Al-Kut University college
Studies, Research and Publishing Center

MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS

Prof. Dr. Hussain F. Alrubeai

Prof. Dr. Ghyath S. Mahamoud Prof. Dr. Abedaljassim M.Aljobory

Assis. Prof. Dr. Samia Kh. Mahmood Assis. Lec. Amin Alkhafaji

Assis. Lec. Bassam Qasim

PART 2

2023

منشورات

مركز البحوث والدراسات والنشر

كلية الكوت الجامعة

620. 0044
R 894 Al-Rubeai, Hussain.
MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS/ Hussain Al-Rubeai with others. - 1ed-Baghdad -Al-Rafaa P.P. 2023.
P2 (404p); 24 Cm.
1- laboratories test (medical laboratory) A Mohammed Ghayth- B- Aljbory, Abedjassim-C- Mohmod, Samiakn-D- AlKhafaji, Amin -E- Qassim, Bassam (co- anothers) F-title.

3138 / 2023

المكتبة الوطنية/الفهرسة اثناء النشر

رقم الايداع في دار الكتب والوثائق ببغداد

٣١٣٨ لسنة ٢٠٢٣ م

الرقم الدولي: **ISBN: 978-9922-685-05-2**

ملاحظة

مركز البحوث والدراسات والنشر في كلية الكوت الجامعة
غير مسؤول عن الافكار والرؤى التي يتضمنها الكتاب
والمسؤول عن ذلك الكاتب او الباحث فقط.

مطبعة الرحاب
07902823204

INTRODUCTION

Clinical pathology tests are carried out on clinical specimens (blood, urine, or other substance from the body) at clinical or medical laboratory to obtain information about the health of a patient to aid in diagnosis, treatment, and prevention of disease.

The menu of laboratory tests available to clinicians constitutes an impressive array that has expanded exponentially since 1920 when the first useful test for the quantification of serum glucose concentration was conducted. The current list of tests offered by one major reference laboratory includes nearly 3,000 analytes, which does not include the additional array of more commonly ordered tests (e.g., complete blood count [CBC], prothrombin time, electrolytes [sodium, potassium, chloride, carbon dioxide], thyroid stimulating hormone [TSH], glucose, hemoglobin A1C etc.) routinely performed on site by most hospital-based clinical laboratories. In addition to vast array of other specialized tests such as cancer detecting tests, enzymes, hormones and immunoassays and serological tests.

This book briefly discusses the techniques in simple and easy-to-understand language, listed all the manual and automated methods concerning blood, urine, stool, cerebrospinal fluid and other body fluids specimens. Principles, methodologies, results, norms, interpretations and diseases concerned have been given for each of the tests. Part one of the book contains the following sections: Haematology, Microbiology/Staining Techniques, Microbiology/Culture and Detection, Microbiology/Immunology and Histopathology. Part two contains the following sections: Biochemistry/Organic, Biochemistry/Enzyme, Biochemistry/Hormones, Clinical Pathology, Other Body Fluids and Cancer Detection.

The editors are confident that medical and technical institutions, clinical laboratories, teachers, technicians and students, especially those at the department of medical laboratory techniques, will find the manual very useful, as well as after graduation when they work at state or public clinical pathology labs, where they will need continuous and daily review for various procedures of clinical pathology tests.

Finally the editors express their sincere thanks to Assis.Prof.Dr Talib Z. Almosawy, Chairman of the board of administration of AlKut University College for his approval of publishing this manual on the expense of AlKut University College.

The Editors

MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS/ PART II

CONTENTS

ERYTHROCYTE SEDIMENTATION RATE (ESR)

- **Wintrobe's method**
- **Westergren's method**
- **Automated Analysis of ESR**

COUNTIG WHITE CELLS (WBC; Leukocyte Count)

DIFFERENTIAL WHITE CELL COUNT

ABSOLUTE EOSINOPHIL COUNT

SEROLOGICAL TECHNIQUE FOR ISOLATION OF LYMPHOCYTE POPULATIONS

PLATELET COUNT

PERIPHERAL BLOOD SMEAR STUDY

- **MAKING, FIXING AND STAINING BLOOD FILMS**

STAINS FOR DIAGNOSE LEUKEMIAS

DETECTION OF NORMAL AND ABNORMAL BLOOD CELLS

COOMBS TEST/ ANTIGLOBULIN TEST (AGT)

- **Direct Coombs test**
- **Indirect Coombs test**

BLEEDING DISORDERS

- **Activated partial thromboplastin time (APTT),**
- **Prothrombin time (PT) and**
- **Thrombin time (TT) tests**

MEASURMENT OF THE BLEEDING TIME: Duke method

OBSERVATION OF CLOT RETRACTION AND MEASURMENT OF LYSIS TIME

EUGLOBULIN LYSIS TIME TEST

FIBRINOGEN ASSAY (CLOT WEIGHT METHOD)

QUALITATIVE ESTIMATION OF FACTOR XIII ASSAY

DIGNOSIS OF IRON DEFICINCY ANEMIA

IRON AND IRON-BINDING CAPACITY TEST

ENZYME IMMUNOASSAY FOR FERRITIN TEST SYSTEM

ENZYME IMMUNOASSAY FOR TROPONIN-I TEST SYSTEM

TEST FOR SICKLE CELL ANEMIA

BLOOD GROUPING AND Rh TYPING

Du BLOOD TYPE TESTING

FLOURSENCE IMMUNOASSAY FOR D-Dimer CONCENTRATION

SECTION 2: MICOBIOLOGY

A- STAINING TECHNIQUES

GIEMSA STAIN

GRAM STAIN FOR BACTERIA

AURAMINE-RHODAMINE STAINING FOR ACID FAST BACILLI

ZIEHL-NEELSEN STAIN

SPECIAL STAINS: WADE-FITE STAINING TECHNIQUE FOR *Mycobacterium leprae*

WARTHIN STARRY STAINING TECHNIQUE FOR SPIROCHETES

GROCOTTS METHENAMINE SILVER NITRATE METHOD FOR FUNGI

LACTOPHENOL COTTON BLUE FOR FUNGAL STAINING

SECTION 2: MICROBIOLOGY

B- CULTURE AND DETACTION

CULTURING BACTERIAL PATHOGENS

CULTURING BLOOD FOR BACTERIAL PRESENCE

CULTURE URINE SPECIMEN

LOWENSTEIN JENSEN MEDIUM: ISOLATION OF MYCOBACTERIUM

BIOCHEMICAL TESTS TO IDENTIFY BACTERIAL SPECIES

- **Bile solubility test, Catalase test, Citrate utilization test, Coagulase test, DNA-ase test, Indole test, Litmus milk decolorization test, Aesculin hydrolysis test, Oxidase test, Urease test**

DIFFERENTIATION OF ENTERIC BACTERIAL SPECIES (Enterobacteriaceae)

- **Methyl red test**
- **Indole test**
- **Voges-Proskauer test**
- **Citrate test**

ANTIMICOBIAL SUCEPTIBILITY TESTING

MANTOUX TEST: SCREEN IN VIVO SENSETIZATION BY M. TUBERCULOSIS

COAGULASE TEST: DETECTION OF S. AUREUS

DETECTION OF MALARIA, PLASMODIUM SPP.

- **Staining blood films with Giemsa stain**
- **Dipstick test**

DETECTION OF LEISHMANIASIS

DETECTION AND IDENTIFICATION OF MICROFILARIAE IN BLOOD

INDIRECT DETECTION OF LEISHMANIASIS: SERUM GAMMA GLOBULIN

DETECTION AND IDENTIFICATION OF MICROFILARIAE IN BLOOD

- **Microscopic examination of capillary blood**
- **Microscopic examination of venous blood concentrated by centrifugation**
- **Alternative method using a microhematocrit centrifuge**

- **Alternative method using saponin lysing solution**
- **Microscopic examination of venous blood concentrated by filtration**

MICROSCOPIC DETECTION OF BACTERIA IN URINE

- **Microscopic examination of cultured specimen**
- **Urine dipsticks test**

MICROSCOPIC EXAMINATION OF UROGENITAL SPECIMENS FOR GONORRHEA

MICROSCOPIC DETECTION OF PARASITES FOUND IN URINE

- **Sedimentation method**
- **Filtration method**

MICROSCOPIC DETECTION OF MOST COMMON INTESTINAL PROTOZOA FOUND IN STOOLS

- **Rapid Field stain for fecal trophozoites**
- **Eosin stain for fecal trophozoites and cysts**
- **Saline and eosin preparations to detect E. histolytica and other parasites**
- **Methylene blue preparation to detect fecal leucocytes when the specimen is unformed**
- **Testing for Cryptosporidiosis causes diarrhea**

MICRESCOPIC DIGNOSIS OF INTESTINAL HELMINTHS

- **Technique for the collection and examination of eggs**
- **Cellophane fecal thick-smear technique for diagnosis of *Schistosoma mansoni* infection**
- **Identification of adult helminths**
- **Techniques for concentrating parasites**

MICRESCOPIC EXAMINATION OF WATERY STOOL SPECIMENS FOR VIBRIO CHOLERAE

- **Basic fuchsin smear to detect campylobacters**

- **Motility test and Gram-stained smear**
- **Use of V. cholerae 0 (group 1) antiserum to immobilize vibrio**
- **Rapid dipstick test**

MICROSCOPIC EXAMINATION OF CSF FOR PARASITES, BACTERIA AND FUNGI DETERMINATION

MICROBIOLOGICAL EXAMINATION OF SYNOVIAL FLUID

MICROBIOLOGIC EXAMINATION OF SEMAN CULTURE

EXAMINATION OF VAGINAL SECREATION

- **Microscopic examination**
- **Gross vaginal secretion examination**

EXAMINATION 0F BRONCHOALVEOLAR LAVAGE AND BRONCHIAL WASHINGS FOR INFECTIOUS AGENTS

MICROSCOPIC EXAMINATION OF SPUTUM SPECIMENS AND THROAT SWABS

EXAMINATION OF PUS, ULCER MATERIAL AND SKIN SPECIMENS

- **Microscopic examination**
- **Culture of specimen**

SECTION 2: MICROBIOLOGY

C- IMMUNOLOGY

SEROLOGICAL METHOD FOR TESTING RHEUMATOID FACTOR

C-REACTIVE PROTEIN TEST FOR DIGNOSIS OF INFLAMMATORY CYTOKINES

ENZYME IMMUNOASSAY FOR ANT-H. PYLORI IgG, IgM, & IgA TEST SYSTEM

ANTI-STREPLYSIN O TEST FOR DETECTION OF STREPTOCOCCI

SEROLOGICAL TESTS FOR SYPHILIS INFECTION

- **The fluorescent treponemal antibody-absorbed FTA-Abs test**

- **The T. pallidum hemagglutination TPHA test**
- **Venereal Disease Research Laboratory (VDRL) Test**
- **Rapid Plasma Regain (RPR) Test**

QUALITATIVE BRUCELLA AGGLUTINATION TEST

TYPHOID AND PARATYPHOID SEROLOGICAL TEST

SEROLOGIC DIAGNOSIS OF RICKETTSIACEAE

SEROLOGIC DIAGNOSIS OF TOXOPLASMOSIS

SARS-CoV-2: AN OVERVIEW OF VIRUS DETECTION

- **NUCLEIC ACID AMPLIFICATION TECHNOLOGY**
- **SEROLOGY TESTING**

RAPID QUALITATIVE DETERMINATION OF HIV 1/2 ANTIBODY

ENZYME IMMUNOASSAY FOR THE DETECTION OF ANTIBODIES TO HIV-1 AND HIV-2

- **ELISA Test**
- **Dipstick test for HIV antibody**

WESTERN BLOTTING TECHNIQUE CONFIRMATION TEST FOR DETECTION OF HIV-1/2 ANTIBODIES

RAPID DETECTION OF HEPATITIS B SURFACE ANTIGEN BY IMMUNOFILTRATION METHOD

ELISA TECHNIQUE FOR HEPATITIS B SURFACE ANTIGEN

- **ELISA Test**
- **Dipstick test for hepatitis B surface antigen**

DETECTION OF HEPATITIS C VIRUS ANTIBODIES BY TRIDOT TECHNIQUE

SEROLOGICAL TEST FOR INFECTIOUS MONONUCLEOSIS

SEROLOGIC METHODS FOR DETACTION OF CYTOMEGALOVIRUS

SECTION 3: HISTOPATHOLOGY

FIXATION OF SPECIMENS

DECALCIFICATION

TISSUE PROCESSING

- **MANUAL PROCESSING SCHEDULE**
- **AUTOMATIC TISSUE PROCESSING**

EMBEDDING

PARAFFIN SECTION CUTTING

FROZEN SECTION

STAINING TECHNIQUES

- **HEMOATOXYLIN AND EOSIN**
- **PERIODIC ACID AND SCHIFF STAIN**
- **ALICIAN BLUE STAINING FOR ACID MUCOPOLYSACCHARIDES**
- **ALKALINE CONGO RED**
- **GOMORI'S ONE STEP TRICHROME STAIN FOR MUSCLE FIBERS**
- **VERHOEFFS ELASTIC STAIN**
- **OIL O RED METOD FOR LIPIDS STAIN**
- **BLEACHING OF MELANIN PIGMENTS**
- **PERL'S IRON STAIN FOR HEMOSIDERIN PIGMENTS**
- **ALIZARIN RED FOR CALCIUM**
- **TOLUIDINE BLUE STAIN FOR MAST CELLS**
- **PAPANICOLAOYU STAINIG FOR CELL DIFFERENTIATION**

IMMUNOHISTOCHEMISTRY

FLUORESCENT ANTINUCLEAR ANTIBODY TEST

MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS/ PART II

CONTENTS

LIPID PROFILE: CHOLESTEROL (HDL-C & LDL-C), TRIGLYCERIDES AND APOLIPOPROTEINS METHODS OVERVIEW

QUANTITATIVE ESTEMATION OF TOTAL CHOLESTEROL USING SEMI-AUTOANALYZER

ESTEMATION OF LOW-DENSITY LIPOPROTEIN CHOLESTEROL

QUANTITATIVE ESTEMATION OF HIGH-DENSITY LIPOPROTEIN CHOLESTEROL USING SEMI-AUTOANALYZER

QUANTITATIVE ESTEMATION OF TRIAYLGLYCEROLES USING SEMI-AUTOANALYZER

QUANTITATIVE ESTEMATION OF TOTAL PROTEIN USING SEMI-AUTOANALYZER

MANUAL DETERMINATION OF TOTAL PROTEIN

QUANTITATIVE ESTEMATION OF ALBUMIN USING SEMI-AUTOANALYZER

MEASURMENT OF CERULOPLASMIN IN SERUM

QUANTITATIVE ESTEMATION OF URIC ACID IN SERUM USING SEMI-AUTOANALYZER

ESTEMATION OF LACTIC ACID IN BLOOD

ESTIMATION OF PYROVIC ACID IN BLOOD

QUANTITATIVE ESTEMATION OF SODIUM, POTASSIUM AND CHLORIDE IN HUMAN PLASMA

QUANTITATIVE ESTEMATION OF CALCIUM IN HUMAN PLASMA

QUANTITATIVE ESTEMATION OF PHOSPHORUS IN HUMAN PLASMA

QUANTITATIVE ESTEMATION OF VITAMIN A IN HUMAN PLASMA

QUANTITATIVE ESTEMATION OF VITAMIN E IN HUMAN PLASMA

QUANTITATIVE ESTEMATION OF VITAMIN C IN HUMAN PLASMA

ENZYME IMMUNOASSAY FOR FOLATE/ VITAMIN B12 ANEMIA PANAL TEST SYSTEM

ENZYME IMMUNOASSAY FOR 25-OH VITAMIN D TOTAL TEST SYSTEM

SECTION 1: BIOCHEMESTRY

B- ENZYMES

DETERMINATION OF ASPARTATE AMINO TRANSFERASE AND GLUTAMIC OXALOACETATE TRANSAMINASE

DETERMINATION OF ALKALINE PHOSPHATASE

ALANINE AMINOTRANSFERASE/SERUM GLUTAMIC-PYRUVIC TRANSAMINASE

QUANTITATIVE ESTIMATION OF ALKALINE PHOSPHATASE

QUANTITATIVE ESTIMATION OF ANGIOTENSIN CONVERTING ENZYME

QUANTITATIVE ESTIMATION OF LACTATE DEHYDROGENASE

MANUAL METHEMOGLOBIN REDUCTION TEST: SCREENING FOR G6PD DEFICINCY

SEMI- AUTOANALYZER DETERMINATION FOR GLUCOSE-6-PHOSPHATE-DEHYDROGENASE

QUANTITATIVE ESTIMATION OF AMYLASE

DETERMINATION OF ORNITHINE AMINOTRANSFERASE ACTIVITY

DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY

QUANTITATIVE ESTIMATION OF SUPEROXIDE DISMUTASE

ENZYME IMMUNOASSAY FOR CREATINE KINASE-MB TEST SYSTEM

SECTION 1: BIOCHIMESTRY

C- HORMONE

ENZYME IMMUNOASSAY FOR CALCITONIN HORMONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR CORTISOL TEST SYSTEM

ENZYME IMMUNOASSAY FOR DEHYDROEPIANDROSTERONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR ADRENOCORTICOTROPIC HOROMONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR ALDOSTERONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR β-HUMAN CHRONIC GONADOTROPIN, FOLLICALE STIMULATING HORMONE, LUTEINIZING HORMONE, PROLACTIN HORMONE SEQUENTIAL: FERTILITY PANAL TEST SYSTEM

SEROLOGY OF HUMAN CHRONIC GONADOTROPIN: URINE PREGNANCY TESTS

URINE PREGNANCY TEST STRIP

ENZYME IMMUNOASSAY FOR ALPHA-FETOPROTEIN, β-HUMAN CHRONIC GONADOTROPIN, UNCOJUGATED ESTRIOL: TRIPLE SCREEN PANAL TEST SYSTEM

ENZYME IMMUNOASSAY FOR ANDROSTENEDIONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR ANTI-MULLERIAN HORMONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR ESTRONE TEST SYSTEM

ENZYME IMMUNOASSAY FOR FREE TESTOSTERONE ELISA TEST SYSTEM

ENZYME IMMUNOASSAY FOR PREGNANCY ASSOCIATED PLASMA PROTEIN-A TEST SYSTEM

ENZYME IMMUNOASSAY FOR FREE THYROXIN, FREE TRIIODOTHYRONINE, THYROTROPIN: FREE THYRIOID PANAL

ENZYME IMMUNOASSAY FOR RAPID THYROXINE TEST SYSTEM

ENZYME IMMUNOASSAY FOR PARATHYROID HORMONE, WHOLE AND INTACT TEST SYSTEM

SECTION 2: CLINICAL PATHOLOGY

FECAL ANALYSIS METHODS

COLLECTING A STOOL SAMPLE

FECAL GROSS EXAMINATION

MICROSCOPIC EXAMINATION OF FECAL SPECIEMENS

- **Fecal leukocytes**
- **Fecal Fat Testing**

- **Meat Fibers Presence**

FECAL OCCULT BLOOD TEST

- **Chemical Methods**
- **Immunochemical methods**

FETAL HEMOGLOBIN (APT TEST)

FECAL CARBOHYDRATES IN MALABSORPTION

EXAMINATION OF URINE SPECIMENS

COLLECTION AND TRANSPORT OF URINE

URINE EXAMINATION-COMPLETE AUTOMATED METHOD

- **Ph, SPECIFIC GRAVITY, TOTAL PROTEIN, Glucose, Ketone, Bilirubin, Urobilinogen, Nitrite, Leukocyte, Blood (erythrocytes, hemoglobin)**

URINE EXAMINATION-MANUAL METHOD

MICROSCOPIC EXAMINATION OF URINE SAMPLE

- **Erythrocytes, leukocytes, epithelial cells, casts, crystals, yeast cells**

SCREENING TESTS FOR BILIRUBIN

- **Dipstick test**
- **Ictotest**

QUALITATIVE IDENTIFICATION OF UROBILINOGEN IN URINE

- **Ehrlich aldehyde test**
- **Dipstick test**

DETECTION AND ESTIMATION OF PROTEIN IN URINE

- **Chemical test**
- **Dipstick test**

DETECTION OF GLUCOSE IN URINE

- **Benedict's Test**

- **Dipstick test**

DETECTION OF KETONE BODIES IN URINE

- **Chemical test**
- **Dipstick test (Multistix^{R})**
- **Acetest^{R} tablet**

QUALITATIVE DETECTION OF BILE PIGMENTS BY FOUCHET'S METHOD

QUALITATIVE DETECTION OF BILE SALTS BY HAY'S TEST

QUALITATIVE DETECTION OF FREE HEMOGLOBIN IN URINE BY BENZIDINE TEST

SECTION 3: OTHER BODY FLUIDS

EXAMINATION OF CEREBROSPINAL FLUID

- **Lumbar puncture**

QUANTITATIVE ESTIMATION OF TOTAL WHITE CELLS IN HUMAN CEREBROSPINAL FLUID

DETERMINATION OF GLUCOSE IN THE CEREBROSPINAL FLUID

DETERMINATION OF PROTEIN IN THE CEREBROSPINAL FLUID

- **Total protein**
- **Globulin (Pandy test)**

SYNOVIAL FLUID

COLLECTION OF SYNOVIAL FLUID

LABORATORY TESTING OF SYNOVIAL FLUID

- ***Volume*, *Color and clarity*, *Viscosity*, *Clotting*, *Mucin clot***

CHEMICAL EXAMINATION OF SYNOVIAL FLUID

- ***Protein*, *Glucose*, *Uric acid*, *Lactic acid*, *Lactate dehydrogenase*, *Rheumatoid factor***

