National University-Sudan

Faculty of Medical Laboratory Sciences

Student Practical Manual-
Haematology and Immunohaematology Department

Second Year, Semester (3)
Basic Haematology(MLS-BHEM-214)

Student Name: …………………………………………………………..

ID: ………………………………………**Batch**………………….....…...

Instructions

- Wear lab coat
- Wear Gloves
- Avoid swallow any chemical
- Follow the procedures provided
- Write your results in this manual

Content

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Blood film preparation and staining

- Blood films should be made on a clean glass slides.
- Use fresh blood or EDTA (avoid heparinized blood).
- Films may be made by hands or by an automated slide spreader.

Method:

- Place a small drop of blood in the centre of a slide about 1 cm from one end.
- Without delay, place a spreader in front of the drop at an angle of 30˚ to the slide and move it back to make contact with the drop.
- The drop should spread out quickly along the line of contact, with a steady movement of the hand; spread the drop of blood along the slide.
- The spreader must not be lifted off until the last track of blood has been spread out.
- The film should be about 3 cm in length.
- Allow the film to dry in air.
- Label the film immediately after spreading by pencil on the frosted end of the slide or on the thick part of the film itself.
- Fix the film by immersing in a jar of methanol for 5-10 min. this is important to preserve the morphology of the cells.

Blood film staining

- Romanowsky stains are used universally for staining of blood films, and satisfactory results can be obtained.
- Leishman´s stain is widely used for routine staining of blood films, preparing by dissolve 3g of leishman stain powder in 1 liter of absolute methanol. Add glass, shake well and incubate at 37˚C overnight.

Staining Method:

- Make thin blood films and air-dry.
- Flood the blood film with leishman's stain and leave for 3 minutes.
- Add twice volume of buffer (6.8 pH) and leave for 7 minutes to stain.
- Wash off stain with tap water, dry and examine.

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Haemocytometry

Haemocytometery is the process of counting blood cells (White blood cells, Red blood cells, and Platelets) using the haemacytometer counting chamber; a device made of heavy glass with accurate specifications.

There are many types of haemacytometers, but the commonest one is the Improved Neubauer Chamber.

Materials:

The needful elements to do a cell count with Neubauer chamber are:

a) Cellular dilution to measure

c) Optical microscope

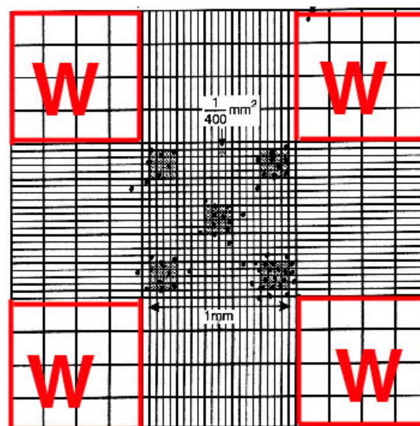
d) Cover glass

e) Pipettes

Calculations of the Cell Counts:

The total number of cells per cubic millimeter of sample can be calculated from:

1. The average number of cells counted.
2. The ruled areas contain an exact volume of diluted sample.
3. The dilution of the sample.



Total White Blood Cells Count

To count white blood cells, whole blood is diluted 1:20 in a weak acid (glacial acetic acid), which lyses (rupture) the red blood cells.

After the haemacytometers is charged with the diluted blood, cells are counted microscopically in the four large squares labeled (W).

Cells should be counting in the two glass platform, and the difference between them should be less than 10, and the difference between the highest and lowest counts should be less than 15.

Requirements:

1. Glacial acetic acid (2%)
2. Counting chamber and cover glass
3. 1 ml pipette
4. 0.02 ml pipette
5. Test tubes
6. Microscope
7. Gauze
8. Blood sample

Method:

1. Add 0.02 ml blood sample to 0.38 ml of the diluting fluid.
2. Mix well for at least 2 mins.
3. Prepare the counting chamber.

4. Smoothly fill the chamber.
5. Leave the chamber on bench for at least 2 mins.
6. Using power 10 objective, count the white cells in the specific area in the chamber.

Counting formula:

Cell count (per cubic mm) = number of cells counted **X** dilution factor \ area of count **X** depth (total volume)

Reference value:

4 – 11 **X** 10^9\L

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Red Blood Cells Count

Principle

The manual method for counting red blood cells involves diluting blood 1:200 in a solution containing formaldehyde and trisodium citrate (formal-citrate). A counting chamber is filling with the diluted blood and placed on the stage of the microscope so RBCs are counted visually.

