Clinical Hematology Lab

BML- 391

Determination of Hemoglobin

CLINICAL SIGNIFICANCE

The Hemoglobin is a protein that contains iron and that the red color to the blood. The Hemoglobin is in red globules and it is the one in charge of oxygen transport by the blood from the lungs to weaves. When the level of Hemoglobin appears underneath the normal levels is describing an anemia that can be of different origins: primary anemia, cancer, pregnancy, renal diseases, and hemorrhages. If the Hemoglobin levels appear high it can be due to. cardiopathies. dehydratation and stays in places of much altitude Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

Method :

Sahli's Hemoglobinometer

Principle - Blood is mixed with an acid solution so that Hb is converted to brown colored acid hematin. Diluted with water till brown colour matches that of brown glass standard. Hb value is read directly from the scale Sahli's Acid Hematin Method.

Equipments:

Sahli hemoglobinometer

Sahli pipette(marked at 20 microlite or 0.02 ml)

Stirrer

Dropping pipette

Reagents :

N/10 hydrochloric acid

Distilled water Sahli's Acid Hematin Method

Procedure

1. Place N/10 HCl into Hb tube upto 2 grams.
2. Blood sample in Sahli's Hb pipette upto 20 micro litre.
3. Add blood sample to acid solution.
4. Mix with a stirrer.
5. Allow to stand for 10 minutes.
6. Add distilled water drop by drop till the colour of the solution matches to brown glass standard.
7. Take the reading of the lower meniscus from the graduated tube in grams.

Screening for Sickle cell Anemia

Specimen: EDTA or Heparinized capillary blood

Principle: A strong reducing agent, sodium metabisulfite is mixed with the blood. If the red cells contain HbS, they become sickle shaped.

Requirements:

1) Microscope
2) Slides or Cover slip
3) Pasteur pipettes
4) Filter papers

Reagents:

2 g/dl sodium metabisulfite in distilled water. Prepare fresh (about 25 ml) before use.

Procedure:

1) Place one drop of blood on the glass slide
2) Add equal drop of sodium metabisulfite
3) Mix carefully with a corner of coverslip, seal edges and place in a petridish that has wet filter paper and incubate for 15 mins.
4) Examine the various part of the preparation under high dry objective of the microscope;
5) If the result is negative re-examine the slide after 2 hrs and after 24 hrs

Osmotic Fragility Test

Clinical Significance: To aid diagnosis of hereditary spherocytosis & Thalassemia.

Material & instruments

1. Test tubes
2. NaCl with different concentrations
3. Heparinized venous blood
4. Distilled Water

Procedure

1- Prepare different concentrations of NaCl start with 0 concentration i.e. put only D.W.

2- then put in the rest of the test tubes the following concentrations of NaCl 0.3, , 0.45, 0.5, 0.9 %2-

3- Put a few drops of heparinized blood in each test tube and read the results visually3- Carefully observe each tube for depth of red color of the supernatant and the mass of red cells at the bottom.4

4- - Place a small drop of each of the following solutions on a separate, clean microscope slides: 5% NaCl, 0.9% NaCl, 0.4% NaCl, and distilled water. Red blood cells have been suspended in the following solutions.

5- 5- Observe each slide and note the appearance of the blood cells. What has happened to the cells?

Observation and Results :

Red cells in hypertonic saline. In hypertonic solutions, the RBCs , like other body cells, shrink (crenate) due to movement of water out of the cells (exosmosis).Red cells in hypotonic saline. In hypotonic saline, water moves into the red cells (endosmosis). They swell up and lose their biconcave shape, becoming smaller and thicker. When they swell and become completely spherical, further increase in volume is not possible without an increase in their surface area.

Investigation of G6PD deficiency

Principle

Glucose-6-phosphate dehydrogenase (G6PDH, D-glucose-6-phosphate) catalyzas the first step in the pentose phosphate shunt ,oxidising glucose-6-phosphate (G-6-P)to 6-phosphogluconate(6-PG) and reducing NADP to NADPH.G-6-P + NADP PG + NADPH + H+G-6PDH

NADP is reduced by G-6-PDH in the presence of G-6-P. The rate of formation of NADPH is directly proportional to the G-6-PDH activity and is measured spectrophotometrically as an increased in absorbance at 340nm. Prodution of a second molar equivalant of NADPH by erythrocyte 6-phosphogluconate dehydrogenase (6-PGDH) according to the reaction :6-PG + NADP+Ribulose-5- phosphate + NADPH + H+ + CO2.