MICROSCOPIC EXAMINATION OF SYNOVIAL FLUID

- **Appearance, Differential cell counts, Crystals, Glucose conc., Protein conc.**

SEMEN ANALYSIS

MACROSCOPIC EXAMINATION

- ***Liquefaction, Appearance, Volume, Viscosity***
- **SPERM CONCENTRATION**
- **MOTILITY**
- **AGGLUTINATION**
- **VIABILITY**
- **PENETRATION**
- **MORPHOLOGY**

CHEMICAL ANALYSIS

- ***pH, Acid Phosphatase, Fructose, Hormones***

IMMUNOLOGIC TESTING

AMNIOTIC FLUID: OVERVIEW

- **AMNIOCENTESIS, SPECIMEN COLLECTION AND HANDLING**
- **DIFFERENTIATION OF AMNIOTIC FLUID FROM MATERNAL URINE**
- **PHYSICAL AND MICROSCOPIC EXAMINATION OF AMNIOTIC FLUID**
- **TESTING AMNIOTIC FLUID FOR GENETIC AND CONGENITAL DISORDERS**
- **FETAL DISTRESS TESTING**

-Infection, Respiratory Distress Syndrome

SECTION 4: CANCER DETECTION

ENZYME IMMUNOASSAY FOR ALPHA-FETOPROTEIN, CARCINOEMBRIONIC ANTIGEN, TOTAL PROSTATIC SPECIFIC ANTIGEN CANCER: PANEL TEST SYSTEM

ENZYME IMMUNOASSAY FOR CANCER ANTIGEN 125 TEST SYSTEM

ENZYME IMMUNOASSAY FOR CARCINOEMBRYONIC ANTIGEN

QUANTITATIVE ESTIMATION OF ACID PHOSPHATASE

ACID PHOSPHATASE (TARTRATE-LABILE) FOR PROSTATIC CANCER

SECTION 1: BIOCHEMISTRY

A- ORGANIC

SEMI-AUTOANALYZER FOR GLUCOSE QUANTITATIVE ESTIMATION

Purpose: Quantitative estimation of glucose in human serum or plasma or Cerebrospinal fluid (CSF) or other body fluids by enzymatic method (GOD-POD). Plasma Glucose determinations are useful in the diagnosis and treatment of diabetes mellitus and in monitoring the response to treatment of diabetes mellitus with insulin or oral hypoglycemic agents. Elevated glucose levels may be associated with pituitary or thyroid dysfunction, renal failure and liver disease, whereas low glucose levels may be associated with insulinoma, hypopituitary neoplasms, or insulin induced hypoglycemia. fluids have increased glucose in diabetic condition.

Principle: Glucose oxidase (GOD) converts glucose to gluconic acid. Hydrogen peroxide formed in this reaction, in the presence of peroxidase (POD), oxidatively couples with 4-aminoantipyrine (AAP) and phenol to produce red quinone-imine dye. This dye has absorbance maximum at 505 nm. The intensity of color complex is directly proportional to the concentration of glucose in specimen.

Glucose ----GOD----> gluconic acid + H_2O_2

H_2O_2 + AAP + Phenol----- POD----> Red dye

Performance specifications

- ***Linearity: Up to 500 mg/dL of plasma.***
- ***Measurement range: 40–500 mg/dL***
- ***Sensitivity: The minimum detection limit by this kit is 40 mg/dL***

Primary sample

- Use only plasma as specimen for the test

- Collect 2 mL of venous blood in a Fluoride-EDTA mixture tube Heparin vacutainer tube.

-Do not use lysed plasma for testing as it may give very high results

-Do not use contaminated/turbid samples for testing

- Process the sample on the same day within 3 hours of collection.

- Type of container and additive: Fluoride-EDTA mixture tube.

Equipment: Semi-autoanalyzer

Reagents: Phosphate buffer; pH 7.5; glucose oxidase; peroxidase; 4 aminoantipyrine; phenol

Procedure:

-Switch on the machine and press "FLUSH" button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.

-Press "PROC". Different test procedures will be displayed

-Select the test to be processed by entering its number and then press "ENTER" key.

-Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

-Feed the blank with each batch and ensure the absorbance of the blank is less than 0.15. If the absorbance of the 'blank is more than 0.15' discard the reagent.

-Then feed the test samples and record the values.

- Check whether the sample is hemolyzed, icteric or lipemic before processing. If the sample is lysed, collect another sample and proceed.

Assay: End point	**Reagent volume: 1.0 mL**
Wavelength: 505 nm (500-550)	**Sample volume: 10 PL**
Temperature: 37°C	**Zero setting with Reagent blank**
Incubation	**5 minutes**

Interference: Turbid, lipemic, hemolyzed samples, high levels of ascorbic acid, and plasma bilirubin will interfere. Oxalate and fluoride do not interfere.

Calculating results:

Sample absorbance / Standard absorbance x Concentration of standard = Sample concentration

Biological reference range:

Glucose Fasting is 60-110 mg/dL.

Glucose PP is 90-140 mg/dL

Glucose Random is 60-130 mg/dL

Critical/Alert level values:

Below 60 mg/dL

Above 400 mg/dL

Laboratory interpretation: Increase of blood glucose usually in diabetes mellitus, decrease in insulinoma. Decrease of CSF sugar in infection. Increase of CSF sugar in hyperglycemia.

Potential sources of variability:

-Do not use if the absorbance of the blank reagent is greater than 0.15 at 500 nm as it indicates deterioration of the reagent.

-Check if the patient has followed the instructions regarding preparation before collecting samples for fasting/post-prandial, plasma glucose/glucose tolerance test.

-The periodic update on the reference ranges needs to be made note of.

ESTIMATION OF GLUCOSE CONSETRATION IN BLOOD: o-toluidine method

(This method is also used for estimating the glucose concentration in CSF).

Estimates of the glucose (sugar) concentration in blood are required to help in the diagnosis of diabetes mellitus or any other condition in which there is abnormal carbohydrate metabolism in the body. In patients with diabetes glucose is usually found in the urine

Principle: The proteins are first precipitated by trichloroacetic acid. The glucose in the filtrate reacts with the *o*-toluidine reagent to give a green color, which is measured using a photoelectric colorimeter.

Materials and reagents

- **Colorimeter**
- **Conical centrifuge tubes and large test-tubes (to hold 20ml)**
- **Test-tube racks**
- **Blood (Sahli) pipettes, 0.2ml**
- **Pipettes, 0.5 ml, 5.0ml**
- **Water-bath at 100°C**
- **Glucose reagents**
- **trichloroacetic acid, 3% solution**
- ***o*-toluidine reagent**
- **benzoic acid, 0.1% solution**
- **glucose stock reference solution (100mmol/l)**
- **glucose working reference solutions (2.5, 5.0, 10, 20 and 25mmol/l)**
- **Whole blood (capillary or venous), plasma or serum, taken from a fasting patient (If venous blood is used, it is advisable to use fluoride oxalate as the anticoagulant. This will prevent the glucose from being destroyed in the blood).**
- **Control serum. A control serum should be used with each batch of tests. If the result of the test with the control serum is correct, it can be assumed that the patient's results will also be correct.**

Method

1. Pipette 1.8 ml of trichloroacetic acid solution into each of three centrifuge tubes.

***Note*: Trichloroacetic acid is corrosive. Use it with care.**

2. With a 0.2-ml blood pipette, deliver 0.2 ml of the blood (When this test is performed using CSF, the volume required in this step is greater (0.8 ml).

specimen to the *bottom* of the first centrifuge tube — i.e. *under* the trichloroacetic acid solution. The trichloroacetic acid solution will become cloudy where it contacts the blood specimen.

3. Raise the pipette and draw clear trichloroacetic acid solution into it in order to wash out all traces of the blood specimen.

4. Expel the trichloroacetic acid solution from the pipette into the centrifuge tube.

5. Mix well (the entire solution will become cloudy) and allow to stand for 5 minutes.

6. Using a clean 0.2 ml blood pipette, deliver 0.2 ml of distilled water and 0.2ml of glucose working reference solution to the second and third centrifuge tubes, respectively, as described in step 2. These tubes will be used to prepare the reagent blank and the glucose working reference standard, respectively.

7. Centrifuge the three tubes at 3000*g* for 5 minutes. The precipitated proteins in the tube containing the blood specimen will sediment and a clear supernatant fluid will be obtained.

8. Take three (or more if needed) large test-tubes and label as:

— blank tube (B)

— reference tube (R)

— patient tube (P).

***Note*: If more than one estimation is being carried out, label each of the P tubes with the name or number of the patient.**

9. Pipette into each tube as follows:

Blank:

— 0.5 ml of fluid from the second centrifuge tube

— 3.5 ml of *o*-toluidine reagent.

Reference:

— 0.5 ml of from the third centrifuge tube

— 3.5 ml of *o*-toluidine reagent.

Patient:

— 0.5 ml of supernatant fluid from the first centrifuge tube

— 3.5 ml of *o*-toluidine reagent.

***Note*: The *o*-toluidine reagent is corrosive.**

10. Mix the contents of each tube. Place all the tubes in the water-bath at 100°C for exactly 12 minutes.

11. Remove the tubes and allow them to cool in a beaker of cold water for 5 minutes.

12. Measure the color produced in a colorimeter at a wavelength of 630nm.

(a) Place the orange-red filter in the colorimeter.

(b) Fill the colorimeter tube or cuvette with the solution contained in the tube marked B (blank) and place in the colorimeter.

(c) Adjust the reading of the colorimeter to zero with the cuvette containing solution B in place.

(d) Pour solution B out of the cuvette, rinse the cuvette with a small amount of solution R (reference), pour this out, and fill the cuvette with solution R; place the cuvette in the colorimeter and read the absorbance, *A*R.

(e) Pour solution R out of the cuvette, rinse the cuvette with a small amount of solution P (patient), pour this out, and fill the cuvette with solution P; place the cuvette in the colorimeter and read the absorbance, *A*P.

Calibration of the colorimeter

Before taking measurements, prepare a calibration graph using the different concentrations of the glucose working reference solution treated as described in steps 6–9. The graph should be linear up to the highest concentration and should pass through the origin. Prepare a new graph whenever the *o*-toluidine reagent is renewed, to confirm the linearity.

Results

Calculation

Calculate the concentration of glucose in the blood specimen using the following formula:

(The calculation given is for SI units. The formula for calculating blood glucose concentrations in traditional units is as follows: concentration of glucose (mg /100ml) = concentration of glucose (mmol/ l) 1/ 0.0555).

concentration of glucose in blood (mmol/ l) = (A_P / A_R) X C

where:

A_P = absorbance reading of the patient's specimen

A_R = absorbance reading of the glucose working reference solution

C **= concentration of the glucose working reference solution.**

Note**: If a control serum has been included, make the calculation for that serum in exactly the same way, substituting A_C (absorbance of the control solution) for A_P in the formula.**

Reference range

The reference ranges of glucose concentrations in the blood of fasting patients are shown in the Table below.

High and low values

If unusually high or low values are observed, the test should be repeated in order to confirm the results, as described below.

Table: Blood glucose concentrations in fasting patients

Fluid	Glucose concentration	
	SI units (mmol/l)	Traditional units (mg/100ml)
Venous blood	3.3–5.5	60–100
Capillary blood	3.9–5.5	70–100
Serum	3.9–6.4	70–115
Plasma	3.9–6.4	70–115

Glucose concentrations higher than 16.5mmol/l

Dilute solutions B (blank) and P (patient) with an equal volume of glacial acetic acid. Using diluted solution B in the cuvette, set the colorimeter reading to zero. Then read the absorbance *A*P with diluted solution P in the cuvette. Recalculate

the glucose concentration, using the new value of *A*P and the value of *A*R that was obtained previously. Multiply the result by two (because solution P has been diluted 1 in 2) to obtain the true glucose concentration.

Glucose concentrations lower than 2.3mmol/l

Repeat the entire test. In step 1, use 1.6 ml of trichloroacetic acid solution (instead of 1.8 ml), and in step 2 use 0.4 ml of blood, serum or plasma (instead of 0.1 ml). Perform the test and calculate the result exactly as before. Divide the result by four to obtain the true glucose concentration.

Medical device for glucose concentration measurement

A glucose meter is a medical device for determining the approximate concentration of glucose in the blood. It can also be a strip of glucose paper dipped into a substance and measured to the glucose chart. It is a key element of home blood glucose monitoring (HBGM) by people with diabetes mellitus or hypoglycemia. A small drop of blood, obtained by pricking the skin with a lancet, is placed on a disposable test strip that the meter reads and uses to calculate the blood glucose level. The meter then displays the level in units of mg/dl or mmol/l.

- GlucoWatch™ (Cygnes, Redwood City, CA)

Diabetology A painless, bloodless, automatic, wristwatch device worn for up to 12hr monitoringof serum glucose, which incorporates an alarm triggered when h ypoglycemic or hyperglycemic levels are detected.

- Portable blood glucose analyzer (Source: HemoCue, Mission Viejo, California, USA.)

Laboratory blood glucose analyzers allow patients to be monitored for conditions such as diabetes, hyperglycemia, or hypoglycemia in settings that require high volume, accuracy, or speed. Such settings include a hospital, sports clinic, or research laboratory. The American Diabetes Association recommends the hemoglobin A1c (HbA1c) test for monitoring a patient's blood glucose levels. It further recommends HbA1c as the preferred diagnostic test for diabetes mellitus.

A glucose analyzer consists of a recognition element that identifies the target molecules, a transducer element to measure the signal from the target molecule, and a processing system so the signal can be read. Transducer elements can be electrochemical, optical, thermometric, piezoelectric, and magnetic. The majority of transducers available today are electrochemical, due

to their high sensitivity and reproducibility with relatively low cost. Additional considerations when choosing a blood glucose analyzer include the software the system uses, throughput, capacity, speed, and footprint.

- Portable, Biosensor Blood Glucose Monitor/Miniaturized

StatStrip® Glucose Xpress are now the only three hospital glucose meters to be cleared by the FDA and proven to be safe and effective for use throughout all hospital and professional healthcare settings, including with critically ill patients. Use of any other strip-based glucose meter with critically ill patients is considered "off-label" by the FDA and Centers for Medicare and Medicaid Services.

- Continuous Glucose Monitoring (CGM) is a method to track glucose levels throughout the day and night. CGM systems take glucose measurements at regular intervals, 24 hours a day, and translate the readings into dynamic data, generating glucose direction and rate of change.

Continuous Glucose Monitoring (CGM) systems track glucose levels throughout the day. CGM users insert a tiny sensor wire just under their skin using an automatic applicator. An adhesive patch holds the CGM sensor housing in place so the sensor can measure glucose readings in interstitial fluid throughout the day and night. A small, reusable transmitter connects to the sensor wire and sends real-time readings wirelessly to a receiver, so the user can view the information. With some systems, a compatible smart device with the CGM system app can serve as the display device. The receiver or compatible smart device displays current glucose levels, as well as historical trends in levels. The CGM receiver and/or compatible smart device can also be set to send custom alerts to the user when certain glucose thresholds are reached.

The Dexcom CGM System consists of three parts:

1. **Simple auto-applicator – a one-touch applicator[||] easily inserts a small sensor just beneath the skin.**
2. **Sensor and transmitter – a slim sensor continuously measure glucose levels just beneath the skin and sends data wirelessly to a display device through a transmitter.**
3. **Display device – a small touch screen receiver or compatible smart device[†] displays real-time glucose data.**

-Blood Glucose Meters (BGM) measure glucose levels at a single moment in time, while Continuous Glucose Monitoring (CGM) systems continually check glucose levels throughout the day and night.

ENZYME IMMUNOASSAY FOR C-PEPTIDE/ INSULIN DIABIATES PANAL TEST SYSTEM

Intended Use: Quantitative determination of Insulin or C- Peptide concentration levels in human serum by a Microplate Enzyme Immunoassay, Colorimetric.

SUMMARY AND EXPLANATION OF THE TEST

Diabetes is one of the leading causes of disability and death in the U.S. It affects an estimated 16 million Americans - about one third of them do not even know they have the disease. The causes of diabetes are not precisely known, but both genetic and environmental factors play a significant role. The disease is marked by deficiencies in the body's ability to produce and properly use insulin. The most common forms of diabetes are type 1, in which the body's ability to produce insulin is destroyed, and type 2, in which the body is resistant to insulin even though some amount of insulin may be produced.

In-vitro determination of insulin and *C-Peptide* levels help in the differential diagnosis of liver disease, acromegaly, Cushing's syndrome, familial glucose intolerance, insulinoma, renal failure, ingestion of accidental oral hypoglycemic drugs or insulin induced factitious hypoglycemia. Both insulin and *C-Peptide* are produced by enzymatic cleavage of proinsulin. Proinsulin is stored in the secretory granules of pancreatic β-cells and is split into a 31 amino acid connecting peptide (*C-Peptide;* MW 3600) and insulin (MW 6000). *C-Peptide* is devoid of any biological activity but appears to be necessary to maintain the structural integrity of insulin. Although insulin and *C-Peptide* are secreted into portal circulation in equimolar concentrations, fasting levels of *C-Peptide* are 5-10-fold higher than those of insulin owing to the longer half- life of *C-Peptide.* The liver does not extract *C-Peptide*, however; it is removed from the circulation by degradation in the kidneys with a fraction passing out unchanged in urine. Hence urine *C-Peptide* levels correlate well with fasting *C-Peptide* levels in serum. The glucagon stimulated *C-Peptide* determination is often used for differential diagnosis of insulin-dependent from non-insulin- dependent diabetic patients.

The circulatory insulin can be found at much higher levels in patients with pancreatic tumors. These tumors secrete abnormally high levels of insulin and thus cause hypoglycemia. Accordingly, fasting hypoglycemia associated with

inappropriately high concentrations of insulin strongly suggests an islet-cell tumor (insulinoma). To distinguish insulinomas from factitious hypoglycemia due to insulin administration, serum C-peptide values are recommended. These insulinomas can be localized by provocative intravenous doses of *tolbutamide* and *calcium*.

PRINCIPLE

Immunoenzymometric assay: The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (Ab), (enzyme conjugated and microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal Insulin antibody. Upon mixing monoclonal biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

REAGENTS

Materials Provided:

A. C-Pep/Ins Calibrators

Six (6) vials of references for Insulin and C-Peptide antigens at levels of 0(A), 5(B), 25(C), 50(D), 100(E), and 300(F) µIU/ml for Insulin and 0(A), 0.2(B), 1.0(C), 2.0(D), 5.0(E), and 10.0(F) ng/ml for C-Peptide. Reconstitute each vial with 2ml of distilled or deionized water.

For C-Peptide, the assay should be performed immediately; reconstituted vials can be stored at 2-8°C for 8 hours then discarded. For Insulin, the reconstituted calibrators are stable for 3 days when stored at 2-8°C. In order to store for a longer period of time aliquot the reconstituted calibrators in cryo vials and store at -20°C. *DO NOT FREEZE THAW MORE THAN ONCE.* A preservative has been added.

Note: The human serum-based calibrators were calibrated using a reference preparation, assayed against the WHO 1st IRP 66/304 for Insulin and WHO 1st IRP 84/510 for C-Peptide.

B. Insulin Enzyme Reagent

One (1) vial containing enzyme labeled affinity purified monoclonal mouse x-insulin IgG, biotinylated monoclonal mouse x-insulin IgG in buffer, dye and preservative. Store at 2-8°C.

C. C-Peptide Enzyme Reagent

One (1) vial containing enzyme labeled affinity purified monoclonal mouse antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

D. Streptavidin Coated Plate

One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate

One (1) vial containing a surfactant in phosphate buffered saline. A preservative has been added. Store at 2-30°C.