Reagent

1 % formal citrate.

Preparation: by adding 1 mL of 40% formalin to 99 mL sodium citrate (3.2 %).

Prepare 3.2% trisodium citrate by dissolving 3.2g in 100ml D.W.

Drop 1ml form the solution.

Add 1ml formalin (40% formaldehyde).

Note: The addition of formalin inhibits the growth of bacteria.

Method

- Add 0.02 mL of well mixed blood to 4mL of diluting fluid.
- Mix well and fill the counting chamber smoothly.
- Place the chamber in a moistened Petri dish for 20 minutes to allow the cells to settle.
- Using x 40 objective, count the red cells in 1 mm^2 in the central area of the chamber.

Counting:

Red blood cells were counted in the central area of the chamber, which consists of 25 groups of 16 small squares separated by closely ruled triple lines (which appear as thick black lines in the figure).

- Count RBCs in 5 squares from the 25; the 4 squares at the corners and the central square.

Calculation:

RBCs count = № of cells **X** D.F. / Area counted (Depth **X** Volume)

- Dilution= 0.02/4=1/200, so D.F.=200
- The volume of the 5 squares is 1/5 of the whole volume.
- Depth=0.1mm.

Normal range

- Male………. 4.7 - 6.1 $x10^6/\mu L$ ($10^{12}/L$)
- Female…….. 4.2 -5.4 $x10^6/ \mu L$ ($10^{12}/L$)

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Platelets Count

Aim:

Diagnosis of the coagulation system disorders.

- Platelets count can be obtained by two methods:

I. Using a counting chamber; a method involves making a suitable dilution of whole blood, filling a counting chamber, and counting the platelets using a microscope.

II. From a thin blood film; prepared from EDTA anticougulted blood.

Platelets Count

"Using the counting chamber"

Principle:

Platelets count are best performed on EDTA – anticoagulated blood which is obtained by a clean venepuncture. The blood is diluted in an isotonic solution to prevent swelling.

Reagent:

1% formal citrate.

Method:-

1) Add 0.02 mL of well mixed blood to 4.0 mL of formal citrate.
2) Mix the suspension well and fill the counting chamber.
3) Place the counting chamber in a moistened Petri dish and leave for 20 minutes to give time for the platelets to settle.

4) Count the platelets in 5 squares in the controlled ruled area using x 40 objective. The platelets appear as small highly refractive particles.

Calculation:

$X \times D_1 \times D_2 \times A$

Normal range:

Platelets count = 150 – 400 (10^9/L)

Platelets Assessment

"From a thin blood film"

Materials

- Microscope
- Immersion oil
- Well-spread blood film stained with a Romanowsky stain.

Microscopic examination

- Using the 100 oil-immersion objective, count the number of platelets in 20 fields and make a rough estimate of the number of erythrocytes per field.
- Calculate the ratio of platelets to erythrocytes. If the erythrocyte number concentration is known, the platelets count can be estimated.
- This method requires that RBCs are counted using a semiautomated counter.

If the RBCs count is not available;

- Platelets counted in 10 fields.
- Take the mean.

- Multiply in 20.

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Haemoglobinometry

(Estimation of Haemoglobin)

Aim:

- To assist in the diagnosis of anaemia.
- To calculate amount of blood to be transfused.

Methods :

A- Measurement of Hb power of combining with O_2:

O_2 combining capacity of the blood is 1.34 ml O2 /g Hb.

Dis advantages:

- Hardly practicable.
- Results are 2% lower than other methods.

B- Measurment of Hb iron content:

0.347 g iron = 100 g Hb.

Disadvantages:

Not suitable for routine purpose.

C- Measurement of its color: using colorimeter or spectrophotometer:

Cyanomet Haemoglobin method:

Internationally Recommended.

Principle:

Dilution of blood in a solution containing potassium cyanide and potassium ferricyanide, Hb, metHb, and carboxy Hb are converted to HiCN. The absorbance of this solution is then measured at 540 nm.

Hb+ Kferricyanide ……………………Met Hb (Hi) (unstable)

Hi+ Kcyanide……………………. HiCN (stable)

Advantages:

- Allow direct comparison with HiCN standard
- All forms of Hb can be measured except sulpha Hb.

Disadvantages:

- Use of cyanide is biohazards
- Diluted blood has to stand for a while to ensure complete conversion of Hb.
- The rate of conversion of carboxy Hb is slow.