Specimen

Whole blood collected with EDTA, heparine or acid citrate dextrose .Red cell G-6-PDH is stable in whole blood for one week refrigrated (2-8°c),but is unsteble in red cell hemolysates.

Procedure prepare reaction mixture:

Add 0.01ml blood directly to vial containing G-6-PDH assay solution and mix throughly to completely suspend erythrocytes, lat stand at room tempreture(18-25°c) for 5-10min.Add 2.0ml G-6-PDH substrate solution directly to vial and mix gently by inverting several times.Transfer contents of vial to cuvet.

Place cuvet in constant tempreture cuvet compartment or water bath and incubate for approximatly 5min to attain therma; equilibrium.Read and record absorbance (A1) of test at 340 nm vs water or ptassium dichromate solution. This is initial A .(if using awater bath or incubator ,return cuvet to it)Exactly 5min later, again read and record (A2), this is final A.To determine G-6-PDH activity do the following calculation.

Calculation = ΔA per min X 4839 / Hb (g/dl) X TCF Where:

100 = factor to convert activity to 100ml3.01 = total reaction volume (ml)0.01 = sample volume (ml)6.22 = mill molar absorptive of NADPH at 340 nmHb (g/dl) = hemoglobin concentration determined for each specimen TCF = temperature correction factor (1 at 30°c)

Qualitative method in G-6-DP determination:

Glucose -6-phosphate dehydrogenase,present in the red blood cell hemoysate, act on glucose -6-phosphate and reduces NADP to NADPH which, with the help of PMS, reduces blue colored 2,6Dichlorophenol Indophenol into acolorless form.the rate of decolorization is proportional to the enzynme activity. The reaction can be represented as:G-6-phosphate +NADP phosphogluconic acid +NADPH

Procedure: Step1:

Preparation of red cell hemolysate:

Purified water : 2.5ml Fresh blood : 0.05mlMix well and allow standing for 5min at R.T.

Step2:

Assay of the enzyme:

Add 1mi of the hemolysate (step 1) to the vial of solution 1 and mix gently.

Add immediately about 1ml of reagent 3.

Seal the vial with aluminium foil and incubate in water bath at 37°c.

Observation: the time taken for the color change from initial deep blue to reddish purple. Follow up to amax. Of 6 hours with 30 min intervals.

Results :

Normal: min.G-6-PD deficient (heterozygous males, homozygous female): 140min-24hrG-6-Pdcarriers (heterozygous females): min-several hours.

Measurement of BT

Clinical Significance: The bleeding time is mainly used in the diagnosis and treatment of the haemorrhagic diseases. The bleeding time is also useful just before operations such as tonsillectomy. In such cases it may point out an abnormal bleeding process. This will aware the physician to take proper precaution.

Name of the Method : Duke,s Method

Specimen: Blood collected by earlobe or figure puncture

Principle: A 1 mm deep incision is made on the ear lobe or finger of the patient. The length of time is required for bleeding to cease is recorded.

Requirements:

Spirit, cotton, needle, piece of filter or bloating paper, stop watch.

Procedure:

1) The finger tip of the subject is sterilized with spirit and a bold prick is made with a sterile needle to have free flow of blood.

2) The stop watch is started and time is recorded.

3) A piece of bloating paper is folded into half and exactly at every 15 seconds interval the blood coming out from the puncture is wiped.

4) The above step is repeated until blood ceases to flow.

5) The time at which blood ceased to flow is recorded.

6) The bleeding time is determined from the recorded time data.

Normal Values:

Normal range of BT by the Duke's method varies from 1 to 5 minutes.

Report

The bleeding time of the subject is found to be mins.

Capillary tube method

Specimen: Capillary blood

Principle: Blood is collected in a capillary tube after a finger prick and the stop watch is started. The formation of fibrin string is noted by breaking the capillary tube at regular intervals. The time is noted at the first appearance of the fibrin string

Requirements:

i. Material for Sterile finger prick
ii. Capillary tubing (10-15 cm in length and 1.5 mm in dm) without anticoagulant

Procedure:

i. Clean a finger with spirit and allow the spirit to dry.
ii. Pricked the finger by lancet. Remove the first drop of blood.
iii. Squeeze the finger to obtain a larger drop of blood and fill the capillary tube with blood.
iv. The capillary tubes are sealed plasticine and immersed in water bath at 37 oC.
v. After one minute start breaking small pieces of the capillary tube every 30 second until a fibrin thread is seen between the two broken ends.