F. Substrate A

One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution

One (1) vial containing a strong acid (1N HCl). Store at 2-30°C

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C except for calibrators.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1.Pipette(s) capable of delivering 0.050 & 0.100ml (50 & 100µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 300µl) volumes with a precision of better than 1.5%.

3.Microplate washer or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability (The 620nm filter is optional)

5.Absorbent Paper for blotting the microplate wells.

6.Plastic wrap or microplate cover for incubation steps.

7.Vacuum aspirator (optional) for wash steps.

8. Timer.

9.Storage container for storage of wash buffer.

10.Distilled or deionized water

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days

2.Working Substrate Solution – Stable for one (1) year.

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20 - 27 C).

****Test procedure should be performed by a skilled individual**

1. Format the microplates' wells for calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.050ml (50µl) of the appropriate calibrators, controls and samples into the assigned wells.

3.Add 0.100ml (100µl) of the Insulin or C-Peptide Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the microwell.

4.Swirl the microplate gently for 20-30 seconds to mix. Cover with a plastic wrap.

5.Incubate for 120 minutes at room temperature (20-27°C).

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see "Reagent Preparation"), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat two (2) additional times.

8.Add 0.100ml (100µl) of working substrate solution to all wells (see "Reagent Preparation").

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Insulin or C-Peptide in unknown specimens.

1. **Record the absorbance obtained from the printout of the microplate reader.**

2. Plot the absorbance for each duplicate serum reference versus the corresponding Insulin or C-Peptide concentration in µIU/ml or ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of Insulin or C-Peptide for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µIU/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.624) (0.405) intersects the dose response curve at 66.8 µIU/ml (0.82ng/ml) for the Insulin (C-Peptide) concentration.

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized the validation of the software should be ascertained

EXPECTED RANGES OF VALUES

C-Peptide values are consistently higher in plasma than in serum; Monobind advises that a serum sample be used for accurate determination. Compared with fasting values in non-obese non- diabetic individuals, C-Peptide levels are higher in obese non-

diabetic subjects and lower in trained athletes. Based on the clinical data gathered by Monobind in concordance with the published literature the following ranges have been assigned. These ranges should be used as guidelines only:

Adult (Normal)	**0.7 – 1.9 ng/ml**

Insulin values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non- obese non-diabetic individuals, insulin levels are higher in obese non-diabetic subjects and lower in trained athletes. Although proinsulin cross reacts with most competitive insulin assays, there is less than 1% cross reaction found with proinsulin using the Insulin test system. Based on the clinical data gathered by Monobind in concordance with the published literature the following ranges have been assigned. These ranges should be used as guidelines only:

Children 12 yrs	**< 10 µIU/ml**
Adult (Normal)	**0.7 – 9.0 µU/ml**
Diabetic (Type II)	**0.7 – 25 µIU/ml**

SEMI-AUTOANALYZER FOR GLUCOSE TOLERANCE TEST

Purpose: To recognize milder cases of diabetes and renal glycosuria. Quantitative estimation of glucose in human plasma by enzymatic method (GOD-POD). Plasma glucose determinations are useful in the diagnosis and treatment of diabetes mellitus and in monitoring the response to treatment of diabetes mellitus with insulin or oral hypoglycemic agents. Elevated glucose levels may be associated with pituitary or thyroid dysfunction, renal failure and liver disease, whereas low glucose levels may be associated with insulinoma, hypopituitarism or insulin induced hypoglycemia. CSF and fluids have increased glucose in diabetic condition.

Principle: Glucose oxidase (GOD) converts glucose to gluconic acid. Hydrogen peroxide formed in this reaction in the presence of peroxidase (POD), oxidatively couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye. This dye has absorbance maximum at 505 nm. The intensity of color complex is directly proportional to the concentration of glucose in specimen.

Performance specifications

-Linearity: Up to 600 mg/dL of plasma.

-Measurement range: 25–600 mg/dL.

-Sensitivity: The minimum detection limit 40 mg/dL.

Primary sample

-Use only plasma as specimen for the test.

-Collect 2 mL of venous blood in a fluoride-oxalate mixture tube.

-Do not use lysed plasma for testing as it may give very high results.

-Do not use contaminated/turbid samples for testing.

-Process the sample on the same day within 3 hours of collection.

Type of container and additive: Fluoride-EDTA mixture tube.

Reagents/Consumables: For patient use, commercially available glucose (75 g) mixed with water.

Instrument: Semi-autoanalyzer

Procedures:

Instructions to be given to the patient: These instructions should be given to the patients by previous day of the investigation.

-The patient should not take any food after 9 pm the previous night till the test is performed.

-The subject should have normal diet for at least 3 days prior to the test.

- He/she should not have taken drugs which affect blood sugar.

- In exceptional cases, when the patient has to come from a distant place, light tea without sugar may be allowed (2 hours before collection)

Method: Upon arrival of the patient, the following should be done:

-Body weight should be noted down.

-Fasting blood sample should be collected and glucose estimation should be performed

-Specimen of fasting urine is collected and test for glucose, albumin and acetone to be done.

-75 g of glucose dissolved in 300 mL of water should be given orally.

-Blood and urine samples will be collected for every half an hour interval for 2 hours after the glucose has been taken.

-It is not always possible to collect urine at every half an hour interval. In such cases urine sample can be collected for every 1-hour interval.

Glucose estimation: As per the method given in this manual.

Normal responses: Fasting glucose within normal limit. Maximum blood glucose is reached either half or one hour after taking the glucose. The blood glucose then returns rapidly to the normal fasting limits, which are often reached in one and a half hour and almost always at two hours. There should be no sugar in any of the urine specimens.

Reference: The GTT curve will be interpreted with the standard curves.

Critical/Alert level values: Below 40 mg/dL, above 400 mg/dl.

Potential sources of variability:

-Do not use if the absorbance of the blank reagent is greater than 0.150 at 500 nm as it indicates deterioration of the reagent.

-Check if the patient has followed the instructions regarding preparation before collecting samples.

MANUAL GLUCOSE TOLERANCE TEST

The Glucose Tolerance Test (GTT) is of two types depending upon the route of glucose administration:

1. **Oral Glucose Tolerance Test (OGTT)**
2. **Intravenous Glucose Tolerance Test (IVGTT)**

OGTT is mostly preferred and is explained in detail here. IVGTT may be chosen for patients who are unable to absorb an oral dose of glucose (eg. malabsorption syndrome).

Principle of Glucose Tolerance Test

Following a standard oral dose of glucose, plasma and urine glucose levels are monitored at regular intervals, in order to measure tolerance under defined conditions.

Procedure for OGTT

Patient Preparation

1. **The patient should be on balanced diet, containing normal daily requirement of carbohydrates, at least 2-3 days prior to the test.**
2. **Patients should avoid drugs likely to influence the blood glucose levels, at least 2 days prior to the test.**
3. **Patient should report to the laboratory after fasting for 12-16 hours. He/She can drink water.**
4. **All samples of blood should be venous preferably. If the capillary blood from 'finger-prick' is used, all samples should be capillary blood.**
5. **Patients should be in a position to wait at the laboratory for at least 2-3 hours, since 5 or more blood samples are collected at the interval of 30 minutes.**

Conduction of OGTT

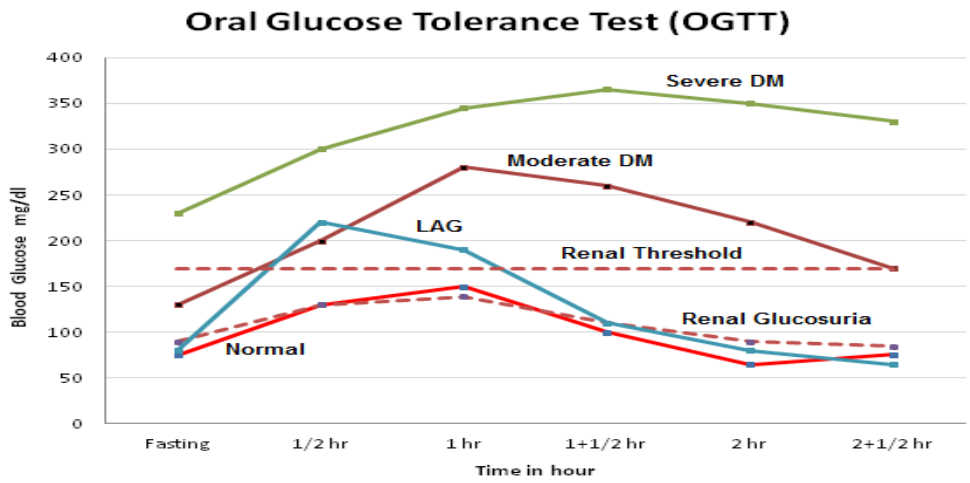
1. **A fasting sample of venous blood is collected in a fluoride vial.**
2. **The bladder is emptied completely and urine is collected for qualitative test for glucose and ketone bodies.**
3. **The individual is given 75 grams of glucose dissolved in water (about 250 ml). Addition of lemon juice lessens the risk of patient vomiting.**
4. **Note the time of oral glucose administration.**
5. **A total of five specimens of venous blood and urine are collected every 1/2 hour (30 minutes) after the oral glucose administration.**
6. **Glucose content of all five samples of blood are estimated by the specific methods used in laboratory. Corresponding urine samples are tested qualitatively for the presence of glucose and ketone bodies.**
7. **A curve is plotted by plotting time on X-axis and plasma glucose level on Y-axis, which is called Glucose Tolerance Curve (GTC).**

Normal Values and Interpretation of OGTT

1. Normal response

- **A typical normal response shows following features.**

- **Initial fasting glucose within normal limits.**
- **The highest peak value is reached within 1 hour.**
- **The highest value does not exceed the renal threshold (160-180 mg/dl).**



- **The fasting level is again reached by 2-2.5 hours.**
- **No glucose or ketone bodies are detected in any specimen of urine.**

2. Response of diabetic patients

- **Fasting blood glucose is definitely raised above 110 mg/dl.**
- **The highest value is reached after 1-1.5 hours.**
- **The highest value exceeds the renal threshold.**
- **The blood glucose level does not return to fasting level within 2.5 hours. This is the most characteristic feature of DM.**
- **Urine sample always contains glucose except in some chronic diabetes or nephritis who may have raised renal threshold.**

	Normal persons	Criteria for Diagnosing Diabetes	Criteria for Diagnosing Impaired Glucose Tolerance (IGT)
Fasting	<110 mg/dl (<6.1 mmol/L)	>126 mg/dl (>7.0 mmol/L)	110 to 126 mg/dl
1 hr (after glucose administration)	<160 mg/dl (<9.0 mmol/L)	Not Prescribed	Not Prescribed
2 hr (after glucose administration)	<140 mg/dl (<7.8 mmol/L)	>200 mg/dl (>11.1 mmol/L)	140 to 199 mg/dl

According to severity, GTC may be:

- **Mildly diabetic curve**
- **Moderately severe diabetic curve**
- **Severe diabetic curve**

3. LAG Curve for oxyhyperglycemia

- **Fasting glucose level is normal.**
- **Rises rapidly in the 1/2 to 1 hour and exceeds the renal threshold so that the corresponding urine specimens show glucose.**
- **The return to normal value is rapid and complete.**

This curve is obtained in:

- **Hyperthyroidism**
- **After gastroenterectomy**
- **During Pregnancy**
- **Also, in early diabetes**

4. Curve for Renal Glucosuria

- **Glucose appears in the urine at levels of blood glucose much below renal threshold.**
- **Patients who show no glucosuria when fasting may have glucosuria when blood glucose is raised.**

It may be seen in:

- **Renal disease and pregnancy**
- **Early diabetes**

HEMOGLOBIN A1C TEST

The A1C test is also known as the hemoglobin A1c test or HbA1c test. Other names for the test include the glycosylated hemoglobin test, glycohemoglobin test, glycated hemoglobin test, or A1C.

A1C tests became available in the 1980s and quickly became an important tool in monitoring diabetes control. A1C tests measure average blood glucose over the past two to three months. So even if you have a high fasting blood sugar, your overall blood sugar may be normal, or vice versa.

A1C measures the amount of hemoglobin in the blood that has glucose attached to it. Hemoglobin is a protein found inside red blood cells that carries oxygen to the body. Hemoglobin cells are constantly dying and regenerating. Their lifespan is approximately three months.

Glucose attaches (glycates) to hemoglobin, so the record of how much glucose is attached to your hemoglobin also lasts for about three months. If there's too much glucose attached to the hemoglobin cells, you'll have a high A1C. If the amount of glucose is normal, your A1C will be normal.

Diagnosis*	A1C Level
Normal	below 5.7 percent
Prediabetes	5.7 to 6.4 percent
Diabetes	6.5 percent or above

The HbA1c test involves a sample blood test that is tested for glycated haemoglobin using any of the following methodologies;

- **High Performance Liquid Chromatography (HPLC)**
- **Immunoassay**
- **Enzymatic**
- **Capillary Electrophoresis**
- **Boronate affinity chromatography**
- **Ion Exchange**

The last method is quantitative in vitro determination of hemoglobin A1C (HbA1C) in human whole blood on photometric systems.

TEST PARAMETERS

Method: Colorimetric, Ion exchange column

Wavelength 415 nm, (405-425 nm)

Temperature: 21 - 26°C

Sample: whole blood Heparin or EDTA may be used as anticoagulants.

Linearity: at least 17.0%

Sensitivity: lower than 4.3%

REAGENT COMPOSITION

COMPONENTS	FINAL CONCENTRATIONS
Reagent 1:	
Potassium phtalate pH 5,0	**50 mmol/L**
Reagent 2:	
Phosphate Buffer pH 6,5	**30 mmol/L**
Reagent 3:	
Phosphate Buffer pH 6,5	**72 mmol/L**
Microcolumns	
Resin equilibr. in PPS pH 6,5	**72 mmol/L**

REAGENT PREPARATION

Reagents are ready to use.

The long-term storage of the columns leads to an excessive packing of the resin diminishing the flow rate and lengthening the elution step. To regain the flow efficiency, it is advisable 10 minutes before starting the test, to invert the columns to resuspend the contents, place them back to their upright position and let the resin settle for a few minutes. Some air bubbles may occasionally appear inside the resin bed. Their presence does not alter the test performance.

REAGENT STABILITY AND STORAGE

Conditions: close immediately after use avoid contamination

Storage temperature: 15 – 30°C

Stability: up to the expiration date

SAMPLE STABILITY AND STORAGE

Stability: at 2 – 8°C 10 days

Discard contaminated specimens.

INTERFERING SUBSTANCES

no interference up to

bilirubin 20 mg/dl

triglyceride 1000 mg/dl

Some drugs and other substances may interfere. In the ionic exchange chromatographic methods, the presence of hemoglobin C or S in the sample may slightly alter results, but differences are not clinically significant5. Other hemoglobin variants like HbE, HbF, carbamyl-Hb and acetyl-Hb can interfere. The incubation with Reagent 1 eliminates the interference due to HbA1c-labile.

In hemolytic anemia, iron deficiency anemia and transfusion, the average age of erythrocytes is altered. Caution should be used when interpreting the HbA1c results from patients with these conditions.

MANUAL TEST PROCEDURE

Bring reagents and samples to room temperature (21-26°C) Use only microcolumns and reagents of the same lot number.

Hemolysate preparation and labile fraction elimination

•Pipette into test tubes

Blood 50 µl

Reagent 1 200 µl

•Shake thoroughly and let it stand at room temperature for 10-15 min. Then prepare the column

Column preparation

• Before placing the column into a tube, keep it standing upside down for 10 min. to improve the fluidity.

• Remove the upper cap of the column.

• Push the upper filter disc down to the surface of the resin by using the flat end of a pipette. Take care not to compress the resin.

• Then snap the tip off the bottom.

• Let the column drain completely to waste.

Separation and reading of HbA1C fraction

- **Pipette on the upper filter**
- **Hemolysate 50 µl**
- **Let the column drain to waste**
- **In order to drain any sample residue left above the upper disc pipette after 1 minute:**
- **Reagent 2 200 µl**
- **Let the column drain to waste and pipette:**
- **Reagent 2 2000 µl**
- **Let the column drain to waste. Then place the column over a new test tube and add:**
- **Reagent 3 4000 µl**
- **Collect the eluate (=HbA1C fraction), shake thoroughly and read the absorbance A (HbA1c) of the HbA1c fraction at 415 nm against dist. water. The absorbance is stable for at least one hour.**

Reading of Hb TOTAL

- **Pipette into a test tube**
  Reagent 3 12000 µl
  Hemolysate 50 µl
- **Shake thoroughly and read the absorb. A (HbTOTAL) of the HbTOTAL fraction at 415 nm against dist. water. The absorbance is stable for at least one hour.**

CALCULATION (light path 1 cm)

HbA1C percentage in the sample:

HbA1C (%) = A (HbA1C) / A (Hb TOTAL) X 100/3

Automated HbA1c System

HemoCue® HbA1c 501 System (HemoCue AB, Sweden) is a fast, easy and reliable point-of-care system for hemoglobin A1c tests. It is a fully automated HbA1c POC test that provides reliable results straight away for efficient diabetes care.

QUNTITATIVE ESTIMATION OF UREA IN SERUM USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of urea in human serum by Urease-GLDH/UV kinetic method. Determination of serum urea nitrogen is an important index of kidney function. Impaired renal function or increased tissue protein breakdown is associated with increased urea nitrogen levels, whereas liver damage or pregnancy is associated with decreased levels.

Principle: Urea is hydrolyzed by urease to form ammonium carbonate. In the second reaction 2-oxoglutarate reacts with ammonium ion in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD+ for each mole of urea hydrolyzed. The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 340 nm.

Urea + $2H_2O$ ------ Urease ------> $2NH_4^+$ + HCO_3

2-Oxoglutarate + NH_4^+ + NADH ---- GLDH ------->L-Glutamate + NAD^+ + H_2O

Performance specifications:

-Linearity: Up to 240 mg/dL of serum

-Measurement range: 2 -240 mg/dL

-Sensitivity: Lower limit of detection is 2 mg/dL

Primary sample:

-Use plasma

-Collect 2 mL of venous blood from a peripheral vein in a heparin vacutainer tube

-Do not use hemolyzed/contaminated plasma for testing

Type of container and additive

Use heparin/plain vacutainer tubes for collecting samples; do not use hemolyzed/contaminated plasma for testing

Instrument: Semi-autoanalyzer

Reagents: The reconstituted reagent contains the following:

-TRIS pH 7.8, 2-Oxoglutarate, ADP, Urease, GLDH

-NADH

-Urea (50 mg/dL)

Procedure:

- Switch on the machine and press "FLUSH" button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.

- Press "PROC", different test procedures will be displayed

-Select the test to be processed by entering its number and then press "ENTER" key.

-Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

-Run the standard with each batch of patient sample.

-Then feed the test samples and record the values.

Assay: 2-point kinetic	**Sample volume: 10 µL**
Wavelength: 340 nm	**Reagent volume: 400 µL**
Start reagent 100 µL	**Temperature: 37°C**

Biological reference range: 15-38.5 mg/dL

Alert/Critical values: Above 80.0 mg/dL

Laboratory interpretation: Increase suggests impaired renal function, acute nephritis, chronic glomerulonephritis.

Potential sources of variability

-Use of only clear, hemolyzed plasma separated from the erythrocytes as soon as possible. Lysed plasma specimens may give falsely elevated values

-On storage, the working reagent may develop a pink color which makes the use of reagent blanks necessary with every run.

-This method is recommended to perform only on mechanized equipment. It is difficult to incubate all samples and reagent blank exactly for the same intervals.

-The scheme may use for adaptation purpose for instruments with no specific adaptation sheet.

MANUAL ESTIMATION OF UREA CONCENTRATION IN BLOOD

Urea is a waste product formed in the liver following the breakdown of protein. It passes into the blood, is filtered out by the kidneys and excreted in the urine. If the kidneys do not remove urea, the concentration in the blood is increased. This can happen if the kidney tubules become damaged or if the volume of blood flowing through the kidneys is reduced.

Principle: The proteins are first precipitated by trichloroacetic acid. The urea in the filtrate reacts with diacetyl monoxime in the presence of acid, oxidizing reagent and thiosemicarbazide to give a red solution. The color is measured using a photoelectric colorimeter.

Materials and reagents

_ Colorimeter

_ Conical tubes and test-tubes (to hold 20ml)

_ Pipettes, 50ml, 0.1 ml, 0.5 ml, 5ml

_ Measuring cylinder, 50ml

_ Water-bath at 100 °C

_ Urea reagents:

— trichloroacetic acid, 5% solution

— diacetyl monoxime stock solution

— color reagent

— urea stock reference solution (125mmol/l)

— urea working reference solution (10 mmol/l)

_ Acid reagent

_ Blank reagent

_ Patient's blood (treated with EDTA dipotassium salt, 10% solution), serum or plasma

_ Control serum.