2- OxyHb method:

Simplest and quickest photometric method.

Reagent: 0.4 ml/l ammonia solution.

Disadvantages:

- Tendency of the solution of HbO2 to fade due to high dilution and high Ph.
- Not possible to prepare stable HbO2 STD.
- Not satisfactory in the presence of CO Hb, Hi, and sulph.Hb.
-

Other methods

Acid haematin method using Sahli apperatus:

Principle:

blood is lysed in 0.1 mol/l HCL_which convert Hb to acid haematin. The solution is then diluted till its color match that of colored glass standard. The Hb conc. is read directly as g/dl from the scale marked on the tube.

Disadvantages:

- Conversion of Hb to acid haematin is slow process, reaching its maximum at the end of one hour, thereafter, the color begins to fade.
- Difficult to be performed reliably.

Alkaline haematin method using 0.1M NAOH.

Acid alkaline Haematin method using 1M/L NAOH& 0.1M/L HCL.

Cyanomet Hb method

Reagent & equipments needed:

- Colorimeter with filter 540 nm.
- Drabkins solution. Consist of :

Potassium ferricyanide………….. 200 mg

Potassium cyanide……………….. 50 mg

Potassium dihydrogen phosphate….140

Non-ionic detergent…………1 ml

D.W………………………to 1 litre

pH……………………7-7.4

Specimen:

EDTA venous blood or capillary blood.

Procedure:

0.02 ml (20 µl) blood + 4 or 5 ml reagent (according to D.F.), mix well.

Incubate for at least 3 min. and read against blank using filter 540 nm.

Calculation:

Hb con (g/l,g/dl)=

$$\frac{\text{O.D. test}}{\text{O.D. STD}} \times \text{conc of STD (g/l,g/dl)}$$

Results:

Expressed as g/dl, g/l, or as %.

14.6g/dl = 100%

Reference values:

Male……………… 15g/dl +/- 2

Female……………14g/dl +/- 2

Note:

- The chief sources of error are failure to mix blood adequately before sampling and inaccurate dilution.

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Haemoglobin Calibration Graph

"STD Graph"

•**Standard curves** are used to determine the concentration of substances. They are obtained by relating a measured quantity to the concentration of the substance of interest in "known" samples, i.e. Standards of known concentration. These standards provide a reference to determine unknown concentrations. Thus amounts chosen of standards need to span the range of concentrations expected to be found in the "unknown" sample concentration.

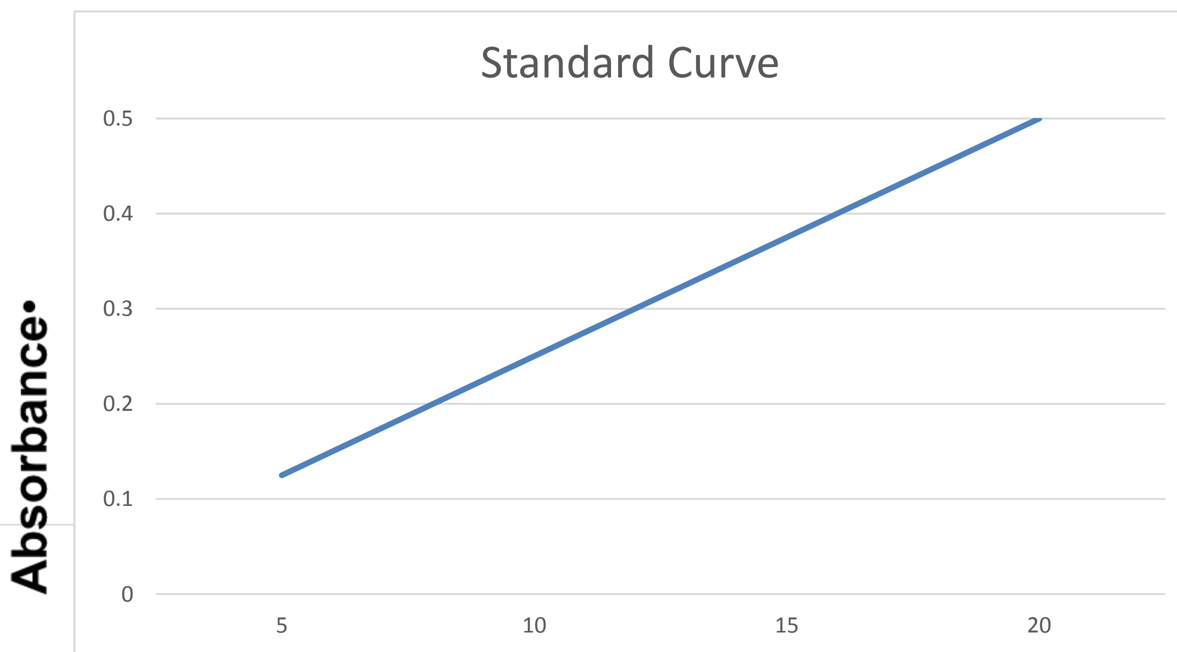
- A standard graph or table relating absorbance readings to haemoglobin in g/l for the individual instrument.
- It is convenient when many blood samples are to be tested.
- Should be prepared each time a new photometer is put in to use or other components are replaced.