Normal Values:

By this method, the normal clotting time is 5 to 10 minutes at 37 oC.

Measurement of CT

Clinical Significance:

1. Clotting Time is advised to find bleeding disorder, most likely due to clotting factors deficiency.
2. To Diagnose hemophilia.
3. Because of other tests, it has lost its importance.

Name of the Method : Lee-white Method

Principle: Venous blood is collected in a clean dry test tube without anticoagulant. The time required for clotting of the blood is noted.

Procedure:

1. Two siliconized tubes with a 10 cms external bore are taken.
2. These tubes are prewarmed at 37 °C in a water bath.
3. Take the blood mostly from the antecubital vein.
4. 2 to 2.5 mL of the blood is taken, and 1 mL of the blood in each test tubes.
5. Start two stopwatches as you see the blood in the syringe.
6. Keep the blood in the water-bath and check for clotting by tilting each tube at intervals of 30 to 60 seconds.

7. Tilt the tube to greater than 90 degrees.
8. Stop the stopwatch as you see the clot in the tube.
9. Clotting time is expressed as the mean of the two stopwatches.

Advantages:

(i) More accurate and standard method.

(ii) Test can be run with control.

Disadvantages:

(i) It is also a rough method.

(ii) There can be contamination of syringe or tube.

Normal clotting time is 5-10 minutes.

1. Capillary Tube Method:

Procedure:

i. Clean the tip of a finger with spirit.

ii. Puncture it upto 3 mm deep with a disposable needle.

iii. Start the stopwatch.

iv. Fill two capillary tube with free flowing blood form the puncture after wiping the first drip of blood.

v. Keep these tubes at body temperature.

vi. After 2 minutes, start breaking the capillary tube at 1 cm distance to see whether a thin fibrin stand is formed between the two broken ends.

vii. Stop the watch and calculate the time from average of the tow capillary tubes.

Disadvantages:

(i) Method is insensitive.

(ii) Method is unreliable.

Advantages:

It can be performed when venous blood cannot be obtained.

Normal clotting time is 1-5 minutes.

Measurement of PT

Clinical Significance

Prothrombin time (PT) is a blood test that measures how long it takes blood to clot.

A Prothrombin time test can be used to check for bleeding problems.

PT is also used to check whether medicine to prevent blood clots is working.

A PT test may also be called an INR test.

Name of the Method:

Quick's Method

Normal Range: 14 sec

Specimen: Citrated Plasma

Principle:

Prothrombin Time (PT) Principle A preparation of rabbit brain emulsion (which contains tissue thromboplastin) is added to plasma in the presence of calcium. This, in the presence of factor VII triggers stage 2 of the coagulation mechanism, & the clotting time is recorded after the addition of calcified thromboplastin to the plasma .

Requirements:

1. Water bath
2. Stop watch
3. Test tube
4. Brain thromboplastin
5. Calcium chloride

Procedure:

6. Centrifuge the blood sample at 2500 -3000 RPM for 15 minutes.
7. Separate the plasma from the cells as soon as possible.
8. Label 2 test tubes as test tube No.1 & 2 .
9. Add 0.1 ml of patient plasma to each
10. Label another test tube as control.
11. Add control plasma 0.1 ml .
12. Incubate at 37oC for 1 min.

13. Add 0.2 ml of prewarmed thromboplastin reagent into the tube.
14. Start the stop watch.
15. Mix the tube and shake it in water bath for 5-6 sec, take out the test tube & observe for clot formation against light.
16. Run the duplicate test & control in the same way.
17. Take the average of the two test readings.

Measurement of PTT

Clinical Significance :

A partial thromboplastin time (PTT) test measures the time it takes for a blood clot to form. Normally, when you get a cut or injury that causes bleeding, proteins in your blood called coagulation factors work together to form a blood clot. The clot stops you from losing too much blood.

Normal Range: 60-80 sec

Specimen: Citrated Plasma

Principle:

The platelet substitute, in the form of partial thromboplastin, is prepared from rabbit brain as chloroform extract. When mixed with test plasma containing excess of calcium, it leads to clot formation.

Requirements:

1. Water bath
2. Stop watch
3. Test tube
4. Chloroform extract of brain
5. Calcium chloride

Procedure:

1. Pipette 0.2 ml of brain extract in a small test tube
2. Add 0.2 ml of plasma
3. Add 0.1 ml of pre warmed calcium chloride and start stop watch
4. Till the tube till fibrin clot appears
5. Note the time

6. Repeat the procedure for control serum.

Measurement of APTT

Clinical Significance :

1. This test is performed to diagnose hemophilias that involve the deficiencies of the factors such as VIII, IX, V, X and XIII.
2. APTT time is increased in the presence of inhibitors of coagulation and in disseminated intravascular coagulation
3. This method is useful in the control of heparin therapy.