A control serum (of known concentration) should be used with each batch of tests. If the result of the test with the control serum is correct, it can be assumed that the patient's results will also be correct.

Method

1. Prepare the color reagent immediately before use, using a 1:1 mixture of the diacetyl monoxime stock solution and acid reagent. Prepare at least 15 ml of color reagent for each test. Mix the color reagent in a large test-tube or small flask.

2. Pipette into a conical centrifuge tube 50ml of whole blood (treated with EDTA dipotassium salt solution), serum or plasma.

3. Add 1ml of trichloroacetic acid solution and mix. Centrifuge at high speed (3000*g)* for 5 minutes to sediment the precipitated proteins and obtain a clear supernatant fluid.

4. Take three (or more if needed) large test-tubes and label as:

— blank tube (B)

— reference tube (R)

— patient tube (P).

***Note*: If more than one estimation is being carried out, label each of the P tubes with the name or number of the patient.**

5. Pipette into each tube as follows:

_ Blank:

— 0.1 ml of blank reagent

— 3.0 ml of freshly prepared color reagent.

_ Reference:

— 0.1 ml of working reference solution

— 3.0 ml of freshly prepared color reagent.

_ Patient:

— 0.1 ml of supernatant fluid

— 3.0 ml of freshly prepared color reagent.

6. Mix the contents of each tube. Place all the tubes in the water-bath at 100°C for 15 minutes to allow the red color to develop.

7. Remove the tubes and place them in a beaker of cold water until they have cooled to room temperature.

8. Measure the color produced in a colorimeter at a wavelength of 520nm.

(a) Place the green filter in the colorimeter.

(b) Fill the colorimeter test-tube or cuvette with the solution contained in the tube marked B (blank) and place in the colorimeter.

(c) Adjust the reading of the colorimeter to zero with the cuvette containing solution B in place.

(d) Pour solution B out of the cuvette, rinse the cuvette with a small amount of working reference solution R (reference), pour this out, and fill the cuvette with solution R; place the cuvette in the colorimeter and read the absorbance, *A*R.

(e) Pour solution R out of the cuvette, rinse the cuvette with a small amount of solution P (patient), pour this out, and fill the cuvette with solution P; place the cuvette in the colorimeter and read the absorbance, *A*P.

Results

Calculation

Calculate the concentration of urea in the blood as follows:

(The calculation given is for SI units. The formula for calculating blood urea concentrations in traditional units is as follows:

urea concentration (mg /100ml) = urea concentration (mmol/ l) X1 0.167

urea concentration (mmol/ l) = (A_P A_R) X*C*

where:

***A*P = absorbance reading of patient's specimen**

***A*R = absorbance reading of urea working reference standard**

***C* = concentration of working reference standard (10mmol/l).**

Reference range

The reference range of urea concentrations in blood is approximately 3–7mmol/l (18–42mg/100ml).

High values

If a value greater than 25mmol/l (150mg/100ml) is obtained, repeat the entire test, using 0.1 ml of whole blood (treated with EDTA dipotassium salt solution), serum or plasma in step 2. Perform the test and calculate the results exactly as before, but divide the result by two to obtain the true urea concentration.

QUNTITATIVE ESTIMATION OF CRATININE IN PLASMA USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of creatinine in human plasma by modified Jaffe's method (Initial rate or fixed time method) Measurement of plasma creatinine is useful in the diagnosis, treatment and follow-up of renal diseases/renal failure. Increase of serum creatinine indicates a definite damage of renal tissue.

Principle: Creatinine reacts with picric acid in alkaline medium to form an orange-yellow colored complex of creatinine picrate. This colored complex absorbs light at 492 nm the rate of increase in absorbance is directly proportional to the creatinine concentration in the sample.

Creatinine + Sodium Picrate ------ Alkali medium----> Creatinine – Picrate complex (yellow-orange)

Performance specifications:

-Linearity: Up to 24 mg/dL in plasma.

-Measurement range: 0.1-24 mg/dL of creatinine in plasma.

-Sensitivity: The minimum detection limit by this kit is 0.1 mg/dL

-Specificity: This method measures a number of other non-creatinine substances also other than creatinine.

Primary sample:

-Use only plasma as specimen for the test

-Collect 4 mL of venous blood in a heparin vacutainer tube.

-Do not use lysed plasma for testing as it may give very high results

-Do not use contaminated/turbid samples for testing

-Process the sample on the same day within 3 hours of collection.

Type of container and additive: Use heparin vacutainer tubes for collecting blood samples.

Instrument: Semi-autoanalyzer

Reagents:

-Creatinine reagent: Picric acid 8.73 mmol/L

-Buffer solution: 300 mmol/L of sodium hydroxide

-25 mmol/L of phosphate.

-Creatinine standard (2 mg/dL) solution containing creatinine in hydrochloric acid with preservative.

Procedure:

-Switch on the machine and press "FLUSH " button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.

-Press "PROC". Different test procedures will be displayed.

-Select the test to be processed by entering its number and then press "ENTER" key.

-Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

-Feed the blank with each batch and ensure the absorbance of reagent blank to zero

-Then feed the test samples and record the values.

Assay: Fixed time (Initial rate)	**Reagent volume: 500 µL/1000 µL**
Wavelength 490 nm	**Sample volume: 25 µL/50 µL**
Temperature: 37°C	**Zero setting with distilled water**
No. of readings: 2	**Time: 60 sec**
Concentration of Std: 2 mg/dL	

Calculating results:

Sample absorbance/ Standard absorbance x Concentration of standard= Sample concentration

Biological reference Range:

Male: 0.7-1.4 mg/dL

Female: 0.6-1.2 mg/dL

Critical/Alert level values: More than 3.0 mg/dL.

Laboratory interpretation: Increase of creatinine in blood suggests kidney damage

Example: Chronic glomerulonephritis.

Potential sources of variability:

-Lysed plasma specimens may give falsely elevated values

-Creatinine remains stable in plasma for up to 2 days.

-Number of substances other than creatinine interfere with the assay.

MANUAL DETERMINATION OF CREATININE

Principle:

Creatinine in alkaline medium react with picrate ions to form yellow orange complex whose color intensity is measured at 492nm

Creatinine + Picric acid $\xrightarrow{\text{alkaline solution}}$ Creatinine - Picric acid complex

Reagent composition

R1= (picric acid) – 35mmol/l

R2= (NaOH) – 0.32mmol

R4 =(Creatinine standard) – 2mg/dl

Reagent preparation

Mix equal volume of R1 and R2: Stable for 10 days at 4 to 8° or 1day at + 20 to 25º.

Specimen

Serum, plasma, urine (diluted with distilled water)

Procedure

	Standard	Sample
Working solution	**1000ul**	**1000ul (1ml)**
Standard	**100ul**	**-**
Sample	**-**	**100ul**

Mix and pour into a cuvette after exactly 20seconds read A1 of sample and standard exactly 80sec after first reading, read A2 of sample and standard.

Ctest= Δ Atest X Δ Cstd/ Δ std

Normal values (mg/ dl) = Man 0.7 -1.2

Conversion of mg/100ml into mmol/l and vice versa

Mmol/1= mg/100ml×10/Molecular wt

Mg/100ml= mmol/1× molecular wt/10

Portable Creatinine Analyzer

StatSensor® Creatinine by Nova Biomedical

Comparable to many clinical assays, time is an important factor for analyzing a patient's creatinine levels and their implication for renal functionality and muscle integrity. Point-of-care creatinine analyzers measure creatinine levels and compute globular filtration rates in under 30 seconds. Samples, obtained by a gentle finger-prick, are collected on a 1.2 micro-liter test strip. These analyzers have a creatinine measurement range of 0.3-12.0 mg/dl (27-1056 µmol/L). A creatinine analyzer is portable and is powered by a Li-ion battery, with computer control established via fast Ethernet connection. Creatine analyzers can operate within a wide range of temperatures, altitudes, and humidity. Additional features include patient and staff identification, bar-code scanning, and an attractive, easy-to-read LCD display.

The Beckman Creatinine Analyzer 2 is a system reagent for the quantitative determination of Creatinine in human serum and urine on Beckham Coulter AU analyzers.

QUNTITATIVE ESTIMATION OF SERUM BILIRUBIN USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of serum bilirubin (Total and Direct) by Jendrassik and Grof Method. Measurement of total bilirubin is useful in the diagnosis of jaundice due to any cause and is an indicator of liver function.

Principle: Bilirubin reacts with diazotized sulfonic acid to form an azo dye which is red in neutral and blue in alkaline solution. Whereas the water-soluble bilirubin glucuronides react "directly" (the free bilirubin). "Indirect" bilirubin reacts only in the presence of an accelerator. The total bilirubin in serum or plasma is determined using by coupling with diazotized sulfonic acid after the addition of caffeine, sodium benzoate and sodium acetate. A blue azobilirubin is formed in alkaline Fehling solution II. This blue compound can also be determined selectively in the presence of yellow byproducts (green mixed coloration) by photometry at 578 nm. The direct bilirubin is measured as the red azo dye at 546 nm using the method of Schellong and Wende without the addition of alkali.

Performance specifications:

-Linearity: Up to 20 mg/dL.

-Measurement range: As low as 0.05 mg in serum.

-Sensitivity: Lower detection limit is 0.05 mg/dL

Primary sample:

- Use only serum as specimen for the test

-Collect 4 mL of venous blood in a plain vacutainer tube

-Do not expose samples for serum bilirubin estimation to tube light/sunlight.

-Do not use hemolyzed, contaminated or lipemic sera.

-Separate serum as soon as possible; Store the serum at -10°C until required, for a maximum up to one month.

Type of container and additive: Use plain vacutainer tubes for collecting samples.

Reagents:

-Sulfanilic acid

-Accelerator: Caffeine, sodium benzoate, sodium acetate

-Sodium nitrite

-Fehling solution II: 930 mmol/L Potassium sodium tartrate, 19 mol/L sodium hydroxide solution.

Instrument: Semi-autoanalyzer.

Procedure:

-Switch on the machine and press "FLUSH" button by keeping the tubing in distilled water for 2 minutes.

Press "PROC". Different test procedures will be displayed.

Select the test to be processed by entering its number and then press "ENTER" key.

-Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

-Feed the reagent blank with each batch of patient samples

-Then feed the standard followed by test samples and record the Values

-Assay: End point assay	**Wavelength: 546 nm**
Temperature 30°C	**Reagent volume 500 µL**
Sample volume: 50 µL	**Incubation time: 15 minutes**
No. of readings:3	**Times: 20.20 sec**

Total Bilirubin

REAGENT	BLANK	TEST
Diazo (Sodium nitrite)	-	50µL
Sulfanilic acid	50 µL	-
Sample	50 µL	50 µL
Accelerator	250 µL Incubate 15 minutes at RT	250 µL
Fehling solution	250 µL Incubate 10 minutes at RT	250 µL

Measure the absorbance of the sample against distilled water or if necessary, against the blank.

Direct Bilirubin

REAGENT	BLANK	TEST
Diazo (Sodium nitrite)	-	50µL
Sulfonic acid	50 µL	-
Sample	50 µL	50 µL
Fehling solution	500 µL Incubate 5 minutes at RT	500 µL

Measure the absorbance of the sample against distilled water or if necessary, against the blank.

Interferences: Turbid lipemic and lysed sera. In patients taking heavy dose of B complex riboflavin and the folate in it may interfere by giving yellow color to the blood and urine.

Calculating results: For measurements against a blank: Total bilirubin concentration = A x 10.5 mg/dL.

Biological Reference Range:

- Total Bilirubin - up to 1.0 mg/dL

-Indirect or unconjugated bilirubin -0.1 to 0.6 mg/dL

-Direct or conjugated bilirubin up to 0.3 mg/dL

Critical/Alert level values: 3.0 mg/dL

Laboratory interpretation: Increase of bilirubin suggests jaundice; increase of both total and direct bilirubin suggests obstructive/hepatocellular jaundice. Increase of total bilirubin alone with normal direct bilirubin suggests hemolytic jaundice. A level of 0.4 mg/dL or more of direct bilirubin suggests liver involvement.

Potential sources of variability:

-Lysed serum specimens may give falsely elevated values

-Dilute the specimen if the bilirubin value is > 10 mg/dL suitable dilution can be done with normal saline. In such a case, the results obtained should be multiplied by dilution factor to be obtained correct bilirubin value.

MANUAL DETERMINATION OF TOTAL AND DIRECT BILIRUBIN

Principle of the test: In the presence of caffeine accelerator, total bilirubin couples with Sulfonic acid to form a red azobilirubin dye

Sulfonic acid + $NaNO_2$ $\xrightarrow{HCl}$ Deionized Sulfonic acid

Bilirubin + Deionized Sulfonic acid $\xrightarrow{HCl}$ Azobilirubin

Direct reaction

• Bilirubin – in the serum from obstructive jaundice reacts immediately with Ehrlich's Diazo reagent, while serum with hemolytic jaundice requires the addition of alcohol and a longer time interval for the reaction

• Measurement of direct 1-min bilirubin demonstrates the glucuronide compound while 30-min bilirubin represents total bilirubin.

The quantity of free (indirect reaching) bilirubin is the difference between the total (30min) and direct (1min) values

Qualitative method

Direct reaction

• Place 1ml of non-hemolyzed serum from a fasting person in a graduated centrifuge tube

• Allow 0.5ml of Ehrlich's Diazo reagent must be prepared each day by mixing 10ml of solution A with 0.3ml solution B below (keep in brown bottle in refrigerator)

Solution A	Solution B
Sulfonic acid ------1gm	**Sodium nitrite --------0.5ml**
Conc. HCl acid ------15ml	**Dist. H2O ---------- 100ml**
Dist. H2O----------1000ml	

• The contact zone between the serum and the reagent is examined against a good back ground for the development of a reddish-purple ring in 30sec

Indirect reaction

• After the determination of the direct reaction shake the tube containing the serum and diazo reagent and add 3ml of 95% alcohol and mix

• If a definite pinkish color fails to appear in 15min and only a white turbidity occurs, the reaction is recorded as Negative. If a color already presents from a positive direct test depends up on the addition of alcohol, the reaction is recorded as positive for the indirect test.

LIPID PROFILE: CHOLESTEROL (HDL-C & LDL-C), TRIGLYCERIDES AND APOLIPOPROTEINS METHODS OVERVIEW

Disorders of lipids is of immense importance to medical practice owing to its strong relations to Arteriosclerosis and thus obesity, HTN, DM and other abnormalities. Good prognosis in the mgt of these conditions are predicated on early detection of deranged blood lipid profile.

Method:

Specimen Collection and Storage:

• Patient should fast for 12hours before sampling.

Venous vs Capillary samples:

– Measurements in the capillary samples seem to be little lower than venous samples.

Anticoagulants:

– Some anticoagulants such as citrate exert large osmotic effect resulting in falsely low plasma lipid and lipoprotein concentration.

– Heparin: because of its high M.W can alter electrophoretic mobility of lipoproteins.

– EDTA is preferred anticoagulant even though TC and TG conc in EDTA plasma are 3 % lower than in serum.

Storage:

– TC, TG, HDL-C can be satisfactorily analyzed in frozen samples.

– Apolipoproteins can also be measured in frozen samples. – Serum or plasma must be stored at – 70oC if stored for long time.

ANALYTICAL METHODOLOGY TOTAL CHOLESTEROL:

• Chemical Method: via Liebermann-Burchardt Reaction – Modified Abell Kendall Method – Bloors Method

• Enzymatic Method: – Cholesterol oxidase (Routine Lab)

• GC–MS Method (Reference Method)

• Isotope Dilution Mass Spectrometry (Definitive Method)

TRIGLYCERIDE:

• Chemical Method – Van Handel and Zilversmith

• Enzymatic Method – Glycerol Kinase

• GC–MS Method (Reference Method)

LIPOPROTEINS:

• Polyanion Precipitation

• Electrophoresis

• Ultra-centrifugation

• Immunochemistry

OTHER METHODS:

– Mass Spectrometry

– Chromatography

– Homogenous assay

Cholesterol Estimation CHEMICAL METHODS:

• Abell Kendall Method (Former Reference Method):

Principle: 3 steps

- **Cholesterol is hydrolyzed with alcoholic KOH**
- **Unesterified cholesterol is extracted with petroleum jelly**
- **Measured using the L-B Reaction**

• Liebermann-Burchardt Reaction (L-B Reaction):

Cholesterol + Sulfuric acid + Acetic anhydride => bluish green solution

• Bloors Method:

Principle: 2 steps

- **Cholesterol is extracted using an alcohol ether mixture**
- **Measured using the L-B Reaction**

ENZYMATIC METHOD:

• Cholesterol Oxidase Method (Routine Lab – Assay of Choice):

Principle: Cholesterol ester + H20 cholesterol esterase-> Free cholesterol Free Cholesterol cholesterol oxidase-> 4 cholestene-3-one + H202

– Trinders Reaction: H202 + 4-aminophenazone peroxidase -> Quinoneimine dye (red) + H_2O

– Read at 500nm wavelength – Linear up to 600 – 700mg/dL (15.54 – 18.13mmol/L)

Advantages (in comparison to the Chemical Method):

– Precise and accurate

– Lesser interferences

– bilirubin, ascorbic acid, Hb

– Smaller sample quantity

– Rapid; does not require preliminary extraction step

– Can be used to measure unesterified cholesterol by omitting de- esterification step

– Mild reagents; better suited for automated analyzers

•GC–MS METHOD (Reference Method):

- **Specifically measures cholesterol and does not detect related sterols**
- **Shows good agreement with the Definitive Method**
- **Isotope Dilution Mass Spectrometry**

Cholesterol Desirable level: < 200mg/dL (< 5.2mmol/L); Conversion factor = 0.026

Estimation of Lipoproteins

POLYANION PRECIPITATION:

• Lipoproteins are precipitated with polyanions (heparin sulfate, dextran sulfate and phosphotungstate)

• Reaction should be in the presence of divalent cations Mg, Ca and Mn

• Most commonly for HDL and is reasonably specific

LIPOPROTEIN ELECTROPHORESIS:

• Used to identify rare familial disorders (e.g Type I, III, V Hyperlipidemia)

• Indications:

– serum TG > 300 mg/dL – fasting serum is lipemic

– significant hyperglycemia, impaired glucose intolerance

– serum uric acid > 8.5 mg/dL

– clinical evidence of CHD or atherosclerosis in patient < 40 years of age.

ULTRA-CENTRIFUGATION:

• Preparative Ultracentrifugation

– Uses sequential density adjustments of serum to fractionate major and minor classes of LP • Density gradient methods (non-equilibrium or equilibrium techniques) permits fractionation or several or all classes of LPs in a single run

IMMUNOCHEMICAL METHODS:

• Use antibody-coated plates specific for epitopes on apolipoproteins both in routine and research lab

HDL–C Estimation:

PRECIPITATION METHOD:

– Precipitating reagents such as divalent cations and polyanions are used to remove all lipoproteins except HDL

– Enzymatic method for total cholesterol (Cholesterol Oxidase) is used to quantitate HDL–C

– Demerit:

• Interference from elevated TG levels causing incomplete sedimentation after centrifuging which results in over estimation of HDL-C

MAGNETIC METHOD

• Similar to the HDL-C precipitation method but uses a precipitant that is complexed to magnetic particle

• This sediment and does not require centrifugation

• Has been adapted for use in automated clinical chemistry analyzers because,

• It allows the supernatant to be analyzed without the need to remove it from the sedimented complex.

HOMOGENOUS ASSAY (Direct HDL-C Assay):

• Enzymatic method:

– First reagent – "blocks" non-HDLs

• Use of Antibodies or Polymers or complexing agent e.g Cyclodextrin

• Modification of cholesterol esterase and oxidase enzymes which makes them selective for HDL-C

• Use of blanking step that selectively consumes cholesterol from non-HDL species – Second reagent – quantifies accessible HDL-C

• Highly precise and reasonably accurate but lacks specificity for HDL in unusual specimens e.g liver or kidney disease

• Does not require pretreatment

• The "Three-step Procedure" (Reference method for HDL-C estimation):

– Ultracentrifugation to remove VLDL

– Heparin manganese precipitation to remove LDL

– Analysis of supernatant cholesterol by the Abell Kendall assay • It is tedious and expensive

LDL–C Estimation INDIRECT METHODS:

• Fridewald Equation (Calculation Method) – Routine.