Preparation:

- Prepare five dilutions of the HiCN STD with the Drabkins solution according to the table below.
- It is essential that the dilutions are performed accurately.

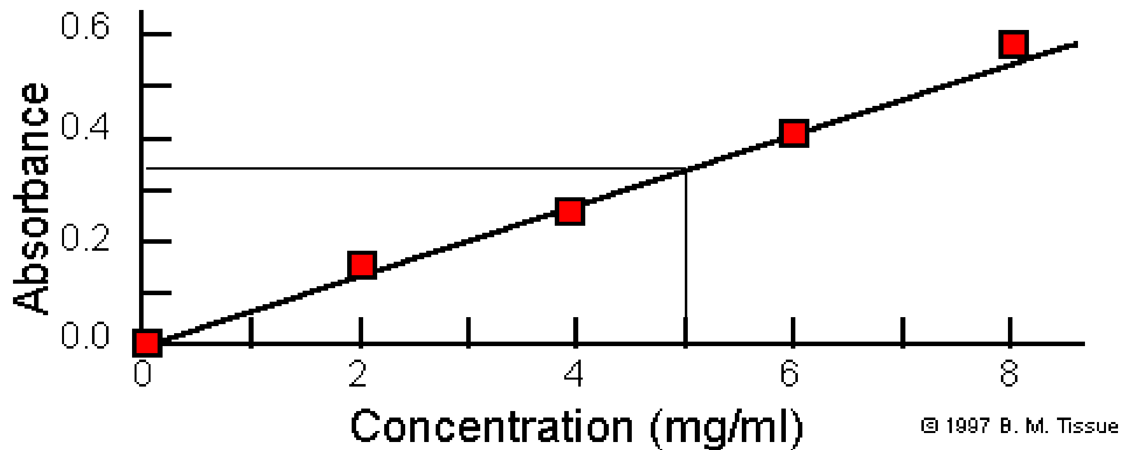
Tube №	HiCN STD Volume (ml)	Drabkins volume (ml)	Hb %	Hb g/dl
1	4	None	100	16
2	3	1	75	12
3	2	2	50	8
4	1	3	25	4
5	none	4	0	0

- Incubate for at least 3 mins., then read the absorbance of each tube against blank at 540 nm.
- Using linear graph paper, plot the absorbance (O.D.) values on the vertical axis and the Hb conc values on the horizontal axis.
- The points should fit a straight line that passes through the origin.



Concentration

- On the upper right portion of the graph (again, such that it doesn't interfere) should be labeled as follows:
    - Your Name , Date
    - Analyte/Procedure
    - Instrument
    - Wavelength
- From the graph, it is possible to construct a table of readings and corresponding Hb values.
- To determine the unknown concentration of a substance in a sample (with same assay as for standards used), intersect across the assay measurement on y with standard concentration, and down to x. The concentration of substance in unknown sample is the value on x.



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***Packed Cell Volume* (PCV)**

- The PCV is the percentage of a volume of blood occupied by red cells.
- (PCV) can be used as a simple screening test for anaemia. It can also be used as a rough guide to the accuracy of haemoglobin measurements, as the PCV should be about 3 times of the haemoglobin value.
- PCV is also used for the calculation of red cells indices (absolute values).

Principle

- The blood (mixed with anticoagulant) is placed in a long capillary tube and centrifuged in a microhaematocrit centrifuge. The level reached by the column of erythrocytes is read with a scale reader.