Normal Range: 35-40 seconds

Specimen: Citrated Plasma

Principle: Partial thromboplastin (brain extract in chloroform) is incubated with kaolin. (factor XII, contact factor activator) The clotting time of plasma is determined after the addition of calcium ions.

Requirements:

1. Water bath
2. Stop watch
3. Test tube
4. Chloroform extract of brain
5. Calcium chloride
6. Normal plasma

Procedure:

1. Pipette 0.1 ml of brain extract, 0.1 ml of kaolin reagent and 0.2 ml of plasma in a test tube
2. Incubate at 37 degree centigrade for one minute
3. Add 0.1 ml 0f calcium chloride and start the stop watch
4. After 20 secs observe the formation of the clot by tilting the test tube. As soon as the clot forms, note the time
5. Repeat the procedure by using a normal control plasma

B12 Assay

This immunoassay kit allows for the in vitro quantitative determination of General Vitamin B12 concentrations in serum, plasma, tissue homogenates, cell culture super nates, and other biological fluids.

Method-Kit Method

Principle of the Assay

The ELISA is based on the competitive binding enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Vitamin B12. During the reaction, Vitamin B12 in the sample or standard competes with a fixed amount of biotin-labeled Vitamin B12 for sites on a pre-coated Monoclonal antibody specific to Vitamin B12. Excess conjugate and unbound sample or standard are washed from the plate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of Vitamin B12 in the samples is then determined by comparing the O.D. of the samples to the standard curve .

Materials Supplied

List of component

1. Assay Plate -1
2. Standard -2
3. Sample Diluent- 1x 20 ml
4. Assay Diluent A 1x 10 ml
5. Assay Diluent B 1x 10 ml
6. Detection Reagent A 1x 60 microliter
7. Detection Reagent B 1 x120 microlitre

8. Wash Buffer (25 x concentrate)-1x 30 ml
9. Substrate 1x10 ml
10. Stop Solution 1x 10 ml
11. Plate sealer for 96 wells -5

Storage Instruction

The Assay Plate, Standard, Detection Reagent A and Detection Reagent B should be stored at -20°C upon being received. After receiving the kit, substrate should be always stored at 4°C. Other reagents are kept according to the labels on vials. But for long term storage, please keep the whole kit at -20°C. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. The test kit may be used throughout the expiration date of the kit (six months from the date of manufacture). Opened test kits will remain stable until the expiring date shown, provided it is stored as prescribed above.

Precautions for Use

1. Abnova is not responsible for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. The kit should not be used beyond the expiration date on the kit label.
3. Do not mix or substitute reagents with those from other lots or sources.
4. If samples generate values higher than the highest standard, further dilute the samples with the Sample

   Diluent and repeat the assay. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

- Precaution

  The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.
- Important note:
- Limited by the current condition and scientific technology, we can't completely conduct the

comprehensive identification and analysis on the raw material provided by suppliers. So there might be
some qualitative and technical risks to use the kit.

- The final experimental results will be closely related to validity of the products, operation skills of the end
  users and the experimental environments. Please make sure that sufficient samples are available.
- Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic
  ones from our website is only for information.
- There may be some foggy substance in the wells when the plate is opened at the first time. It will not
  have any effect on the final assay results.
- Do not remove microtiter plate from the storage bag until needed.
- A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or
  greater at 450 nm wavelength is acceptable for use in absorbance measurement.
- Use fresh disposable pipette tips for each transfer to avoid contamination.
- Do not substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
- Even the same operator might get different results in two separate experiments. In order to get better
  reproducible results, the operation of every step in the assay should be controlled. Furthermore, a
  preliminary experiment before assay for each batch is recommended.
- Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with
  our in-house data due to some unexpected transportation conditions or different lab equipments.
  Intra-assay variance among kits from different batches might arise from above factors, too.

- Kits from different manufacturers for the same item might produce different results, since we haven't compared our products with other manufacturers.