– LDL–Chol(mmol/L) = [TC – HDL-Chol] – Plasma TG/2.175; or, LDL-Chol(mg/dL) = [TC – HDL-Chol] – Plasma TG/5

-VLDL (mg/dL) = [TAG]/5 or VLDL (mmol/L) = [TAG]/2.175

-The factor [TAG]/5 is an estimate of the VLDL cholesterol and is based on the average ratio of triglyceride to cholesterol in VLDL

-Equation assumes patient fasted and plasma [TAG] does not exceed 5.0mmol/L

– Limitations: not appropriate in

• Samples with TG > 400mg/dL

• Patients with suspected Dysbetalipoproteinaemia

– Other limitations:

• Does not account for cholesterol associated with IDL and Lp(a)

• Underestimate LDL-C in chronic alcoholics

• Unsuitable for monitoring

• Mis-classifies 15 – 40 % of patients when TG levels are between 200 to 400 mg/dL

Beta-Quantification (Reference method):

– Tedious

– reserved for samples where Fridewald equation is inappropriate

– 2 steps

• Ultracentrifugation to remove VLDL leaving behind LDL and HDL as well as IDL and Lp(a)

• Chemical Precipitation of HDL-C from either the whole serum or the infranate obtained from the ultracentrifugation

– LDL-C is calculated as difference btw Cholesterol measured in infranate and in the HDL fraction

– VLDL-C is usually calculated as the difference btw that in whole serum and the amount in the infranate fraction

• VLDL-C/Plasma TG ratio: – may be useful in evaluation of type III hyperlipoproteinemia – Expressed in mol/mol or mass/mass

– Ranges 0.230-0.575 in samples without beta VLDL

– Type III subjects have ratio > 0.689, usually in range of 0.689 – 0.0919

Triglycerides:

CHEMICAL METHOD:

• First, Lipids are extracted using chloroform and phospholipids and removed by zeolite absorption

Van Handel and Zilversmith Method (former Reference Method):

Principle:

• TAG alcoholic KOH -> Glycerol + Fatty acids

• Glycerol + periodic acid ----------> Formaldehyde

• Formaldehyde + Chromotropic acid----------> Blue solution

Demerit:

– Tedious, poorly characterized

GC–MS METHOD (Reference Method):

• Hydrolysis of fatty acids on TGs and measurement of Glycerol

NB: Accuracy in TG is less relevant than that for Chol, due to very large physiologic variation with CV of 25 – 30%, thus, contribution of analytical variation is insignificant

ENZYMATIC METHOD:

Glycerol Kinase Method:

Principle:

• TAG + $3H_20$ -----lipase-----> Glycerol + 3fatty acids

• Glycerol + ATP -------glycerol kinase-----> Glycerophosphate + ADP

• Glycerophosphate ----Glycerophosphate Oxidase---> Dihydroxyacetone + H_2O2

Trinder's reaction:

• H_2O2 + Chromogen --------peroxidase----> Pink compound + H_2O

• Read absorbance at 500nm wavelength, and linear up to 700mg/dL

Merits: – Fairly specific

Apolipoproteins

• Apolipoproteins of clinical importance are:

– Apo B: an indicator of combined LDL and VLDL concentration

– Apo A-1: major protein of HDL

– Lp(a): the variant of LDL, an independent indicator of CHD risk. Commonly measured by Immunoassays of different types

IMMUNOTURBIDIMETRY:

• Most common method;

advantages:

– Easily adapted spectrophotometric analyzers

– allows the use of commercially available antisera and reference sera.

• Immunonephelometric can also be employed but requires a Nephelometer and so not commonly used

• These light scattering assays are subject to interferences from larger TG- rich lipoproteins and VLDL

• Other immunochemical methods available include:

– Enzyme-linked Immunosorbent Assay (ELISA)

– Radial Immunodiffusion (RID)

– Radioimmunoassay (RIA)

• Antibodies used may be either monoclonal or polyclonal

QUANTITATIVE ESTEMATION OF TOTAL CHOLESTEROL USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of total cholesterol in human serum by CHOD-PAP method (enzymatic photometric method measurement of serum cholesterol is useful in the screening of the lipid status of the individual to detect atherosclerotic risks and in monitoring the response to lipid lowering measures and also in the diagnosis and classification of hyperlipidemias. Other conditions such as hepatic and thyroid diseases also influence cholesterol levels.

Principle: Cholesterol esters are hydrolyzed by cholesterol esterase to produce free cholesterol and fatty acids. Hydrogen peroxide is then produced from the oxidation of cholesterol by cholesterol oxidase. In a coupled reaction catalyzed by peroxidase (POD), red quinonimine dye red is formed from 4-aminoantipyrine, phenol and hydrogen peroxide. The absorption at 500 nm of the solution of this dye is proportional to the concentration of cholesterol in the sample. (Trinder's reaction)

Cholesterol ester --- Chol. Esterase----→ Cholesterol + Fatty acids

Cholesterol ----Chol. oxidase ------→ 2 H_2O_2 + Cholesten-4-en 3-one

2 H_2O_2 + 4-Aminoantipyrine + Phenol----- POD ----> Red quinonimine (Red dye) +H_2O

Performance specifications:

-Linearity: Up to 1000 mg/dL of serum

-Measurement range: 1 - 1000 mg/dL of cholesterol in serum

-Sensitivity: The minimum detection limit by this kit is 1 mg/dL

-Specificity: Cholesterol oxidase is not totally specific for cholesterol. Other analogs of cholesterol (dehydrocholesterol, 7-dehydrocholesterol, 20 hydroxycholesterols, etc.) are also oxidized. However, these analogs do not normally occur in any appreciable amounts in serum.

Primary sample:

-Use only plasma as specimen for the test

-Collect 4 mL of venous blood in a heparin vacutainer tube.

-Centrifugation at 2500 rpm for 10 minutes

-Do not use lysed plasma for testing as it may give very high results

-Do not use contaminated/turbid samples for testing

-Process the sample on the same day within 3 hours of collection

-If analysis is not done on the same day/within 3 hours of collection

Type of container and additive: Use plain/heparin vacutainer tubes for collecting samples.

Instrument: Semi-autoanalyzer

Reagents:

-Cholesterol reagent: 4 Amino antipyrine

-Phenol

-Cholesterol esterase

-Cholesterol oxidase buffer pH 6.8.

-Cholesterol standard: 200 mg/dL cholesterol in alcohol.

Procedure:

- **Switch on the machine and press "FLUSH" button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.**
- **Press "PROC". Different test procedures will be displayed.**
- **Select the test to be processed by entering its number and then press "ENTER" key.**
- **Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used.**

- **Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.300. If the absorbance of the 'blank is more than 0.300' discard the reagent**
- **Then feed the test samples and record the values.**
- **Check whether the sample is hemolyzed, icteric before Processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay: End point	**Reagent volume: 1000 µL**
Wavelength: 510 nm	**Sample volume: 10 µL**
Temperature: 37°C	**Zero setting with distilled water**
Incubation time 5 minutes	**Conc. of standard: 200 mg/dL**

Interference: Check whether the sample is hemolyzed or icteric before processing. If the sample is lysed collect another sample and proceed. Do not shake vigorously. Over the time the reagent may develop a light pink color. This is expected but it does not affect the reagent performance. Discard the reagent if the absorbance of the same exceeds 0.3 OD against distilled water at 510 nm.

Calculation of results:

Sample absorbance/ Standard absorbance x Concentration of standard = Sample concentration

Biological reference range

- **Serum: 135–220 mg/dL**
- **Risk classification total cholesterol in blood (mg/dL)**
- **Desirable < 200 mg/dL**
- **Borderline higher risk 200-240 mg/dL.**

High-risk > 240 mg/dL.

Critical/Alert level values: More than 300 mg/dL

Laboratory interpretation: Hypercholesterolemia in hypothyroidism (Myxedema), nephrotic syndrome, atherosclerosis, arteriosclerosis, uncontrolled diabetes and obstructive jaundice. Hypocholesterolemia in hyperthyroidism and acanthocytosis.

Potential sources of variability:

- **Lysed plasma specimens may give falsely elevated values**
- **Cholesterol in plasma remains stable for up to 7 days at room temperature and at -20° C for up to 6 months.**
- **Do not use if the reagent is turbid as it indicates contamination of the reagent.**

ESTEMATION OF LOW-DENSITY LIPOPROTEIN CHOLESTEROL

Purpose: The estimation of LDL cholesterol in human serum is done by calculation using a formula. Measurement of serum LDL cholesterol is useful in the screening of the lipid status of the individual to detect atherosclerotic risks and in monitoring the response to lipid lowering measures and also in the diagnosis and classification of hyperlipidemias. Relationship exists between serum LDL cholesterol and the risk of coronary heart disease. LDL cholesterol value above 130 mg/dL is considered as a risk factor for coronary and cerebral vascular disease, it is also useful for lipoprotein phenotyping.

Principle: By calculation

Procedure: Calculation of results:

Total Cholesterol-(Triglycerides/5 + HDL)

Reference range:

Desirable level: < 130 mg/dL

Border line elevation: 130-159 mg/dL

Elevated: > 160 mg/dL

Calculation carried out up to the range 400 mg/dL TGL

QUANTITATIVE ESTEMATION OF HIGH-DENSITY LIPOPROTEIN CHOLESTEROL USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of HDL cholesterol in human serum by precipitation method-Precipitation of VLDL and LDL (by Magnesium ions and Phosphotungstic acid) followed by estimation of HDL cholesterol by cholesterol esterase oxidase method. Measurement of serum HDL cholesterol is useful in the screening of the lipid status of the individual to detect atherosclerotic risks and in monitoring the response to lipid lowering measures and also in the diagnosis and classification of hyperlipidemias. An inverse relationship exists between serum HDL cholesterol and the risk of coronary heart disease. An HDL cholesterol value below 30 mg/dL is considered as a risk factor for coronary and cerebral vascular disease it is also useful for lipoprotein phenotyping.

Principle: Phosphotungstate/Mg^{2+}precipitate all VLDL IDI DL and chylomicron (CM) fractions in serum. The HDL fraction remains unaffected in the supernatant. Centrifugation leaves the cholesterol in the supernatant. The supernatant is then treated as a sample for cholesterol assay. The cholesterol content in the supernatant HDL is determined enzymatically by cholesterol esterase-choles oxidase method:

Serum/Plasma -----Phosphotungstate/ Mg^{2+} ----→ HDL fraction (in supernatant) + (LDL+ VLDL + CM in

the precipitate)

Performance specifications:

- Linearity: Up to 400 mg/dL of serum

- Measurement range: 1-400 mg/dL of HDL cholesterol in serum

- Sensitivity: The minimum detection limit is 1 mg/dL

- Specificity: Cholesterol oxidase is not totally specific for cholesterol. Other analogs of cholesterol (dehydrocholesterol 7-dehydrocholesterol, 20 hydroxycholesterol, etc.) are also oxidized. However, these analogs do not normally occur in any appreciable amounts in serum.

Primary sample:

- **Use only fasting serum as specimen for the test**
- **Collect 4 mL of venous blood in a heparin vacutainer tube**
- **Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500-3000 rpm for 5-10 minutes**
- **Do not use icteric/lysed plasma for testing as it may give very high results**
- **Do not use contaminated/turbid samples for testing**
- **Process the sample on the same day within 3 hours of collection.**
- **If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 2 -8° C for up to 7 days**

Type of container and additive: Heparin vacutainer. No additive/ Preservative is needed to be added.

Reagents:

- **Precipitating reagent phosphotungstic acid (2.4 mM) and magnesium chloride (40 mM)**
- **Cholesterol reagent: 4-Aminophenazone**
- **Phenol**
- **Cholesterol esterase**
- **Cholesterol oxidase**
- **Horseradish peroxidase, buffer pH 6.8, non-reactive stabilizers, and fillers.**
- **HDL-cholesterol standard: 50 mg/dL**

Instrument: Semi-autoanalyzer

Procedure:

- **Bring the reagents to room temperature before use. Add 500 µl of serum and 500 µl of HDL precipitating reagent. Mix well and centrifuge at 4000 rpm for 10 minutes to obtain a clear supernatant**

- **Assay the supernatant for HDL cholesterol using cholesterol reagent (as for total cholesterol)**
- **Switch on the machine and press "FLUSH "button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.**
- **Press "PROC". Different test procedures will be displayed.**
- **Select 'Absorbance' mode**
- **Select the test to be processed by entering its number and then press "ENTER" key. Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used**
- **Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.100. If the absorbance of the blank is more than 0.100', discard the reagent**
- **Then feed the test samples and record the values**
- **Check whether the sample is hemolyzed, icteric before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay: End point	**Reagent volume: 1000 L**
Wavelength: 510 nm	**Sample volume: 50 µL.**
Temperature: 37°C	**Zero setting with reagent blank**
Incubation time 10 minutes	**Conc. of standard: 50 mg/dL**

Interferences: Blood collection in fed state to be avoided, separate plasma immediately. Samples kept above 2 -8°C and aged 1 day or more should not be used.

Calculating results:

Sample absorbance/ Standard absorbance x Concentration of standard = Sample concentration

Biological reference range: 30 - 60 mg/dL

Alert/Critical values: Below 30 mg/dL

Laboratory interpretation: HDL cholesterol/Total cholesterol ratio less than 0.2 indicates a risk factor for coronary heart disease: If it is Total cholesterol/HDL cholesterol the ratio is 5.

Potential sources of variability

- Lysed serum specimens may give falsely elevated values

- Do not use if the reagent is turbid as it indicates contamination of the reagent or if the absorbance of the blank reagent is more than 0.100

QUANTITATIVE ESTEMATION OF TRIAYLGLYCEROLES USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of triglycerols in human serum by enzymatic method using Glycerol -3 Phosphate Oxidase (GPO) Measurement of triglycerides in conjunction with other lipid assays is used in screening the lipid status of an individual to detect atherosclerotic risks and in monitoring the response to lipid lowering measures triglyceride determinations when performed are useful in the diagnosis of primary and secondary hyperlipoproteinemia. They are also of interest in following the course of diabetes mellitus, nephrotic syndrome, biliary obstruction and various metabolic abnormalities due to endocrine disturbances

Principle: The procedure involves hydrolysis of triglycerides by lipoprotein lipase. The glycerol concentration is then determined by enzymatic assay coupled with Trinder reaction that terminates in the formation of a quinoneimine dye which is generated from 4-aminoantipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase. The amount of the dye formed, determined by its absorption at 500 mm, is directly proportional to the concentration of triglycerides in the sample

Triglycerides + H_20------ Lipase ----> Glycerol + Fatty acids

Glycerol + ATP-----Glycerol kinase-----> Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 ----GPO----> DAP-H_2O

2H2O + 4-AA + Chlorophenol -----Peroxidase-----> Red quinone dye + 4H_2O

Performance specifications:

- Linearity: Up to 1000 mg/dL of serum

- Measurement range: 1-1000 mg/dL of cholesterol in serum

- Sasitivity: The minimum detection limit by this kit 1 mg/dl.

Primary sample:

- Use only fasting serum as specimen

- Collect blood sample after an overnight fast of 12-14 hours when testing is a part of lipid profile

- Collect 4 mL of venous blood in a plain vacutainer tube.

- Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500-3000 rpm for 5-10 minutes

- Do not use lysed serum for testing as it may give very high results

- Do not use contaminated/turbid samples for testing

- Process the sample on the same day within 3 hours of collection

- If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 20-25°C for up to 2 days or at +8 °C for up to 7 days

Type of container and additive: Use plain vacutainer tubes for collecting samples. No additive/preservative is needed to be added.

Reagents: Lipoprotein lipase, magnesium acetate, 4 aminoantipyrine, glycerol-3-phosphate oxidase, glycerol kinase, peroxidase, triglyceride standard 200 mg/dL triglycerides as triolein.

Instrument: Semi-autoanalyzer

Procedure:

- **Switch on the machine and press "FLUSH "button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.**
- **Press "PROC. Different test procedures will be displayed.**

- **Select the test to be processed by entering its number and then press "ENTER" key.**
- **Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used.**
- **Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.300 at 520 nm if the absorbance of the blank is more than 0.300, discard the reagent.**
- **Then feed the test samples and record the values.**
- **Check whether the sample is hemolyzed, icteric before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or highly lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay: End point	**Reagent volume: 1000 L**
Wavelength: 546 nm	**Sample volume: 10 µL**
Temperature: 30°C	**Zero setting with distilled water**
Incubation time: 5 minutes	**Conc. of standard: 200 mg/dL**

Calculating results:

Sample absorbance/ Sample concentration x Concentration of standard = Sample concentration

Biological reference range:

Male: 60-165 mg/dL

Female: 40-140 mg/dL

Critical/Alert level values: 400 mg/dL

Laboratory interpretation: Triglycerolemia is a risk factor for myocardial infarction. TGA is phenomenally increased in an eye disease lipemic retinitis.

Potential sources of variability:

- Lysed serum specimens may give falsely elevated values

- Do not use if the reagent is turbid as it indicates contamination of the reagent and if the absorbance of the blank reagent is more than 0.300.

QUANTITATIVE ESTEMATION OF TOTAL PROTEIN USING SEMI-AUTOANALYZER

Purpose: Estimation of total protein in serum/body fluids by Biuret method. Low protein levels are observed in malnutrition, acute or chronic liver diseases, nephrotic syndrome, water intoxication, salt retention syndromes, and massive intravenous infusions. Elevated protein levels are observed in dehydration due to vomiting, diarrhea, Addison's disease and diabetic ketoacidosis. High protein levels of over 2 g/dL in body fluids are suggestive of inflammation or malignancy and are called exudates.

Principle: Peptide bonds of proteins in serum react with cupric ions in alkaline solutions to form a blue colored complex, the absorbance of which is measured at 578 nm. The intensity of the blue color is proportional to the amount of protein present. The reaction sequence employed in the assay of total proteins is as follows:

Protein + Cu^{2+} ------Alkaline pH----> Cu-Protein complex (Blue color complex)

Performance specifications:

- Linearity: Up to 12 g/dL

- Measurement range: This method has a measurement range of 5.3-8.4 g/dL of total protein in serum and body fluids.

- Sensitivity: The minimum detection limit by the kit is 5.3 g/dL.

Primary sample:

- Use serum/body fluids (Pleural, Pericardial, Ascitic fluid) as specimen for the test.

- Collect blood sample in a red color vacutainer tube, separate serum within 30 minutes of collection.

- Process the sample on the same day within 1 hour of collection. If analysis is done on the next day, separate the serum and store it at 2–8°C for up to 30 days.

Type of container and additive: Use plain vacutainer tubes for collecting samples. No additive/Preservative is needed to be added

Reagents:

- Biuret reagent

- Total protein standard

Instrument: Semi-autoanalyzer

Procedure:

- **Switch on the machine and press "FLUSH "button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.**
- **Press "PROC". Different test procedures will be displayed.**
- **Select the test to be processed by entering its number and then press "ENTER" key.**
- **Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used.**
- **Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.150, if the absorbance of the 'blank is more than 0.150' discard the reagent at 546 nm.**
- **Then feed the test samples and record the values.**
- **Check whether the sample is hemolyzed or icteric before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay type: End point, Wavelength: 546 nm

Sample volume: 10 µL, Reagent volume 1000 L

Incubation time: 20 min at RT, Temperature: 37°C

Interferences:

-Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.150, If the absorbance of the 'blank is more than 0.150, discard the reagent at 546 nm.

-Keep the reconstituted reagent at 2–8°C. Discard the same if it develops precipitate.

-Highly hemolytic or icteric samples, prepare sample blank by adding 1 mL of 0-9% saline to 10 microliter samples. The value of the blank is subtracted from the corresponding sample value.

Calculating results:

Sample absorbance/ Standard absorbance x Concentration of standard = Sample concentration

Biological reference range: Adults: 6.6-8.4 g/dL

Critical/Alert values: Below 5.0 g/dL and above 9.0 g/dL

Laboratory interpretation: Increase of proteins in dehydration, multiple myeloma and chronic infections (gammopathy); decrease in malnutrition, liver diseases, nephrotic syndrome.

Potential sources of variability:

-The reagent is linear to 12.0 g/dL. Samples with values above 10 g/dL should be diluted 1:1 with 0.9% saline, re-run, and the result multiplied by two (2)

-The biuret procedure is not sensitive at low ranges (< 1 g/dL).Do not use for urine or spinal fluid.

