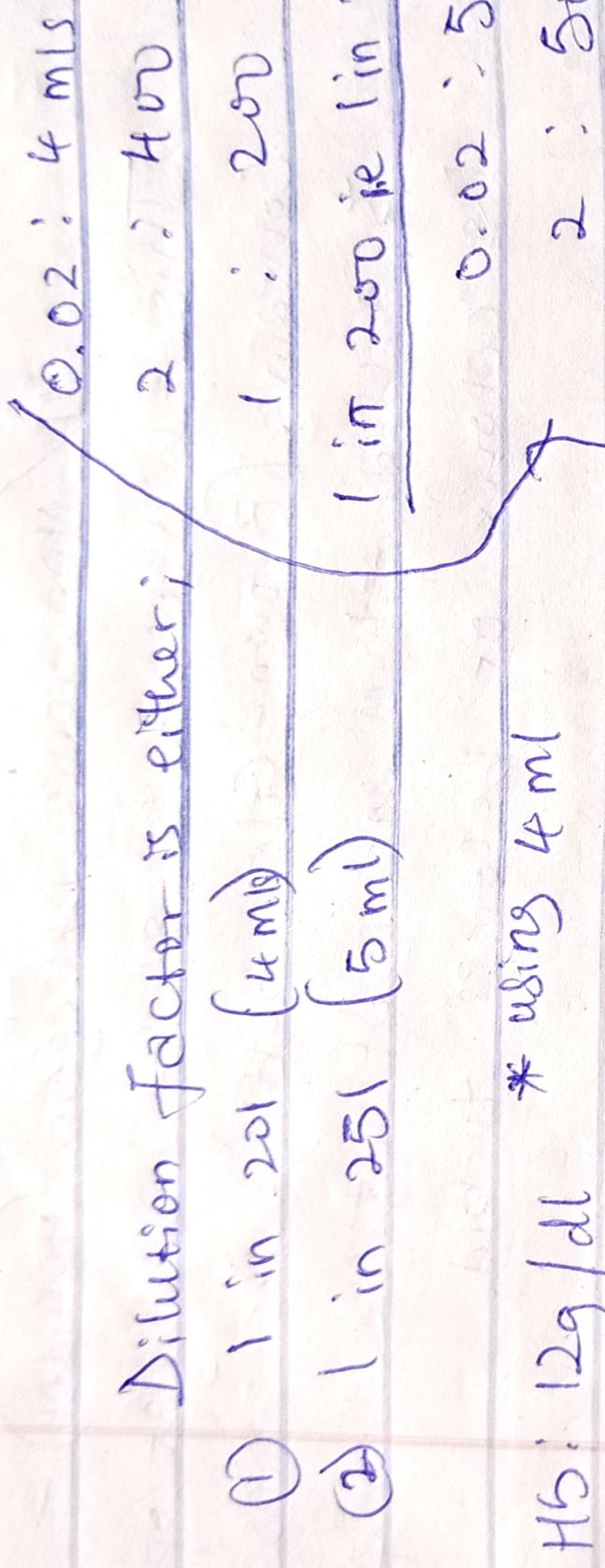
## ⇒ Preparing a Calibration Graph / Curve



## ANALYST: Emanuele

- ① Allow the standard to warm to room temperature.
- ② Place a yellow-green filter (540 nanometer)
- ③ Zero the colorimeter or the spectrophotometer with your Drabkins Neutral diluting fluid.
- ④ Read the absorbance of each standard beginning from the lowest.
- ⑤ Take a sheet of graph paper and plot the

absorbance of each Standard  
 6 Draw a straight line with zero, rule the plotted points. Extend the lines to obtain readings up to 20 points