Materials

- Microhaematocrit centrifuge
- Scale reader (usually provided with the centrifuge)
- Capillary tubes, 75mm long with a 1.5-mm bore, containing dried heparin (if capillary blood is used; if venous blood mixed with EDTA is used, "heparinized" tubes are not required).
- Soft wax (or a Bunsen burner)

Method

- Allow the blood to enter the tube by capillary, Fill about three-quarters of the tube.
- Seal the tube by heating the dry end of the tube rapidly in a fine flame with rotation (or Plug the other end of the tube (i.e. the end that has not come into contact with the blood) with soft wax.
- Place the capillary tube in the centrifuge.

5. Centrifuge for 5 minutes.

6. Read the PCV in the haemotocrit reader by placing the base of the red cells column on the zero line and the plasma on 100 line, then moving the silver line on the adjuster until it is touching the red cell interface and read the PCV from the scale.
7. Express the result as percentage.

- ***Reference range:***

Male……….0.40 - 0.51 l/l

Female……..0.36 - 0.48 l/l

(l/l: liters of red cells per litre of blood)

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Red cell indices "Absolute values"

- The red cell indices include the mean cell volume (**MCV**), the mean cell hemoglobin (**MCH**), and the mean cell hemoglobin concentration (**MCHC**). More recently; modern analyzers also record red cell distribution width (**RDW**) and haemoglobin distribution width (**HDW**).
- These indices are the basis of classifying anaemias and distinction between different types of them.

Calculation of Erythrocyte indices

Mean cell volume (MCV):

This is the mean volume of the red cell expressed as femtolitre. It is measured directly using an automated systems, or is calculated by dividing PCV by RBC.

$$\textbf{MCV} = \frac{\text{Hematocrit (l/l)}}{\text{RBCs count }(10^{12}/\text{l})} \quad \text{femtoliter(fl)}$$

Femtoliter= 10^{-15} of a litre

Ex:

PCV = 45% = 0.45

RBCs count = $5 \times 10^6/\mu l = 5 \times 10^{12}/l$

Volume of one cell (MCV) = $\frac{0.45}{5 \times 10^{12}}$

= 90 fl

Normal range: 82 – 99 fl

Mean cell haemoglobin (MCH):

MCH is derived from the Hb divided by RBC (in picograms per a red cell).

It is calculated using the following formula:

$$MCH\ (pg) = \frac{Hemoglobin\ (g/l)}{RBCs\ count\ (10_{12}/l)}$$

Picogram (pg) = 10^{-12} of a gram

Ex:

Hb = 150g/l

RBCs count = 5 x 10^6/µl = 5 x 10^{12}/l

$$MCH = \frac{150}{5 \times 10^{12}}$$

= 30 picograms (pg)

Normal range: 27 – 33 pg

Mean cell haemoglobin concentration (MCHC):

MCHC is refers to the amount of haemoglobin in 100 mL of packed red cells . It is determined using this formula:

$$MCHC\ (g/l) = \frac{Hb\ concentration\ (g/l)}{PCV}$$

Ex:

Hb = 150g/l

PCV = 45% = 0.45

$$MCHC = \frac{150}{0.45}$$

= 333g/l

Normal range: 320 – 360 g/l

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Reticulocytes count

Aim: To detect the erythropoietic activity of the bone marrow.

Principle: Remnants or ribosomal nucleic acid (RNA) inside the immature RBCs are stained with basic dyes by supra vital stain.

Sample: EDTA anticoagulated blood.

Reagents: Basic dye, e.g. new methylene blue, azure B, or brilliant cresyl blue. Prepared as follows:

Dissolve 1g of the dye in 100ml of iso-smotic phosphate buffer pH 6.5 (56ml of 23.4g/l $NaH_2PO_4.2H2O$ + 44ml of 21.3g/l Na_2HPO_4)

Method:

- In a small test tube take two drops of the sample and two drops of a basic dye.
- Mix gently and incubate at 37˚C for 15-20 mins.
- After the incubation mix again gently and prepare thin blood films from the preparation.
- Dry well and examine by (x100) oil immersion.
- Count the reticulocytes and the RBCs in ten fields in the ideal area of the film.

Results:

The most immature retics are those with the large amount of precipitate material; while the least immature show only few dots or short strands of RNA.

Calculations:

$$\text{Reticulocyte count} = \frac{\text{№ of retics}}{\text{№ of RBCs}} \times 100$$

Notice: Number of RBCs includes also the reticulocytes count.

Absolute reticulocytes count = Reticulocytes% X total RBCs

Normal range: Adults: 0.5-2.5 %

Newborn: 2-5 %

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Evaluation (carried out by the instructor):
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Name and signature of the instructor:
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Date: ………..\.........\............