MANUAL DETERMINATION OF TOTAL PROTEIN

Principle: In the biuret reaction, a chelate is formed between the Cu^{2+} ion and the peptide bonds in protein in alkaline solution to form a violet colored complex whose absorbance is measured to the concentration of protein in the sample

Cu^{2+} serum $\xrightarrow{pH>12/\ 25\text{-}37\ C}$ copper protein sample

Reagent composition

R1: Biuret reagent (cupric sulphate 6mmol/l, sodium – potassium tartrate 21mmol, potassium iodide 6mmol, sodium hydroxide 0.75mol/l, CAL protein standard [Bovine Serum Albumin 7g/dl (70g/l)]

Samples

Serum, EDTA or heparinized plasma and exudates. Total protein is stable in serum for one week at room temperature, for at least 1 month refrigerated at 2 – 8°C, for up to 2 months at -20°C

Interferences: Grossly haemolytic or lipemic samples result in positive interference

Materials required

- **Photometer or colorimeter capable of measuring absorbance at 540 ± 20nm**
- **Constant temperature incubator set at 37°C**
- **Pipettes to measure reagent and samples**

Procedure

- **Pipette in to labeled tubes**

Tubes	Blank	Sample	Standard
Biuret	1ml	1ml	1ml
Sample	-	20ul	-
Standard	-	-	20ul

- **Mix and incubate the tubes for 10 min at 37°C**
- **Read the absorbance (A) of the sample and standard 540nm against the reagent blank**

Calculation

A sample/ A standard X C standard = g/dl total protein

If the results are to expressed as SI units apply g/dl × g/l.

QUANTITATIVE ESTEMATION OF ALBUMIN USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of albumin in human serum by photometric method using bromocresol green (BCG) dye binding. Elevated serum albumin levels are associated with possible dehydration. Low serum albumin levels are indicative of potential malnutrition, liver diseases, kidney disorders chiefly nephroti syndrome, and rheumatoid arthritis.

Principle: Albumin acts as a cation at a pH of 3.8 and selectively binds to the anionic dye, bromocresol green forming a green colored complex. The colored complex absorbs light at 630 nm. The increase in absorbance is directly proportional to the concentration of albumin in the sample.

Performance specifications:

- *Linearity*: Up to 6.0 g/dL in serum

- *Measurement range:* 0.5-6.0 g/dL of albumin in serum

- *Sensitivity:* The minimum detection limit is 0.5 g/dL

- *Specificity:* Ampicillin and other medications interfere with the dye-binding properties of albumin. As the dye-binding properties of albumin from various species have been found to differ widely, only standards and controls containing human albumin should be employed with this procedure as standards. Controls and standards from other species will interfere with the results.

Primary sample:

- **Use only serum, as specimen for the test and fasting specimen is advisable as lipemia interferes with the assay**
- **Avoid venostasis during sample collection to avoid hemo-concentration which will increase albumin concentration**
- **Collect 4 mL of venous blood in a plain red vacutainer tube. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes**
- **Do not use lysed serum for testing as it may give very high results**
- **Do not use contaminated/turbid samples for testing**
- **Process the sample on the same day within 3 hours of collection.**

- **If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 2-8 °C for up to 30 days.**

Type of container and additive: Use plain vacutainer tube for collecting samples. No additive/preservatives needed to be added

Reagents:

-Albumin Reagent: Bromocresol green (BCG), buffer pH 3.68

-Standard: Bovine albumin fraction V with stabilizer (5)

Instrument: RA 50 or any semi autoanalyzer

Procedure:

- Switch on the machine and press "FLUSH" button by keeping the tubing by distilled water for 2 minutes.

- Press "PROC". Different test procedures will be displayed.

- Select the test to be processed by entering its number and then press "ENTER" key.

- Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

- Feed the reagent blank with each batch of patient samples

- Then feed the standard followed by test samples and record the values

- Check whether the sample is hemolyzed, lipemic before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.

Assay: End point Reagent volume: µ500 L/1000 µL

Wavelength: 628 nm Sample volume: 10 µl

Temperature: 37°C Zero setting with distilled water

Incubation time: 10 minutes Conc. of Standard: 5.0 g/dL

Path length: 1 cm

Interferences: Highly hemolytic or icteric samples prefer sample blank by adding 1 mL of 0-9% saline to 10 microliter samples. The values of the blank is subtracted from the corresponding sample value.

Calculating results:

Sample absorbance / Standard absorbance x Concentration of standard = Sample concentration

Biological reference range: Adults: 3.5-5.0 g/dL

Critical/Alert level values: 2.0 g/dL

Laboratory interpretation: Hypoalbuminemia in liver diseases, nephrotic syndrome, malnutrition, chronic diseases, severe hemorrhage and pregnancy; lower albumin is reflected in lowered A/G ratio. Increase of albumin in dehydration.

Potential sources of variability:

- Lysed serum specimens may give falsely elevated values.

- Albumin reagent should be a clear, yellow-green solution. If turbidity or precipitation has occurred, discard the reagent.

- Ampicillin and other medications interfere with the dye-binding properties of albumin. As the dye-binding properties of albumin from various species have been found to differ widely only standards and controls containing human albumin be employed with this procedure, as standards and controls from other species will interfere with results.

MEASURMENT OF CERULOPLASMIN IN SERUM

Purpose: To measure the amount of ceruloplasmin in serum. Lower levels have been reported in Wilson's disease and in cases of nephrotic syndrome. It has been found to be very useful in differentiating chronic liver diseases from Wilson's disease.

Principle: Ceruloplasmin, ferro-oxidase, catalyzes the oxidation of some polyamines and its action on p-phenylene diamine is measured as the amount present in serum.

Performance specifications:

- *Linearity:* Up to 60 mg/dL of serum

- *Measurement range:* 16-60 mg/dL ceruloplasmin activity in serum

- *Sensitivity:* The minimum detection limit by this method is 16 mg/dL.

Primary sample:

- Use only serum as specimen for the test.

- Collect 4 mL of venous blood in a plain red color vacutainer tube. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes.

- Do not use lysed serum for testing as it may give very high results

- Do not use contaminated/turbid samples for testing.

- Process the sample on the same day within 3 hours of collection. 4.6. If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at -20 °C for up to 7 days.

Type of container and additive: Use plain vacutainer tubes for collecting samples. No additive/preservative is need to be added.

Reagents:

- **p-Phenylenediamine hydrochloride, 5 g in 1-liter solution in water. To purify dissolve p-phenylene diamine dihydrochloride in a minimum volume of hot distilled water, decolorize with charcoal, filter and allow to crystallize, keep the dried crystals over calcium chloride. This can be kept in sealed vials for several weeks.**
- **Acetic Acid (1 M): Make up 60 mL glacial acetic acid to 1 lit with water.**
- **Sodium acetate (1 M): 136 g per liter of distilled water.**
- **Acetate Buffer, 400 mM pH 5.5: Add approximately 1.2 mL of the acetic acid to 20 mL of sodium acetate to bring pH 5.5 and dilute to 50 mL store at 4°C.**
- **Sodium azide: Five gram per liter or sodium fluoride 20 liter can be used.**

Instrument: Spectrophotometer.

Procedure: Water blank

	Reag blank	*Test blank*	*Test*
Serum	--	0.1 mL	0.1 mL
Sodium azide	1 mL	1 mL	--
Acetate buffer	8 mL	8 mL	8 mL
Phenylenediamine	1 mL	1 mL	1 mL
	Incubate the tubes at 37°C for 1 hr		
Sodium azide	--	--	1 mL
	Cool for 30 minutes at 4°C and read at 530 nm		

Calculation: $T_{OD} - C_{OD}$ X 60* X 100/0.1 mg/dL

* A conversion factor of 60 (0.06 x 1000) of King (1965) is used to express ceruloplasmin values as mg/dL.

Reference range value: 20–40 mg/dL.

Potential sources of variability:

- Lysed serum specimens may give falsely elevated values.

- If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at -20 °C for up to 7 days.

QUANTITATIVE ESTEMATION OF URIC ACID IN SERUM USING SEMI-AUTOANALYZER

Purpose: Quantitative estimation of uric acid in human serum by enzymatic uricase method. The quantitation of uric acid is an aid in the diagnosis of gout, decreased renal function, and myeloproliferative disorders. The quantitation of uric acid is also an aid in the diagnosis of hyperuricemia due to any cause. Serum uric acid levels will increase with urea and creatinine under conditions of elevation of NPN substances in serum.

Principle: Uric acid is converted by uricase into allantoin and hydrogen peroxides. The hydrogen peroxide initiates the coupling of 4-aminoantipyrine to 3,5-dichloro-2-hydroxybenzene sulfonic acid (DCHBS) to form the chromogen which is measured at 520 nm and which is proportional to the amount of hydrogen peroxide generated from uric acid.

Uric acid $+H_2O + O_2$ ----- Uricase-----► Allantoin $+H_20_2 + CO_2$

$2H_2O_2$ + 4-AAP + DCHBS-----Peroxidase/ HCl + H_2O----► Red colored complex

Performance specifications:

- ***Linearity:*** **25 mg/dL of serum**

- ***Measurement range:*** **1-25 mg/dL**

- ***Sensitivity:*** **The minimum detection limit by this kit is 1 mg/dL**

Primary sample:

- Use serum/plasma
- Collect 4 mL of venous of venous blood from a peripheral vein in a plain red-topped vacutainer tube.
- Do not use hemolyzed / contaminated serum for testing.

Type of container and additive: Use plain vacutainer tubes for collecting samples, do not use hemolyzed/contaminated serum for testing.

Reagents: The reconstituted reagent contains the following:

- **Uric acid reagent: 4-Aminoantipyrine, 3, 5 dichloro-2 hydroxybenzenesulfonate, stabilizer and surfactant, uricase peroxidase (horseradish), buffer pH 7.5**
- **Uric acid Standard 6 mg/dL.**
-

Instrument: Semi-autoanalyzer

Procedure:

- **Switch on the machine and press "FLUSH" button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.**
- **Press "PROC", different test procedures will be displayed.**
- **Select the test to be processed by entering its number and then press "ENTER" key.**
- **Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used**
- **Feed the blank with each batch and ensure the absorbance of the blank is less than 0.4 if the absorbance of the 'blank is more than 0.4', discard the reagent.**
- **Run the standard with each batch of patient sample.**
- **Then feed the test samples and record the values.**
- **Check whether the sample is hemolyzed, icteric or lipemic before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay: End point **Sample volume: 50 µL**

Wavelength: 520 nm **Reagent volume: 500 µL**

Temperature: 37°C **Incubation time: 5 minutes**

Concentration of standard: 6 mg/100 mL

Calculation of results:

Sample absorbance/ Standard absorbance x Concentration of standard = Sample concentration

Biological reference range

Male: 3.4-70 mg/d1

Female: *2.4 5.7 mg/l*

Critical/Alert level values: Above 7 mg/dL.

Laboratory interpretations: Specific for gout in which there is increase of blood level. Increase is also found in leukemia, polycythemia, renal dysfunctions, lactic acidosis alcoholics, atherosclerosis, uncontrolled diabetes, hypothyroidism and glycogen storage diseases. There is decrease in Wilson's disease.

Potential sources of variability

- **Use of only clear, unhemolyzed serum, separated erythrocytes as soon as possible, Lysed serum specimen give falsely elevated values.**
- **On storage, the working reagent may develop a pink color, a makes the use of reagent blanks necessary with every run**
- **Do not use if the absorbance of the blank reagent is greater as it indicates deuteriation of the reagent.**
- **Uric acid remains stable in serum for up to 7 days if this specimen is stored at 2-8° C. Hence if analysis is not done the same day within 3 hours of collection, separate the serum and store it at 2-8° C.**

Interference: Icteric, hemolyzed and turbid sera should not be used.

ESTEMATION OF LACTIC ACID IN BLOOD

Purpose: To estimate lactic acid in blood. The blood lactate concentration is dependent on the extent of metabolism in the liver and muscle and excretion by the kidneys. The purpose of determination of lactate in blood is to diagnose lactic acidosis which is associated with shock, hypovolemia, diabetes mellitus, liver disease, etc.

Principle: Lactic acid is converted quantitatively to acetaldehyde on heated with concentrated sulfuric acid. This acetaldehyde reacts with p-hydroxydiphenyl in the presence of copper sulfate forms violet colored compound, which can be estimated spectrophotometrically.

Performance specifications:

- *Linearity:* Up to 5 mg.

- *Measurement range:* 0.5-5.0 mg.

- *Sensitivity*: 05 mg.

Primary sample:

- **10% TCA precipitated blood sample.**
- **To 2 mL of ice-cold TCA add equal amounts of blood, which is collected without using tourniquet. Mix properly. Centrifuge the sample at 2500 rpm for 10 min. Use the supernatant for analysis, 4.3. Transport the sample in ice. 44. Process the sample on the same day. If analysis is not done on the same day, separate the supernatant and store at -20°C for up to 7 days.**
- **The blood should be collected by vein puncture without using tourniquet.**

Type of container and additive: 15 mL falcon tube is used for collecting sample, containing 2 mL of 10% cold TCA

Reagents:

- **10% TCA: 10 g in 100 mL**
- **4% $CuSO_4.5H_2O$: 4 g in 100 mL**
- **Concentrated sulfuric acid.**
- **p-hydroxy diphenyl reagent: Dissolve 1.5 g of p-hydroxy diphenyl in 95% ethanol,**
- **Stock standard: Lithium lactate-21.3 mg of lithium lactate in 100 mL**
- ***Working standard:* Dilute the stock standard with water to get a solution containing 10 ug/mL.**
- ***Sample preparation:* To 20 mL of 10% cold TCA added 2.0 mL of blood sample, which was collected without using tourniquet. Mixed and centrifuged, the supernatant was used for the estimation**

Instrument: Spectrophotometer.

Procedure:

S.No.	Regents (ml)	Test	Control	Blank
1.	Supernatant	0.05	0.05	-
2.	Distilled water	0.45	0.45	0.5
3.	Conc. sulfuric acid	3	3	3
	Keep the tubes in 95-100" for 10 minutes. Cool it in running water. After cooling add			
4.	4% $CuSO_4$ (drop by drop)	0.05	0.05	0.05
5.	p-hydroxy diphenyl reagent (drop by drop)	0.1	0.1	0.1
	Keep the tubes at room temperature for 30 min.			
	Read at 570 nm against water as blank using spectrophotometer.			

Standardization

S. No.	*Reagents (mL)*	*B*	*S_1*	*S_2*	*S_3*	*S_4*	*S_5*
1.	Std. lithium lactate	--	0.1	0.2	03	04	0.5
2.	Concentration (mg)	--	1	2	3	4	5
3.	Distilled water	0.5	0.4	0.3	0.2	0.1	–
4.	Conc. H_2SO_4	----------3.0----------					

Keep the tube at 95-100" for 10 min. Cool it in running water. After cooling add

---

5. $CuSO_4$ (drop by drop) ----------0.05----------

6. p-hydroxy diphenyl reagent (drop by drop) ----------0.1----------

---

Keep the tubes at room temperature for 30 minutes. Read at 570 nm against water as blank using spectrophotometer.

Calculation: Concentration from graph = X mg in 0.05 mL supernatant

= X/ 0.05x 2

where 90 M met of locate to convert to Mm;

Multiplied by 2 – 1; 2 dilution

1000 - to convert to liter

Reference range: 05-1.3 mM

Potential sources of variability:

-After the blood collection, it should be immediately precipitated with cold TCA

-If the supernatant obtained is turbid, it should not be used for the analysis.

-Sample to be transported in ice

ESTIMATION OF PYROVIC ACID IN BLOOD

Purpose: To estimate pyruvic acid in blood. It is useful in diagnosing diseases like congestive heart disease diarrhea, other digestive disturbances, liver damage, acute infections, beriberi and some neurological diseases.

Principle: Pyruvate reacts with 2,4-dinitrophenylhydrazine forms 2,4-dinitrophenylhydrazone, which reacts with strong alkali to forma reddish compound, which can be estimated spectrophotometrically.

Performance specifications:

- *Linearity:* Up to 50 ug

- *Measurement range:* 70-210 ng

- *Sensitivity:* The minimum detection limit 5 ug

Primary sample:

- **10% TCA precipitated venous blood sample.**
- **To 2 ml of ice-cold TCA add equal amounts of venous blood, which is collected without using tourniquet. Mix properly. Centrifuge the sample at 2500 rpm for 10 min. Use the supernatant for analysis.**
- **Process the sample on the same day. If analysis is not done on the same day, separate the supernatant and keep at -20°C at the maximum of 7 days.**
- **Sample to be transported in ice.**

Type of container and additive: 15 ml falcon tube for collecting blood sample. Equal volume of 10% cold TCA should be added in whole blood.

Reagents:

- **200 mg of DNPH in hot 1000 ml of 1 NHC**
- **NaOH: 16 g/liter.**
- **Standard: Pyruvic acid 1 mg/mL.**

Instrument: Spectrophotometer

Procedure:

S.No.	Reagents	*Blank*	*Test*	S_1	S_2	S_3	S_4
1.	Standard (mL)	-	-	5	10	15	20
2.	Concentration (µg)			5	10	15	20
3.	H_2O(mL)	0.5	-	0.495	0.490	0.485	0.80
4.	Supernatant (mL)	-	0.5	-	-	-	-
5.	DNPH (mL)	1.0	1.0	1.0	1.0	1.0	1.0
	Keep at 37°C for 30 minutes						
6.	NaOH (mL)	10 ml	10 ml	10 mL	10 mL	10 mL	10 ml

Read at 440 nm against blank.

Calculation: **Concentration from graph = X µg in 0.5 mL supernatant**

Calculate for the total volume of the supernatant = 2.0 mL blood

Calculate for 1000 mL blood say = Y µg/L

= Y/ 110 x 1000 x 2 = Z mM

where molecular weight of pyruvate = 110

1:2 dilution with TCA = 2

Reference range: 90-200 µM/L

Potential sources of variability:

- **After the blood collection it should be immediately precipitated with cold TCA.**

- **If the supernatant obtained is turbid, it should not be used for the analysis.**
- **Sample to be transported in ice.**

QUANTITATIVE ESTEMATION OF SODIUM, POTASSIUM AND CHLORIDE IN HUMAN PLASMA

Purpose: Quantitative estimation of sodium, potassium and chloride in human plasma by using ion sensing electrodes in electrolyte analyzer. The levels are useful is assessment of water and electrolyte in diseases with acidosis or alkalosis and endocrine diseases. Knowledge of hypokalemia is used for prompt therapy to save the heart.

Principle: Its methodology is based on the selective electrode measurement (SLE) principle to precisely determine measurement values. Three different electrodes used in the AVL 9180 electrolyte analyzer, sodium, potassium, chloride and a reference electrode. Each electrode has an ion selective membrane that undergoes a specific reaction with the corresponding ions contained in the sample being analyzed. The membrane is an ion exchanger, relating to the electrical charge of the ion causing a change in the membrane potential or measuring voltage, which is built up in the film, between the sample and the membrane

Performance specifications

- **Linearity: 51-196m Eq/L, potassium: 2.0-12.6m Eq/L., Chloride: 56-194 m Eq/L**
- **Measurement range: This method has a measurement range of sodium: 116-150 m Eq/L, potassium 2.0-70 m Eq/L,. chloride: 94-115 m Eq/L**
- **Sensitivity: The minimum detection limit by the kit. Sodium 51 m Eq/L, potassium 1.5 m Eq/L., chloride: 50 m Eq/L.**

Primary sample:

- Use plasma/body fluids as specimen for the test.

- Collect blood sample in 4 mL heparin vacutainer tube

- Separate plasma within 30 minutes of collection

- Process the sample on the same day within 1 hour of collection. If analysis is done on the same day, keep it 2-4 °C. 5.

Type of container and additive: Use 4 mL heparin vacutainer tub for collecting samples.

Reagents:

- **ISE SNAP PAKIM® consists of Std A solution (350 mL), Std B solution (85 mL), Std C solution (85 mL)**

Reference solution: A salt bridge for calibration and measurement in the AVL 9180 electrolyte analysis.

- **Separately packed reagents:**

  Cleaning solution A: For cleaning the measuring system.

  Conditioning solution B: For daily conditioning of the std-electrode and sample sensor in the AVL 9180

- **Electrolyte analyzer: 100 mL**

Instrument: AVL 9180 electrolyte analyzer

Analyzer components: The AVL electrolyte analyzer is a fully automatic microprocessor-controlled medical instrument that measures sodium, potassium and chloride. The analyzer consists of several major components that are important for us to communicate with the analyzer through a keypad with Yes/No keys. With these keys, we can perform all analyzer functions, including sample measurement data input, programming and quality control testing. The measuring chamber consists of the movable left locking device that holds the electrode in place, the electrodes, the right electrode holder, with sample sensor connector and the measuring chamber bare.

Electrodes are labeled: Reference electrolyte, sodium (Na^+), potassium (K^+), chloride (Cl^-).

Operation: Running a sample with the AVL electrolyte analyzer: The AVL electrolyte analyzer provides fast easy operation.

Whenever READY appears on the display, the unit is prepared to conduct sampling measurement.