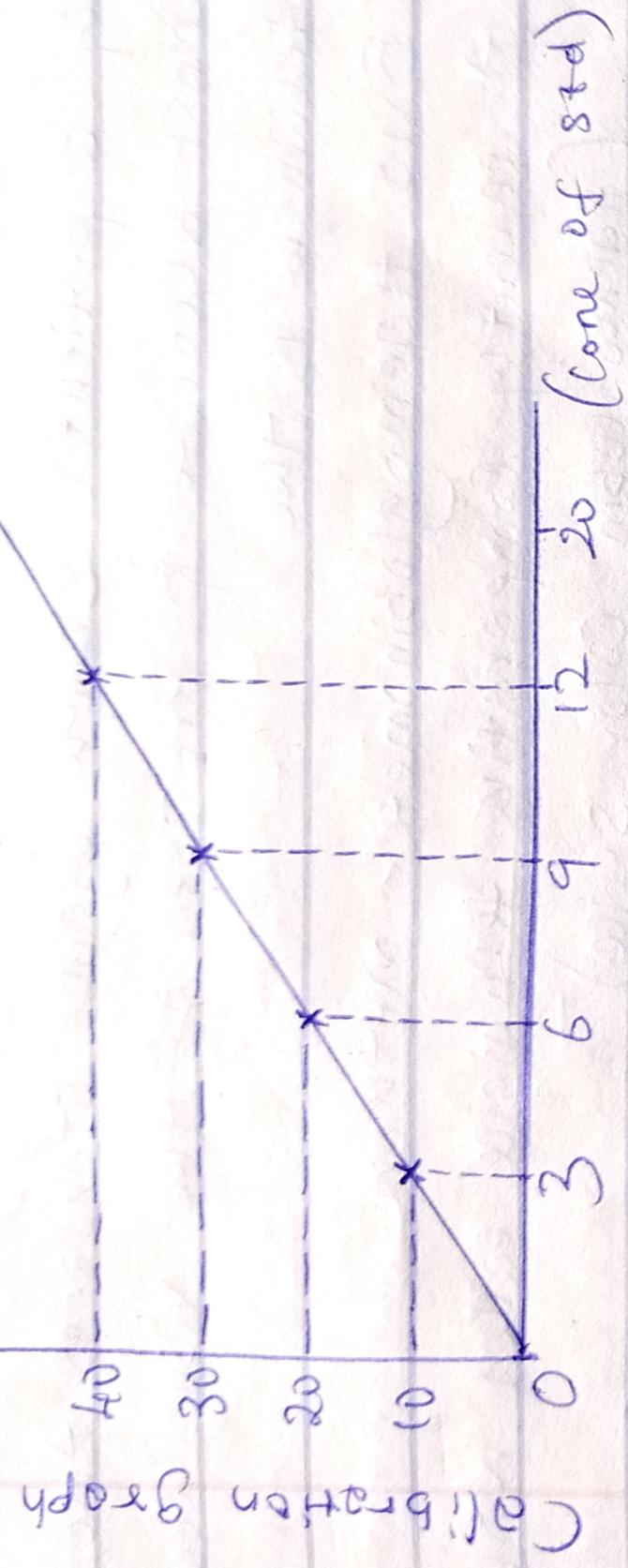


$$\frac{4}{5} \times \frac{12}{1} = 9.6$$

$$\frac{3}{5} \times \frac{12}{1} = 7.2$$

ANSWER Emmanuel

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Example of chart

OD	Hb Conc	OD	Hb conc
1	-	40	13.333333333333333
2	-	-	61
3	-	-	-
4	-	-	-
5	-	0.5	-

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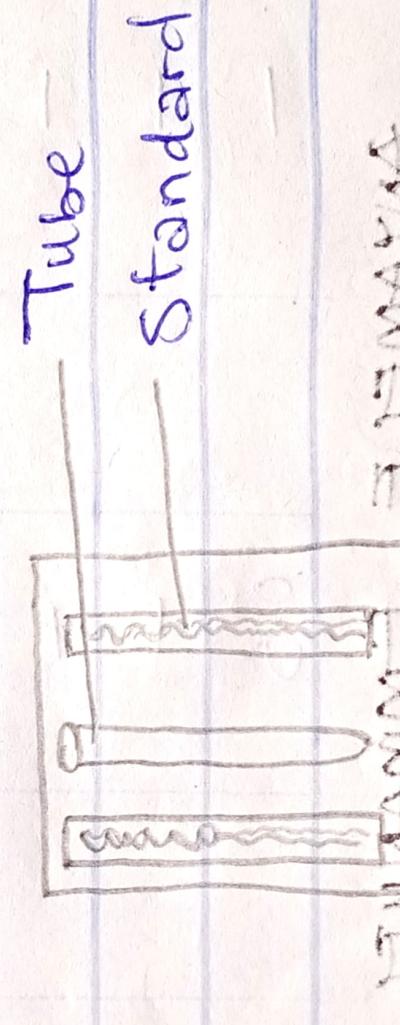
- \* Visual Techniques of haemoglobin Estimation:  
When it is not possible to measure Hb accurately using electronic method for example; in a health centre in a typical village, where it is impossible to measure the Hb electronically, a visual Comparative technique can help to detect Anaemia and assess its severity, though it might not be accurate.