Assay Protocol

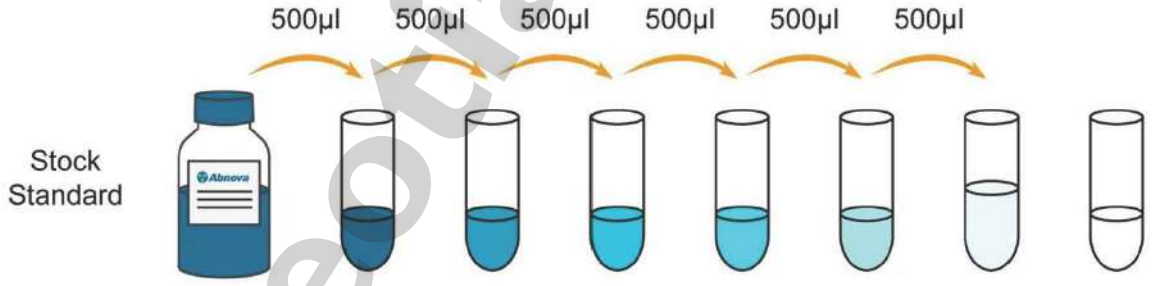
Reagent Preparation

- Wash Buffer

  If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

- Standard

  Reconstitute the Standard with 1.0 mL of Sample Diluent. This reconstitution produces a stock solution of 1,000 pmol/L. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions (Making serial dilution in the wells directly is not permitted). The undiluted standard serves as the high standard (1,000 pmol/L). The Sample Diluent serves as the zero standard (0 pmol/L).



pmol/mL 1000 500 250 125 62.5 31.2 1.56 0

- Detection Reagent A and B

Dilute to the working concentration using Assay Diluent A and B (1:100), respectively.

Sample Preparation

- Serum

  Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C.

- Plasma

  Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2 - 8°C within 30 minutes of collection. Store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
- Note:
  - Samples to be used within 5 days may be stored at 2-8°C , otherwise samples must stored at -20°C (1

    month) or -80°C (2 months) to avoid loss of bioactivity and contamination.
  - Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results

    due to the impacts of certain chemicals.
  - Influenced by the factors including cell viability, cell number and also sampling time, samples from cell

    culture supernatant may not be detected by the kit.
  - Sample hemolysis will influence the result, so hemolytic specimen cannot be detected.
  - When performing the assay slowly bring samples to room temperature.
  - Do not use heat-treated specimens.

Assay Procedure

Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37°C directly.). All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be resealed and stored at 4°C until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

- Add 50 µl of Standard, Blank, or Sample per well.

- Immediately add 50 µl of Detection Reagent A working solution to each well. Cover with the Plate sealer.

  Gently tap the plate to ensure through mixing. Incubate for 1 hour at 37°C.

3. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µl of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 45 minutes at 37°C.
5. Repeat the aspiration/wash process for five times as conducted in step 3.
6. Add 90 µl of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30

   minutes at 37°C. Protect from light.
7. Add 50 µl of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to

   ensure thorough mixing.
8. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

Note:

1. *Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended*

   that all reagents should be freshly prepared prior to use and all required strip-wells are secured in the

   microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
2. *Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. The reconstituted Standards Detection Reagent A and B can be used only once. This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is*

recommended to suck more than 10 µl for once pipetting.

3. *To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay.*
4. *For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.*
5. *To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.*
6. *The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.*
7. *Duplication of all standards and specimens, although not required, is recommended.*
8. *Substrate Solution is easily contaminated. Please protect it from light.*

Data Analysis

Calculation of Results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the KS concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use some related software to do this calculation, such as curve expert 1.3. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Results of a typical standard curve are provide for demonstration only and should not be used to obtain test results. A standard curve must be run for each set of samples assayed.

Performance Characteristics

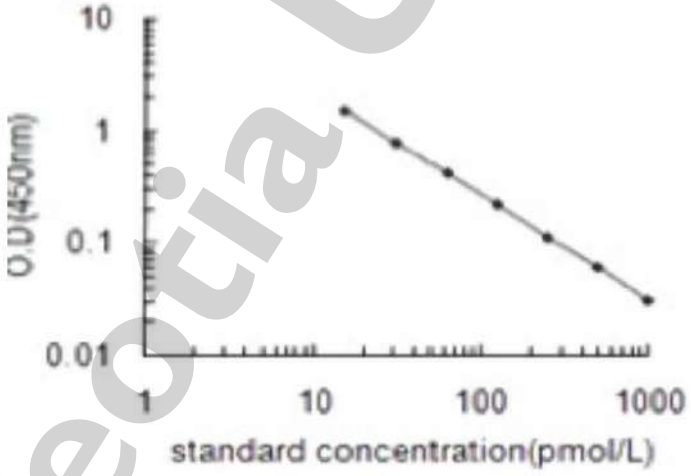
- Specificity

  This assay recognizes recombinant and natural General Vitamin B12. No significant cross-reactivity or interference was observed.

  Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between General Vitamin B12 and all the analogues, therefore, cross reaction may still exist.

- Sensitivity

  The minimum detectable dose of Vitamin B12 is typically less than 7.8 pmol/L. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.



Detection Range 15.6 - 1000 pmol/L.

Folate Assay

Folic Acid ELISA Kit (Cell biolab)

Introduction

Folic acid is a B vitamin also known as Vitamin B9. Since humans don't synthesize folic acid, it is required from the diet and is therefore considered to be an essential vitamin. In cells, folic acid is required for amino acid metabolism as well as to carry one-carbon groups used for methylation reactions and synthesis of nucleic acids (such as thymine and purine bases). Therefore, deficiency in folic acid disrupts DNA synthesis and cell division, affecting mostly hematopoietic cells and abnormal tissue growths because of their higher rate of cell division.

Folic acid is used to supplement folic acid deficiency. This deficiency can cause anemia. Symptoms of anemia can include fatigue, heart palpitations, difficulty breathing, open sores observed on the tongue, and color changes of the hair or skin. Deficiency can occur in children after only a month of consuming a folic acid deficient diet. In adults, normal total body folic acid levels are between 10,000–30,000 micrograms (μg) with plasma levels of greater than 7 nM (3 ng/mL) (Table 1). Women also take supplemental folic acid during pregnancy to prevent fetal neural tube defects (NTDs). Insufficient levels of dietary folic acid in early pregnancy are thought to be the cause of over half of babies born with neural tube defects. As a result, over 50 countries add folic acid to certain foods as a way to decrease NTD incidents in the population.

Normal Value:
Adults – **3-20 ng/mL**
Children - 5-21 **ng/mL**
Infants -14-51 **ng/mL**

The Folic Acid ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of folic acid in serum, cell or tissue samples. The quantity of folic acid in unknown samples is determined by comparing its absorbance with that of a known folic acid standard curve. The kit has detection sensitivity limit of 24 pg/mL folic acid. Each Folic Acid ELISA Kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

Assay Principle

The Folic Acid ELISA kit is a competitive ELISA for the quantitative measurement of folic acid. The unknown folic acid samples or folic acid standards are first added to a Folic Acid Conjugate preabsorbed microplate. After a brief incubation, an Anti-Folic Acid polyclonal antibody is added, followed by an HRP conjugated secondary antibody. The folic acid content in unknown samples is determined by comparison with a predetermined folic acid standard curve.

Related Products

1. MET-5054: L-Amino Acid Assay Kit
2. MET-5056: Branched Chain Amino Acid Assay Kit
3. MET-5151: S-Adenosylhomocysteine (SAH) ELISA Kit
4. MET-5152: S-Adenosylmethionine (SAM) ELISA Kit
5. STA-670: Homocysteine ELISA Kit
6. STA-674: Glutamate Assay Kit

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Protein Binding Plate (Part No. 231001): One strip well 96-well plate.
2. Anti-Folic Acid Antibody (500X) (Part No. 50681C): One 10 µL vial.
3. Secondary Antibody, HRP Conjugate (Part No. 231009): One 20 µL vial.
4. Assay Diluent (Part No. 310804): One 50 mL bottle.
5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
7. Stop Solution (Part. No. 310808): One 12 mL bottle.
8. Folic Acid Standard (Part No. 50682C): One 100 µL amber vial of 10 µg/mL Folic Acid in water.

Box 2 (shipped on blue ice packs)

1. 100X Folic Acid Conjugate (Part No. 50683C): One 100 µL amber vial.

Materials Not Supplied

1. Folic acid samples such as serum, plasma, or folic acid extracted from cells or tissues
2. Tissue Homogenizer
3. 1X PBS
4. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
5. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
6. Multichannel micropipette reservoir
7. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Upon receipt, aliquot and store 100X Folic Acid Conjugate at -20oC and avoid multiple freeze/thaw cycles. Store all other components at 4oC. The 100X Folic Acid Conjugate and Folic Acid Standard are light sensitive and must be stored accordingly.