To analyze a std. sample, press No. To get QC/STD/DIALYSATE/URINE SAMPLE? appears press Yes. To analyzer a sample lift the sample door. The promptly,

"Introduce sample", will be displayed and the pump will begin to aspirate. Introduce the sample to the probe. Hold the sample, under the probe will wipe probe close sample door is displayed. Use a lint-free tissue to clean the probe, and then close the sample door when prompted. The analyzer will display Thank You! and a brief countdown will begin. Upon completion of analysis, the test results will be displayed and printed.

Note: **Values that is higher or lower than the programmed normal range will be indicated by an arrow pointing up or down. The process involves cleaning and conditioning the sampling path, including the probe and electrodes. You will need to have ready the bottles of cleaning solution. A and electrolyte conditioning solution B and a package of lint-free tissues to use in drying sample probe. Check the bottles to ensure that the expiration date has not been reached.**

Standby Mode: The AVL electrolyte analyzer is designed to calibrate automatically every four hours during normal operation. If sampling will be displayed for an extended period of time, such as evening and weekends, you may place the analyzer into standby mode to suspend automatic calibration. Important points to be noted when operating the electrolyte analyzer: It is very important that the main door is closed during sampling, since it provides shielding from sources of electromagnetic interference.

Procedure:

- **Sample size: 95 mL**
- **Sample types: whole blood, serum, plasma**
- **Sample container: Capillary, AVL microsampler, syringe, collection tube, sample cup.**
- **Ambient temperature: +15 to +32° C (60-90° F)**
- **Relative humidity: 5 to 85% (non-condensing)**
- **Type of measurement: Direct potentiometry.**
- **Calibration: The analyzer contains software, which permits one of three configurations. (Na^+, K^+, Cl^-) Each of the configurations is done with the same calibration solutions.**
- **A two-point calibration is performed automatically every 4 hours.**
- **In ready mode and a one-point calibration is automatically performed with every measurement.**

- **Automatic calibration procedure is also performed shortly after power on or reset. A calibration cycle can also be initiated manually at times when no sample measurements are performed.**

Interference: Lysed sample should not be used.

Calculating results: Automatic calculation done by the machine.

Biological reference range: Sodium 136-145 m Eq/L

Potassium 3.5-5.1 m Eq/L

Chloride 97-111 m Eq/L

Critical/Alert values: Plasma sodium < 120-150 m Eq/L

Plasma potassium < 2.0-6.0 mEq/L

Plasma chloride < 97-120 mEq/L

Laboratory interpretation: Hyponatremia and hyperkalemia show abnormal cardiac functions. Hyponatremia in Addison's disease and salt-losing nephrites and hyper natremia its Cushing's syndrome and primary aldosteronism.

QUANTITATIVE ESTEMATION OF CALCIUM IN HUMAN PLASMA

Purpose: Quantitative estimation of Calcium in human serum/by Direct Photometric Method using Arzenazo III. Hypercalcemia (increased serum calcium) is observed in hyperparathyroidism, hypervitaminosis D, sarcoidosis, multiple myeloma, and certain cancers of the bone. Hypocalcemia can cause osteoporosis and tetany. Monitoring of serum calcium level is useful during calcium supplementation in the treatment of osteoporosis

Principle: Calcium reacts with Arzenazo III at neutral pH to form blue colored complex that absorbs at 650 nm. The intensity of the color is directly proportional to the calcium concentration in specimen. Interference by magnesium is eliminated by the addition of 8-hydroxy quinoline 5 sulfonic acid.

Calcium + Arzenazo III--- Neutral Ph----->Blue colored complex

Performance specifications:

- ***Linearity:*** **This method is linear for calcium concentrations up to *16 mg/dL of serum***
- ***Measurement range:*** **This method has a measurement range of 5 16 mg/dL of calcium in serum**
- ***Sensitivity:*** **The minimum detection limit by this kit is 5 mg/dL.**

Primary sample:

- **Use only serum as specimen**
- **Collect 2 mL of venous blood in a plain vacutainer tube. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes**
- **Do not use lysed serum for testing as it may give very high results**
- **Do not use contaminated/turbid samples for testing**
- **Do not collect blood in EDTA as calcium is chelated by DTA**
- **Do not use tourniquet while collecting vinous blood sample may lead to falsely elevated levels of calcium in the sample**
- **Process the sample on the same day within a hours or collection**
- **If analysis is not done on the same day within 3 hours of collection separate the serum and store it at 20 25 for up to 7 days or at 4-8°C for up to 21 days.**

Type of container and additive: For serum calcium, use a plain Vacutainer tube for collecting venous sample. No additive/ preservative is needed to be added

Reagents:

- The reconstituted reagent contains Arzenazo III

- Calcium Standard: 10 mg/dL

Instrument: Spectrophotometer

Procedure:

Assay: End point **Reagent volume: 1000 µl**

Wavelength: 650 nm **Sample volume 10 µl**

Temperature: 37°C **Zero setting with distilled water**

Incubation time 5 minutes **Conc of standard: 10 mg/dL**

Interferences: Turbid, lipemic, icteric and lysed samples should not be used. Tourniquet should not be used, because tissue fluids will affect the value

Calculating Results:

Sample absorbance/ Standard absorbance X Concentration of standard= Sample concentration

Biological reference range: Serum: 8.8-10.2 mg/dL

Critical/Alert level values: <8 mg/dL >12.0 mg/dL

Laboratory interpretation: Hypercalcemia is found in sarcoidosis, hyperparathyroidism and multiple myeloma; hypocalcemia is found in hypoparathyroidism. It can cause tetany and irritability

Potential sources of variability:

- **Lysed serum specimens may give falsely elevated values.**
- **Do not use tourniquet while collecting venous sample as it may result in falsely elevated values**
- **If plasma is used as specimen use heparinized plasma only. If EDTA is used for collecting blood samples it may give very low values due to chelation of calcium by EDTA**
- **As calcium is an ubiquitous ion, to prevent accidental contamination, all glassware should be rinsed in diluted hydrochloric acid and water before use. Even water and glassware containing calcium will react with the reagent.**

QUANTITATIVE ESTEMATION OF PHOSPHORUS IN HUMAN PLASMA

Purpose: Quantitative estimation of phosphorus in serum UV End Point Method. Measurement of Serum Phosphorus is useful in the diagnosis of bone disorder. Increased serum phosphorus levels are seen in hypervitaminosis D, hyperparathyroidism, and renal failure. Reduced serum phosphorus levels are seen in rickets (vitamin D deficiency) hypoparathyroidism, and Fanconi syndrome.

Principle: Inorganic phosphorus reacts with ammonium molybdate in an acid medium to form a phosphomolybdate complex, which absorbs light at 340 nm. The absorbance at this wavelength is directly proportional to the amount of inorganic phosphorus present in the sample.

Phosphorus + Ammonium molybdate----- Acid pH---> Phosphomolybdate complex

Performance specifications:

- *Linearity:* Up to 20 mg/dL in serum

- *Measurement range:* 1-20 mg/dL in serum

- *Sensitivity:* The minimum detection limit is 1 mg/dL

Primary sample:

- **Use only serum as specimen for the test**
- **Collect 4 mL of venous blood from a peripheral vein in a plain vacutainer tube**
- **Do not use hemolyzed/contaminated serum for testing**
- **Process the sample on the same day within 3 hours of collection.**
- **If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 2-8° C for up to 7 days.**

Type of container and additive: Use plain vacutainer tubes for collecting. No additive/Preservative is needed to be added

Instrument: Semi-autoanalyzer

Reagents:

- **Inorganic phosphorus reagent: Ammonium molybdate 0.3 mM, sulfuric acid 1% with surfactant.**
- **Inorganic phosphorus standard: 5.0 mg/L**

Procedure:

- **Switch on the machine and press "FLUSH" button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes,**
- **Press "PROC". Different test procedures will be displayed.**
- **Select the test to be processed by entering its number and then press "ENTER" key.**
- **Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used**
- **Feed the blank with each batch and ensure the absorbance of the blank is less than 0.3 if the absorbance of the 'blank is more than 0.300' discard the reagent.**
- **Run the phosphorus standard with each batch of patient sample**
- **Then feed the test samples and record the values.**
- **Check whether the sample is hemolyzed, icteric or lipemic before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic, dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.**

Assay: UV- End point	**Reagent volume: 1000 µL**
Wavelength: 340 nm	**Sample volume: 10 µL**
Temperature: 37°C	**Conc. of Standard: 5 mg/dL**
Incubation time: 5 min	**Zero setting with: Distilled water**
No. of readings: 1	**Time: 30 sec 9.**

Interferences: Turbid, lipemic, icteric and lysed samples should not be used.

Feed the blank with each batch and ensure the absorbance of the blank is less than 0.300 if the absorbance of the 'blank is more than 0.300' discard the reagent.

Calculating results:

Sample absorbance/ Standard absorbance x Concentration of standard = Sample concentration Biological reference range

Male: **2.1-5.6 mg/dL**

Female: **1.5-6.8 mg/dL**

10 days-24 months: 4.5-6.7 mg/dL

24 months-11 years: 4.5-5.5 mg/dL

Critical/Alert level values: 6.0 mg/dL

Laboratory interpretation: Lowered phosphorus in hyper- parathyroidism and vice-versa. In rickets, osteomalacia, renal rickets and Fanconi syndrome there is lowered phosphorus. Increase of phosphorus could cause tetany.

Potential sources of variability:

- **Detergents used in glassware washing and disposable wipes used in the laboratory contain phosphates, and the use of improperly rinsed glassware may result in elevated inorganic phosphorus values**
- **Use only clear, unhemolyzed serum, separated from the erythrocytes as soon as possible. Lysed serum specimens may give falsely elevated values, as erythrocytes contain organic phosphates that can hydrolyze on standing or can be enzymatically cleaved by phosphatases. Inorganic phosphates can then leak through the cell walls, increasing the concentration.**
- **Phosphorus remains stable in serum for up to 7 days if the serum specimen is stored at 2-8°C hence if analysis is not done on the same day within 3 hours of collection, separate the serum and store it at 2-8°C.**
- **Do not use if the absorbance of the blank reagent is greater than 0.300 as it indicates deterioration of the reagent.**

QUANTITATIVE ESTEMATION OF VITAMIN A IN HUMAN PLASMA

Purpose: Quantitative estimation of vitamin A in human serum or plasma by high performance liquid chromatography (HPLC) at 280 nm. Vitamin A estimation is useful in diseases with possible weak antioxidant defences like atherosclerosis, cancer, Eale's disease, uveitis. etc. HPLC analysis provides a quantitative and sensitive detection of vitamin A in serum samples. The advantages include small sample size requirement, non-destructive nature and speed of analysis and highly accurate and reproducible separation.

Principle: The serum proteins are precipitated by ethanol and the vitamin A is extracted with hexane, the organic solvent is evaporated under the nitrogen gas atmosphere and re-dissolved in the ethanol and injected in the HP-HPLC and detected at 280 nm.

Performance specifications:

1. Linearity: Up to 250 ug/dL in human serum or plasma.

2. Measurement range: 20 ng to 250 ug for vitamin A in serum.

3. Sensitivity: Detection limit of vitamin A is 20 ng.

Primary sample:

1. Use serum as specimen for the test

2. Collect 4 mL of venous blood in a plain vacutainer

3. Do not use hemolyzed sample.

Type of container and additive:

1. Use plain vacutainer tubes for collecting samples

2. No additive/preservative is needed to be added

Reagents:

1. Vitamin A stock standard -Retinol (1 mg/ml)

2. Working standard (20-100 ng)

3. Solvent - 100% methanol

4. Hexane

5. Ethanol

Instrument: Reverse-phase high performance liquid chromatography (RP-HPLC)

Procedure:

1. Take 100 uL of the serum sample and to it add equal volume of ethanol.

2. Vortex the mixture well for 2 minutes.

3. Add 400 uL of hexane and again vortex for 2 minutes and centrifuge at 2500 rpm for 10 minutes.

4. Remove 300 uL of the clear supernatant.

5. Pass nitrogen gas until completely gets dried.

6. Add 100 uL of ethanol to the dry tube and vortex for 1 minute

7. Load 50 uL into HPLC and the values are calculated against the standard area.

8. Prior to the analysis degas the methanol solvent and keep the column for equilibrium/Flow rate/min.

Reference Range: 30-120 ug/L

Potential sources of variability:

1. Lysed serum samples may give falsely increase values.

2. Freeze thaw of the sample may give false values.

QUANTITATIVE ESTEMATION OF VITAMIN E IN HUMAN PLASMA

Purpose: Quantitative estimation of Vitamin E in human serum or plasma by HPLC at 280 nm. Vitamin E has got protective effects as biological antioxidant against environmental and drug toxicity as well as carcinogenesis. Its estimation is useful in diseases with possible weak antioxidant defences like atherosclerosis, cancer, Eale's disease, uveitis etc. HPLC analysis provides a quantitative and sensitive detection of vitamin E in serum samples. The advantages include small sample size requirement, non-destructive nature and speed of analysis and highly accurate and reproducible separation.

Principle: The serum proteins are precipitated by ethanol and the vitamin E is extracted with hexane, the organic solvent is evaporated under the nitrogen gas atmosphere and re-dissolved in the ethanol and injected in the HP-HPLC and detected at 280 nm.

Performance specifications:

1. Linearity: Up to 30 mg/L in human serum or plasma

2. Measurement range: 1-30 mg/L for vitamin E in serum

3. Sensitivity: The minimum detection limit is 1 mg/L.

Primary Sample:

1. Use serum as specimen for the test

2. Collect 4 mL of venous blood in a plain vacutainer tube.

3. Do not use hemolyzed sample.

Type of container and additive:

1. Use plain vacutainer tubes for collecting samples

2. No additive/Preservative is needed to be added

Reagents:

1. Vitamin E stock standard (1 mg/mL)

2. Working standard (200-1800 ng)

3. Solvent - 100% methanol

4. Hexane

5. Ethanol

Instrument: Reverse-phase high performance liquid chromatography (RP-HPLC)

Procedure:

1.Take 100 uL of the serum sample and to it add equal volume of ethanol

2. Vortex the mixture well for 2 minutes.

3. Add 400 uL of hexane and again vortex for 2 min and centrifuge at 2500 rpm for 10 minutes.

4. Remove 300 uL of the clear supernatant.

5. Pass nitrogen gas until completely gets dried.

6. Add 100 uL of ethanol to the dry tube and vortex for 1 minute.

7. Load 50 uL into HPLC and the values are calculated against the standard area.

8. Prior to the analysis degas the methanol solvent and keep the column for equilibrium/Flow rate/min.

Reference range: 5-15 mg/L.

Potential sources of variability:

1. Lysed serum samples may give falsely increased values.

2. Serum sample unused can be stored in at -20°C with occasional freeze-thaw cycles for 3 to 5 weeks.

QUANTITATIVE ESTEMATION OF VITAMIN C IN HUMAN PLASMA

Purpose: To estimate the vitamin C in plasma. It is an effective antioxidant and is estimated in oxidative stress conditions like Eales disease. It is decreased in scurvy.

Principle: Ascorbic acid in plasma is oxidized by Cu (II) to form dehydroascorbic acid, which reacts with acidic 2.4-dinitrophenylhvdrazine to form a red bis-hydrazine, which is measured at 520 nm.

Performance specifications:

1. Linearity: This method is linear up to 15 mg/L

2. Measurement range: This method has a measurement range of 5-15 mg/L

3 Sensitivity: the minimum detection limit by this method is5 mg/L

Primary Sample:

1. Use only heparinized plasma as specimen for the test

2. Do not use lysed plasma for testing as it may give very high results

3. Do not use contaminated/turbid samples for testing

4. Process the sample immediately on the same day.

5. As soon as received, the plasma is treated with 6% meta-phosphoric acid and processed immediately, if not the supernatant is stored at -20°C.

Type of container and additive: Collect 4 mL of blood in heparin vacutainer tube.

Reagents:

1. 6% Metaphosphoric acid solution: Dissolve 30.0 g of metaphosphoric acid (HPO) in distilled water and bring to a final volume of 500 mL. Prepare immediately before use.

2. 4.5 mol/L sulfuric acid: Add slowly 250 mL of concentrated sulfuric acid, reagent grade, to 500 mL of cold water in a 1 L flask and fill to mark with distilled water. *Caution:* Since significant heat is generated when concentrated sulfuric acid is diluted; the flask should be placed in an ice bath. The concentrated acid should be added slowly to water in small quantities at a time and the resulting solution mixed constantly.

3. 12 mol/L Sulfuric acid: Add 650 mL of concentrated sulfuric acid to 300 mL of cold water in a 1 L flask, cool, and fill to mark with distilled water. The concentrated acid should be added slowly to water in small quantities at a time and the resulting solution mixed constantly and refrigerate.

4. 2% 2,4-dinitrophenylhydrazine (DNPH) reagent: 4.5 mol/L sulfuric acid. Dissolve 2 g (DNPH) in 100 mL 4.5 mol/L sulfuric acid. Let it stand in the refrigerator overnight, and then filter.

5. 5% Thiourea solution: Dissolve 5 g of thiourea in distilled water and dilute to a final volume of 100 mL. This reagent is stable for 1 month at 4°C

6. 6% Copper sulfate solution: Dissolve 0.6 g of anhydrous copper sulfate in distilled water and dilute to a final volume of 100 mL

7. Dinitrophenylhydrazine-thiourea-copper sulfate (DTCS) reagent: Mix 5 mL of the thiourea solution, 5 mL of the copper sulfate solution, and 100 mL of the 2. 4-dinitrophenylhydrazine reagent. Store in a bottle at 4°C for a maximum of 1 week

8. Calibrators: All ascorbic acid calibrators should be prepared daily.

9. 50 mg/dL Ascorbic acid stock calibrator. Dissolve 50 mg of ascorbic acid in metaphosphoric acid (6.0 g/dL) and bring to a final volume of 100 mL with metaphosphoric acid.

10. 5 mg/dL Intermediate ascorbic acid calibrator. Pipette 100 of stock calibrator into a 100 ml colorimetric flask and dilute to mark 6% with metaphosphoric acid.

11. Working calibrators: In a series of 25 ml volumetric flask, pipette the following amounts of intermediate calibrator: 2.0, 4.0, 6.0, 10.0, 15.0 and 20.0 ml. Bring to a final 25 ml with 6% metaphosphoric acid to yield working calibrator of 0.10, 0.40, 0.80, 1.20, 2.00, 3.00 and 4.00 mg/dL.

Instrument: Spectrophotometer.

Procedure:

1. Add 0.5 mL of heparinized plasma to 2.0 mL of freshly pre metaphosphoric acid in a 13 x 10 mm test tube and mix well in a vortex mixer. Centrifuge the plasma-metaphosphoric acid mixture for 10 min at 2500 rpm. Pipette 1.2 mL of the supernatant into a 13 x 100 mm Teflon-lined, screw-cap test tube. 2. Add 1.2 mL of each concentration of working calibrator in 13 x 100 mm screw-cap test tubes. Prepare calibrators in duplicate. Add 1.2 mL of metaphosphoric acid to two tubes for use as blank.

3. Add 0.4 mL of DTCS reagent to all tubes. Cap the tubes, mix the contents, and incubate the tubes in a water bath at 37°C for 3 hrs.

4. Remove the tubes from the water bath and cool for 10 min in an ice bath. While mixing, slowly add to all tubes 2.0 mL of cold sulfuric acid, 12 mol/L, cap, and mix in a vortex mixer (The temperature of the mixture must not exceed room temperature 8.5. Adjust the spectrophotometer with the blank to read

zero at 520 nm and read the calibrators and unknowns. Plot the concentration of each working calibrator versus absorbance values. The calibration curve obeys Beer's law up to an ascorbic acid concentration of 2.0 mg/dL.

Calculation: From the standard graph the test absorbance plotted and the value will be calculated for 100 ml.

Standardization protocol

Reagents (mL)	Blank	S1	S2	S3	S4	S5
Intermediate calibrators		0.5	2.0	4.0	6.0	10.0
6% Metaphosphoric acid	1.2	24.5	23.0	21.0	19.0	15.0
Working calibrators	_	1.2	1.2	1.2	1.2	1.2
Vit C conc. in mg/dl.	_	0.1	0.4	0.8	1.2	2.0
DTCS	0.4	0.4	0.4	0.4	0.4	0.4
Mix and Incubate at 37 °C for 3 hrs, then cool the tubes in ice bath for 10 min						
12 M H_2SO_4	2.0	2.0	2.0	2.0	2.0	2.0
OD at 520 nm						

Reference range: 5-15 mg/L

Potential sources of variability:

1. Do not use lysed plasma samples it may give false elevated values.

2. Immediately add metaphosphoric acid to the collected sample as it may get oxidized.

ENZYME IMMUNOASSAY FOR FOLATE/ VITAMIN B12 ANEMIA PANAL TEST SYSTEM

Intended Use: The Quantitative Determination of Vitamin B12 and Folate Concentration in Human Serum and Plasma by a Microplate Enzyme Immunoassay, Colorimetric.