There are several techniques available for estimating Hb based on matching visually the colour of a patient's sample against standards. Among the most accurate & precise of the visual comparatory technique is the

- (1) BMS Haemoglobinometer method
- (2) A recently comparative technique called "WHO Haemoglobin Color Scale"
- (3) Sahli - which is the most common in our area.

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#### SAHLI ACID HAEMATIN METHOD



It is called Sahli acid. Haematin because Hydrochloric Acid is used (diluted HCl) - 1 in 10 ml

This visual comparative method of estimating Hb, although still used in some health centres & hospitals is not recommended because of its unacceptable inaccuracy and unprecision.

In the Sahli's method, we use 20 µL (micro litre) of blood (i.e.  $0.02 \times 1000$ ) is mixed in a tube containing

0.1 mol per litre HCl which converts Hb to acid Haematin. After 10 minutes, add drop by drop (in small volume) the 0.1 mol. HCl, with mixing (and watching of the colour) until the colour of the solution matches with the colour of the glass Standard (Brown) positioned along side the dilution tube.

The Concentration of the Haemoglobin is Read from the graduated Scale on the dilution tube. Most of the problems associated with this method is due to;

- 1 Inability of free acid Haematin
- 2 Fading Colour of the Standard

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#### Assignment

- Mention the various types of visual Haemoglobin Estimation and describe one of them in detail.

#### Haemoglobin Normal Range

Children at birth (Neoninth)	= 13.5 - 19.5 g / dl
Children 2 yrs - 5 yrs	= 11 - 14 g / dl
Children 6-12	= 11.5 - 15.5 g / dl
Adult male	= 13 - 18 g / dl
Adult female	= 12 - 15 g / dl
Pregnant woman	= 11 - 13.8 g / dl

## PACKED CELL VOLUME (PCV)

This is also called HAEMATOcrit. It is used to calculate mean cell Haemoglobin Concentration (MCHC) and mean cell volume (MCV). It is the ratio of white blood cell to the fluid. This red cell indices (absolute values) are used in the investigation of Anaemia. The PCV is also used in screening for Anaemia when it is not possible to measure Hb and to diagnose poly cythaemia vera and thalassemia. It is suitable in screening population.