Preparation of Reagents

• Folic Acid Conjugate Coated Plate: Dilute the proper amount of 100X Folic Acid Conjugate 1:100 into 1X PBS. Add 100 µL of the diluted 1X Folic Acid Conjugate to each well and incubate at 37oC for two hours or overnight at 4oC. Remove the coating solution and wash twice with 200 µL

of 1X PBS. Blot plate on paper towels to remove excess fluid. Add 200 µL of Assay Diluent to each well and block for 1 hr at room temperature. Transfer the plate to 4oC and remove the Assay Diluent immediately before use.

Note: The Folic Acid Conjugate-coated wells are not stable and should be used within 24 hrs after coating. Only coat the number of wells to be used immediately.

- 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.

- Anti-Folic Acid Antibody and Secondary Antibody: Immediately before use dilute the Anti-Folic Acid Antibody 1:500 and Secondary Antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve

1. Use the provided stock Folic Acid Standard 10 µg/mL solution to prepare a series of the remaining standards according to Table 1 below.

Standard Tubes	0 µg/mL Folic Acid Standard (µL)	Assay Diluent (µL)	Folic Acid Folic Acid (ng/mL) (nM)	Folic Acid Folic Acid (ng/mL) (nM)
1	10	990	100	227
2	100 of Tube #1	300	25	56.75
3	100 of Tube #1	300	6.25	14.19
4	100 of Tube #1	300	1.56	3.55
5	100 of Tube #1	300	0.391	0.887
6	100 of Tube #1	300	0.098	0.222

7	100 of Tube #1	300	0.024	0.055
8	0	300	0	0

Preparation of Samples

- Serum: Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Aliquot samples for testing and store at -80oC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.
- Plasma: Avoid hemolyzed and lipemic blood samples. Collect blood with heparin or citrate and centrifuge at 2000 x g and 4oC for 10 minutes. Remove the plasma layer and store on ice. Avoid disturbing the white buffy layer. Aliquot samples for testing and store at -80oC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.

  Note: This assay is not compatible with rabbit serum or plasma due to high levels of rabbit IgG that will cross react with the secondary antibody.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each folic acid sample including unknown and standard should be assayed in duplicate.
2. Add 50 µL of unknown sample or Folic Acid standards to the wells of the Folic Acid Conjugate coated plate. Incubate at room temperature for 10 minutes on an orbital shaker.
3. Add 50 µL of the diluted Anti-Folic Acid antibody to each well, incubate at room temperature for 1 hour on an orbital shaker.

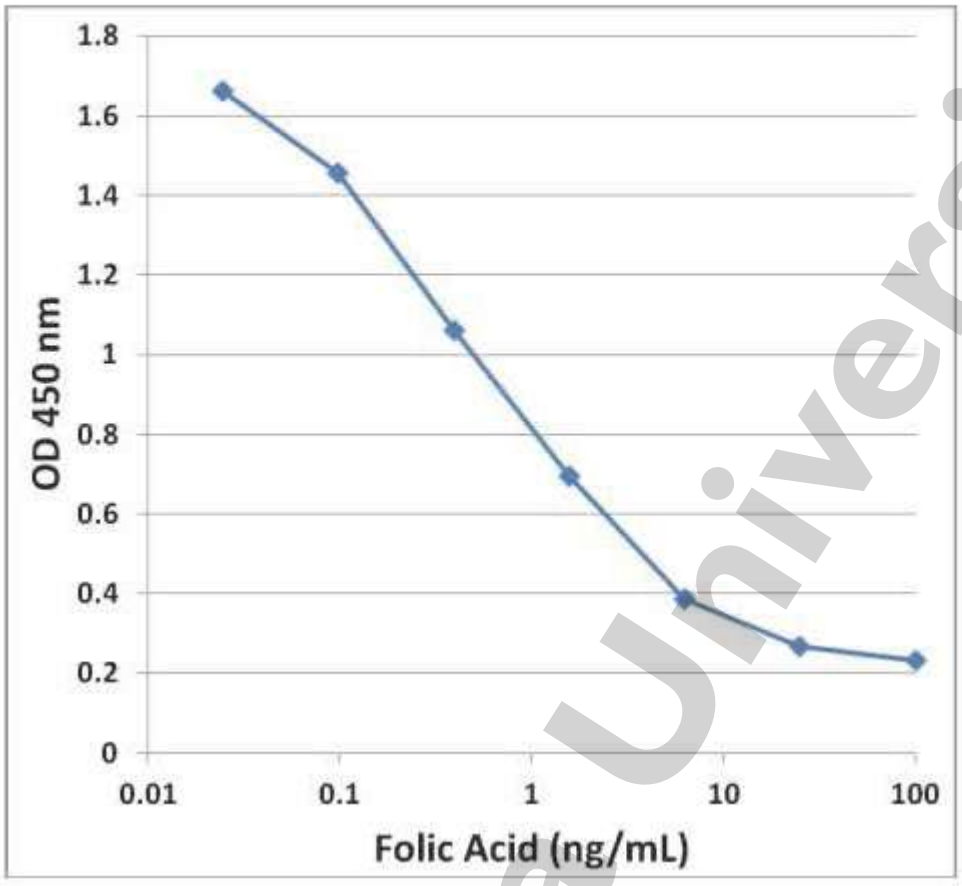
4. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
5. Add 100 µL of the diluted Secondary Antibody-HRP Enzyme Conjugate to all wells.
6. Incubate at room temperature for 1 hour on an orbital shaker.
7. Wash microwell strips 3 times according to step 4 above. Proceed immediately to the next step.
8. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

   Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.

9. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
10. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical Folic Acid ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.



IRON

IRON BINDING CAPACITY

Colorimetric method

ENDPOINT

CLINICAL SIGNIFICANCE

Iron deficiency is a common disorder, particularly a disease of children, of young women, and older people. Although measurement of serum iron, TIBC, and transferrin saturation are commonly used for iron deficiency, they are in appropriate to diagnose the cause of the disease but useful for confirmation and monitoring of acute iron poisoning in children. Iron toxicity is usually defined a serum iron result that exceeds the TIBC.

Though measurements of SI concentration and TIBC have been used as aids, the most reliable method of diagnosis of iron deficiency is the cytochemical staining of bone marrow aspirate that demonstrates the presence or absence of hemosiderin.

TIBC varies in disorders of iron metabolism and it is often *increased* in iron-deficiency anaemia and *decreased* in chronic inflammatory disorders, malignancies and also in hemochromatosis (iron overload) presenting a high serum iron and transferrin saturation and normal or low TIBC.

PRINCIPLE

Serum iron is bound to transferrin, but only about one third of the iron binding sites are saturated with iron. The unsaturated iron-binding capacity of transferrin (UIBC) denotes the available iron-binding sites of serum. The amount of iron that serum transferrin can bind when completely saturated with an excess of Fe^{+3} is the total iron-binding capacity (TIBC).

The method[1,2] measures the TIBC by first saturating the transferrin with excess of Fe^{+3}. The remaining iron is adsorbed with magnesium carbonate, and once the binding process is complete the quilter is removed by centrifugation, and an assay for iron content performed in the supernatant. From this measurement the TIBC value is obtained. When the serum iron (SI) determination is performed concurrently with the TIBC and the result subtracted from the TIBC value, the

difference yields the unsaturated iron-binding capacity (UIBC), or seric transferrin not bound to iron.

- Pipettes with disposable plastic tips.
- – Centrifuge tubes, iron-free.
- – Vortex mixer.
- – Desktop centrifuge.

REAGENT COMPOSITION

1. **R1 Iron solution**. 500 µg/dL Fe^{+3} (89.5 µmol/L)
2. **R2 Magnesium carbonate**. Magnesium hydroxide carbonate. Powder.
3. **REAGENT PREPARATION**
4. All the kit compounds are stable until the expiry date stated on the label. Do not use reagents over the expiration date.
   Store the vials tightly closed, protected from light and prevented contaminations during the use.
5. **Discard If appear signs of deterioration:** - **R1**.- Presence of particles and turbidity. - **R2**.- Presence of moisture.
6. **SAMPLES**
7. Serum or heparinized plasma free of hemolysis. Centrifuge the specimen as soon as possible. The assay must be done from an early morning sample, and after at least several days ´abstention from iron medication or contraceptives.
8. Serum iron-binding capacity is stable for 7 days at 2-8oC.

Procedure

Colorimetry

1. Bring the Total Iron kit reagents to room temperature and proceed with the determination of iron from an aliquot of the supernate.

CALCULATIONS

Once performed the serum iron (SI) test as described in the technical insert of the kit, figured out the results as follows:

total iron-binding capacity(TIBC)

TIBC = μg/dL supernat x 3 (Dilution Factor). *Unbound iron-binding capacity (UIBC)*

Reference value:

Men 65 - 75 μg/dL (11.6 - 31.3 μmol/L)

Women 50 - 170 μg/dL (9.0 - 30.4 μmol/L)