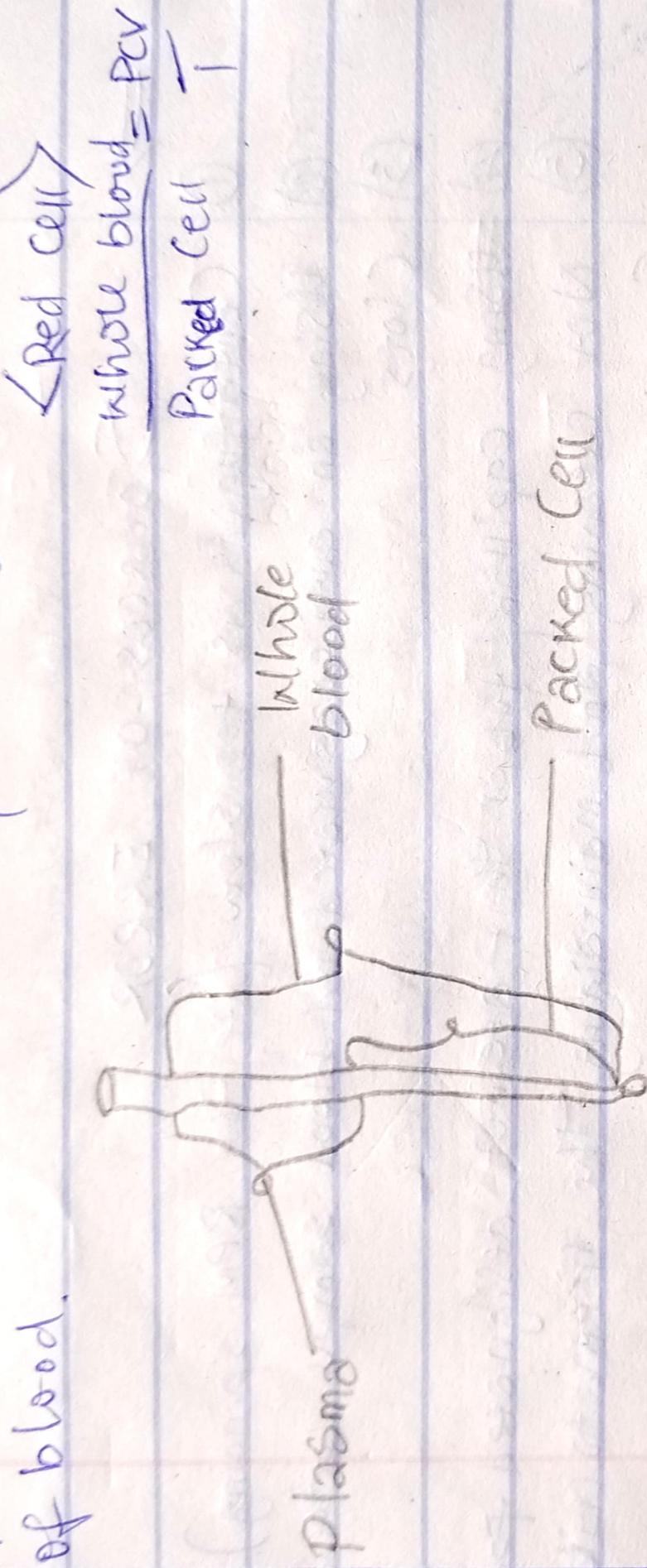
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### The Principle of the Test of PCV Count

The packed cell volume is the proportion of whole blood occupied by red cells expressed as a ration (litre per litre). An aliquoted blood in a glass capillary of specified length bore, size (width) of wall thickness is centrifuged in a micro-haemato crit centrifuge at 12,000 to 15,000 RPM (Revolution Per Minute) for 3-5 minutes to obtain constant packing of red cell. A small amount of plasma remains trapped between the packed

red cell.

The PCV value is read from the scale of a haemocrit reader or calculated by dividing the height of the red cell column by the height of the total column of blood.



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### METHODS STAMMIS: STAMMIS

- ① About three-quarters, fill your capillary tube (three-quarter) either in a plane capillary tube with well mixed EDTA anti coagulated blood or with a heparinized tube.
- ② Seal the unfilled end using a sealant.
- ③ Carefully locate the sealed capillary in one of the numbered slots of the micro-haematoцит reader with the sealed end against the ring-gator (to prevent breakage).
- ④ Centrifuge for 3-5 minutes at 12,000 - 15,000 rpm
- ⑤ Immediately after centrifuging, read the PCV with your

Haemato crit reader.

- The heparinized has red ring
- The non-heparinized (plain) has blue ring.

#### ⇒ SOURCES OF ERROR

- ① Centrifuging is too slow (i.e the RPM spinning)
- ② Using an anti coagulated red blood sample containing ED Clots
- ③ Using capillary tubes that are not designated for PCV
- ④ Not cleaning and maintaining the haemato crit Centri fuge as recommended.

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- ⇒ Normal PCV Value
- |                              |           |
|------------------------------|-----------|
| - Children at birth (Neonet) | - 44 - 54 |
| - 2 - 5 years                | - 34 - 40 |
| - 6 - 12 years               | - 35 - 45 |
| - Adults male                | - 40 - 54 |
| - Women                      | - 36 - 46 |

## PHOTOMETRIC METHODS