SUMMARY AND EXPLANATION OF THE TEST

Folate plays an important role in brain development and is therefore vital during growth. The most common defects resulting from folate deficiencies are neural tube defects. With a vital role in nucleic acid synthesis, folate supplementation has been found to be beneficial during pregnancy and other times of rapid tissue growth. Folate also plays a vital role in maintaining proper balance of homocysteine, a contributing factor in occurrences of occlusive vascular diseases and stroke.

Individuals with susceptibility to heart disease and several forms of cancer may also benefit from supplementation. Major sources of folate include green leafy vegetables, legumes, beans and fortified cereals. Foods fortified with folate are actually fortified with folic acid because of the higher bioavailability for absorption by the body. In circulation, folate is present in several different forms, some of which are more stable than others. Folic acid and N-methyltetrahydofolate are two common forms, the latter being more stable and found in higher concentrations in serum. Due to the stability of the molecule, methytetrahydrofolate is very often used as the form focused on during methods of analysis.

Folate binding proteins are responsible for folate metabolism. Two types exist in circulation: one type aids in binding to the cell surface and the other soluble form exists in circulation. These folate binding proteins also have the capability of binding several different folate derivatives including folic acid and N-methytetrahydrofolate. The interaction between folic acid and folate binding protein is greater than methyltetrahydrofolate. Current assays on the market require an extraction step to release the folate derivatives from the folate binding protein.

Vitamin B12 is one of the nine water soluble vitamins important for healthy body functioning. The most important roles Vitamin B12 plays in the human body are in the formation of red blood cells and the formation of the myelin sheath around the nerves. Since the effects are seen in body systems with a large range of function, the symptoms of Vitamin B12 deficiency can sometimes

be very ambiguous. A deficiency may also take from months to years to manifest depending on the cause and severity.

Two of the most common causes of Vitamin B12 deficiency are diet and age. Because most sources of dietary Vitamin B12 come from animals, vegans who do not efficiently supplement their diet are at risk. The elderly community is also at high risk because of their diet, as well as the less efficient functioning of their digestive system.

Two very useful tests to distinguish between Vitamin B12 deficiency and folate deficiency are methylmalonyl CoA (MMA) and homocysteine (hcy). Both deficiencies are represented by similar symptoms; however, even though both show increased levels of homocysteine, only Vitamin B12 deficiency causes an increase in methylmalonyl CoA. The increase in levels of methylmalonyl CoA and homocysteine is thought to be the root cause of any symptoms that accompany a Vitamin B12 deficiency. High levels of these two analytes in the blood stream causes increased oxidative stress to cells therefore causing increased apoptosis. In turn, vascular disease results in the form of atherosclerosis, coronary heart disease and/or neurodegeneration (ex. Parkinson's Disease).

PRINCIPLE

Folate - Competitive Binding Protein Assay:

The essential reagents required for a competitive binding assay include specific binding protein, enzyme-antigen conjugate and native antigen. Upon mixing enzyme-antigen conjugate, biotinylated binding protein and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of binding sites.

A simultaneous reaction between the biotin attached to the binding protein and the streptavidin immobilized on the microwell occurs. This effect the separation of the binding protein enzyme bound fraction after decantation or aspiration.

The enzyme activity in the protein binding protein bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Vitamin B12 - Delayed Competitive Enzyme Immunoassay:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated

antibody with a serum containing the antigen, a reaction results between the antigen and the antibody.

After a short incubation, the enzyme conjugate is added (this delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binging sites (not consumed in the first incubation).

MATERIALS

Reagents for 96 well Microplate, provided

A.Combi-Cal® Folate/Vit B12 Calibrators

Six (6) vials containing references for markers at levels indicated below. A preservative has been added. The calibrators, human serum based, were calibrated using a reference preparation indicated in the chart.

Analyte	Folate (ng/ml)	Vitamin B12 (pg/ml)
A	0.0	0
B	1.0	125
C	2.5	250
D	5.0	500
E	10.0	1000
F	25.0	2500

B.Folate Enzyme Reagent

One (1) vial containing Folate (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with dye. Store at 2-8°C.

C.Folate Biotin Reagent One (1) vial containing biotinylated purified folate binding protein conjugate in buffer, dye and preservative. Store at 2-8°C.

D.Vitamin B12 Enzyme Reagent – 7.0 ml/vial – Icon

One (1) vial containing Vitamin B12 (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

E.Vitamin B12 Biotin Reagent

One (1) vial containing anti-Vitamin B12 biotinylated purified rabbit IgG conjugate in buffer, dye and preservative. Store at 2-8°C.

F.Wash Solution Concentrate

One (1) vial containing surfactant in buffered saline. A preservative has been added. Store at 2-8°C for up to 60 days.

G.Streptavidin Coated Microwells

One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

H.Substrate Reagent

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

I.Stop Solution

One (2) vial containing a strong acid (0.5M H_2SO_4). Store at 2-8°C.

J.Releasing Agent

One (1) vial containing a strong base (sodium hydroxide) and potassium cyanide. Store 2-8°C.

K.Stabilizing Agent

One (1) vial containing tris (2-carboxyethyl) phosphine (TCEP) solution. Store at 2-8°C.

L.Neutralizing Buffer

One (1) vial containing buffer with dye that reduces the pH of sample extraction. Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.

Note 3: Above reagents are for a single 96-well microplate

Materials Required but Not Provided:

1. Pipette capable of delivering 0.050ml (50µl) and 0.100ml (100µl) with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.

4. Glass test tubes for serum reference, control, and patient sample preparation.

5. Microplate washer or a squeeze bottle (optional).

6. Microplate Reader with 450nm and 620nm wavelength absorbance capability.

7. Absorbent Paper for blotting the microplate wells.

8. Plastic wrap or microplate covers for incubation steps.

9. Vacuum aspirator (optional) for wash steps.

10. Timer.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml (100µl) of the specimen is required for each anemia marker assayed.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at room temperature (2-30°C) for up to 60 days.

2. EXTRACTION AGENT

Add an aliquot of the stabilizing agent in order to prepare a 1/40 (stabilizing agent / releasing agent) dilute solution. For example, to make 4ml (4000µl), add 0.100ml (100µl) stabilizing agent to 3.9ml (3900µl) releasing agent.

3. SAMPLE EXTRACTION (*See Note 3)*

Obtain enough test tubes for preparation of all patient samples, controls, and calibrators. Dispense 0.10ml (100µl) of all samples into individual test tubes. Pipette 0.050ml (50µl) of the prepared extraction agent to each test tube, shaking *(see note 3)* after each addition. Let the reaction proceed for 15 min. At end of the 15 min, dispense 0.050 ml (50µl) of the neutralizing buffer, vortex *(see note 3)*.

***For Folate:* after the neutralization buffer is added and mixed, let the reaction go to completion by waiting an additional 5 min before dispensing into the microwells. Wait time is not needed for Vitamin B12.**

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

Note 3: Use of multiple (3) touch vortex is recommended.

Note 4: It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the glass tubes at an angle while touching the side of the tubes.

Note 5: Samples with high protein concentration should be diluted 1:1 with a saline solution before performing the extraction.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (*20 - 27° C*).

*****Test Procedure should be performed by a skilled individual or trained professional*****

For Folate:

1. Prepare all samples according to the "Sample Extraction" procedure in section "8.0 Reagent Preparation"; it is important to wait 5 min before proceeding to allow the neutralization reaction to go to completion (see above).

2. Format the microplates' wells for each calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

3. Pipette 0.050 ml (50 µL) of the appropriate extracted Folate calibrator, control or specimen into the assigned well.

4. Add 0.050 ml (50 µl) of Folate Enzyme Reagent to all wells

5. Mix the microplate gently for 20-30 seconds.

6. Add 0.050 ml (50 µl) of the Folate Biotin Reagent to all wells.

7. Mix the microplate gently for 20-30 seconds.

8. Cover and incubate for 45 minutes at room temperature.

9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

10.Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill

each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

11.Add 0.100 ml (100 µl) of substrate reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

12.Incubate at room temperature for twenty (20) minutes.

13.Add 0.050 ml (50 µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

14.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within thirty (30) minutes of adding the stop solution.

Note 1: Dilute the samples suspected of concentrations higher than 25ng/ml 1:5 with Folate '0' ng/ml calibrator and re-assay.

Note 2: It is very important to dispense all reagents in the center of the coated well. Always add reagents in the same order to minimize reaction time differences between wells.

For Vitamin B12:

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.050 ml (50 µL) of the appropriate extracted Vitamin B12 calibrator, control or specimen into the assigned well.

3. Add 0.050 ml (50 µl) of the Vitamin B12 Biotin Reagent to all wells.

4. Mix the microplate gently for 20-30 seconds to mix.

5. Cover and incubate for 45 minutes at room temperature.

6. Add 0.050 ml (50 µl) of Vitamin B12 Enzyme Reagent to all wells. Add directly on top the reagents dispensed in the wells.

7. Mix the microplate gently for 20-30 seconds to mix.

8. Cover and incubate for 30 minutes at room temperature.

9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

10.Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

11.Add 0.100 ml (100 µl) of substrate reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

12.Incubate at room temperature for twenty (20) minutes.

13.Add 0.050 ml (50 µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

14.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note 3: Dilute the samples suspected of concentrations higher than 2000pg/ml 1:5 and 1:10 with Vitamin B12 '0' pg/ml calibrator and re-assay.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of each corresponding marker in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Examples 1 and 2.

2. Plot the absorbance for each duplicate serum reference versus the corresponding marker concentration in appropriate units on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of corresponding cancer marker for an unknown, locate the average absorbance of the duplicates for each unknown on

the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph.

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 'A' should be > 1.8.

2. Four out of six quality control pools should be within the established ranges.

EXPECTED VALUES

In agreement with established reference intervals for a "normal" population the expected ranges for the Folate/Vit B12 VAST AccuBind® ELISA Test System are detailed in Table 1 and 2.

TABLE 1

Expected Values Folate
Normal Adult Population > 3.0 ng/ml

TABLE 2

Expected Values - Vitamin B12

Population	**pg/ml**	**poml/l**
Newborn	**160-1300**	**118-95 9**
Adult	**200-835**	**148-616**
Adult >60 yr	**110-800**	**81-590**

ENZYME IMMUNOASSAY FOR 25-OH VITAMIN D TOTAL TEST SYSTEM

Intended Use: The Quantitative Determination of 25-OH Vitamin D Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Vitamin D is a fat soluble secosteroid hormone that is important in the management of calcium and phosphorus concentrations required in the mineralization of bone. Vitamin D has two important forms: cholecalciferol (D3) formed in the skin from ultraviolet light and ergocalciferol (D2) found in dairy products. However, these forms do not have significant biological activity. The hormonal active form, 1, 25-dihydroxylcholecalciferol, is produced through transformations in the liver and kidney. The first step in this conversion is an enzymatic reaction of D2 or D3 into 25OH-D2 or 25OH-D3. These 25OH D forms are not freely circulating in blood, but are primarily bound to vitamin D binding protein (VDBP). The high binding affinity of the 25OH D(2 or 3) compared to other derivatives of vitamin D leads to a long half-life in blood and its use as an accurate indicator of Vitamin D status. Vitamin D deficiency has been associated to diseases related to bone damage such as osteomalacia and rickets. Vitamin D can be dietarily supplemented through the use of Vitamin D2 or vitamin D3. The sum of the 25OH D(2 and 3) in serum or plasma is referred to as total 25OH Vitamin D. The accurate measurement of total vitamin D is necessary in monitoring deficient vitamin D patients to achieve the optimum dosage and avoid excessive levels, which are considered toxic.

PRINCIPLE

Sequential Competitive Method:

The essential reagents required for a solid phase sequential enzyme immunoassay include immobilized antibody, enzyme- antigen conjugate and native antigen. Upon mixing immobilized antibody, and a whole blood sample containing the native antigen, a binding reaction results between the native antigen for a limited number of insolubilized binding sites.

After removing any unreacted native antigen by a wash step, the enzyme-conjugated antigen is introduced. The conjugate reacts with sites of the antibody unoccupied by the native antigen.

REAGENTS

Materials Provided:

A.Vit D Calibrators

Seven (7) vials containing human serum albumin reference calibrators for 25-OH Vitamin D at approximate* concentrations of 0 (A), 5 (B), 10 (C), 25 (D), 46 (E), 85 (F), and 150 (G) in ng/ml. A preservative has been added. Store at 2-8°C.

*** Exact levels are given on the labels on a lot specific basis. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 2.5. For example: 10ng/ml x 2.5 = 25nM/L**

B.Vit D Controls – 1ml/vial

Two (2) vials containing human serum reference controls at concentration established (exact value listed on label). A preservative has been added. Store at 2-8°C.

C.Vit D Releasing Agent

One (1) vial containing vitamin D binding protein releasing agents. Store at 2-8°C.

D.Vit D Enzyme Reagent

One (1) vial containing 25-OH Vitamin D3 (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

E.Vit D Antibody Coated Plate

One 96-well microplate coated with < 1.0 µg/ml anti-Vitamin D sheep IgG and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

F.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

G.Substrate Reagent

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H.Stop Solution

One (1) vial containing a strong acid (H_2SO_4). Store at 2-8°C

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1.Pipette capable of delivering 0.025 & 0.100ml (25 & 100µl) with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.

3.Microplate washer or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

5.Absorbent Paper for blotting the microplate wells.

6.Plastic wrap or microplate cover for incubation steps.

7.Vacuum aspirator (optional) for wash steps.

8.Timer.

9.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirements.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) with no anti-coagulants. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25 µL) of the appropriate extracted 25-OH Vitamin D calibrator, control or specimen into the assigned well.

3.Add 0.100 ml (100 µl) of the 25-OH Vitamin D Releasing Agent to all wells.

4.Mix (Note 3) the microplate for 20-30 seconds until homogeneous.

5.Cover and incubate for 30 minutes at room temperature

6.Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used.

manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100 ml (100 µl) of 25-OH Vitamin D Enzyme Reagent to all wells.

DO NOT SHAKE THE PLATE AFTER ADDITION

9. Cover and incubate for 30 minutes at room temperature.

10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

11. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

12. Add 0.100 ml (100 µl) of substrate reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE (MIX) THE PLATE AFTER SUBSTRATE ADDITION

13. Incubate at room temperature for twenty (20) minutes.

14. Add 0.050 ml (50 µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

15. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

Note 3: Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.

Note 4: It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the microwells at an angle while touching the side of the well.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of 25-OH Vitamin D in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader

2. Plot the absorbance for each duplicate calibrator versus the corresponding 25-OH Vitamin D concentration in ng/ml on linear graph paper (do not average the duplicates of the calibrators before plotting).

3. Connect the points with a best-fit curve.

4. To determine the concentration of 25-OH Vitamin D for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.033) intersects the dose response curve at 39.9 ng/ml 25-OH Vitamin D concentration.

Note: Computer data reduction software designed for ELISA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXPECTED RANGES OF VALUES

Based on the published literature the following ranges have been assigned. <u>These ranges should be used as guidelines only:</u>

TABLE 1
Expected Values for the Vit D-Direct ELISA

LEVEL	RANGE (ng/ml)
Very severe vitamin D deficiency	< 5
Severe vitamin D deficiency	5-10
Vitamin D deficiency	10-20
Suboptimal vitamin D provision	20-30
Optimal vitamin D level	30-50
Upper norm	50-70
Overdose, but not toxic	70-150
Vitamin D intoxication	> 150

SECTION 1: BIOCHEMESTRY

B- ENZYMES

DETERMINATION OF ASPARTATE AMINO TRANSFERASE AND GLUTAMIC OXALOACETATE TRANSAMINASE

Purpose: Quantitative estimation of AST activity in human serum by optimized UV kinetic (Modified IFCC Method). Measurement of AST (SGOT) is useful in diagnosis and treatment of heart and hepatobiliary diseases such as cirrhosis, metastatic carcinoma, and viral hepatitis. Increased AST (SGOT) levels indicate damage to heart/skeletal muscle. Injury to these tissues results in the release of the AST (SGOT) enzyme to general circulation. Following myocardial infarction, serum levels of AST (SGOT) are elevated and reach a peak in 48 to 60 hours after onset.

Principle: AST catalyzes the transfer of an amino group between L-aspartate and 2-oxoglutarate. The oxaloacetate formed in the first reaction reacts with NADH in the presence of malate dehydrogenase (MDH) to form NAD+. AST activity is determined by measuring the rate of oxidation of NADH at 340 nm. Lactate dehydrogenase is included in the reagent to convert endogenous pyruvate in the sample to lactate during the lag phase prior to measurement. Addition of pyridoxal 5-phosphate stabilizes the transaminase and avoids falsely low values in samples containing insufficient endogenous pyridoxal phosphate. The enzymatic reaction-sequence employed in the assay of aspartate aminotransferase is as follows:

L-aspartate + α-ketoglutarate------ GOT--- > Oxaloacetate + L-Glutamate

Oxaloacetate + NADH + H^+ -----MDH--->L- Malate + NAD^+

Performance specifications:

1. Linearity: Up to 500 IU/L of serum

2. Measurement range: 2 - 500 IU/L of AST activity in serum

3. Sensitivity: The minimum detection limit by this kit is 2 IU/L of serum

Primary sample:

1. Use only serum as specimen for the test

2. Collect 4 mL of venous blood in a plain vacutainer tube.

3. Allow the tube to stand for 30–45 min and separate the serum by centrifugation at 2500 rpm for 10 min.

4.. Do not use lysed serum for testing as it may give very high results

5. Do not use contaminated/turbid samples for testing.

6. Process the sample on the same day within 3 hours of collection

7. lf analysis is not done on the same day/ within 3 hours of collection, separate the serum and store it at 2-8 C for up to 7days.

Type of container and additive: Use plain vacutainer tubes for collecting samples. No additive/ preservative is needed to be added

Reagents:

Reagent 1: NADH, α-ketoglutarate

MDH, LDH

Reagent II: Tris buffer and L. aspartate

Discard the reagent if the initial absorbance, read against water at 340 nm is below 0.800

Instrument: Semi-autoanalyzer

Procedure:

1. Switch on the machine and press “FLUSH” button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes

2. Press "PROC. Different test procedures will be displayed

3. Select the test to be processed by entering its number/name of the test and then press “ENTER” key 4. Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used

5. Feed the blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.800. If the absorbance of the blank is more than 0.800 discard the reagent

6. Then feed the test samples and record the values.

7. Check whether the sample is hemolyzed, icteric or lipemic before processing. If the sample is lysed collect another sample and proceed. If it is icteric or lipemic dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed b dilution factor 10.

Assay: Kinetic assay **Reagent volume: 500 µL**

Wavelength: 340 nm **Sample volume: 50 µL**

Temperature: 37C **Zero setting with distilled water**

Number of readings:4 **Time: 60 sec**

Interferences: Samples with a very high SGOT activity cause an excessive consumption of NADH, resulting in very low initial absorbance and/or nonlinear reaction. When this occurs, the assay should be repeated with a diluted sample.

Pyridoxal phosphate can elevate AST values by activating the apoenzyme of the transaminase Pyridoxal phosphate may be found in diluent water. contaminated with microbial growth High levels pyruvate may also interfere with assay performance.

Calculating results: AA/min x factor given - (Factor 1746 given in the kit).

Biological references: Up to 46 IU/L at 37°C. 12.

Critical/Alert level values: Above 100 IU/L

Laboratory interpretation: Damage of the cells of heart, liver skeletal muscle. Peak values of AST on the second day of myocardial infarction. Increase also in pancreatitis and mononucleosis.

Potential Sources of Variability: Lysed serum specimens may be falsely elevated values as erythrocytes contain fifteen times the AS activity in serum.

AST activity remains stable in serum for up to 7 days if the serum specimen is stored at 2-8°C hence if analysis is not done on the same day within 3 hours of collection, separate the serum and store it at 2-8°C.

Do not use if the absorbance of the blank reagent is greater than 0.800 as it indicates deterioration of the reagent.

Pyridoxal phosphate can elevate AST values by activating the apoenzyme form of the transaminase. Pyridoxal phosphate may be found in diluent's water contaminated with microbial growth. High levels of pyruvate may also interfere with assay performance.

DETERMINATION OF ALKALINE PHOSPHATASE

Principle: Alkaline phosphatase (ALP, ALKP, ALPase, Alk Phos) or basic phosphatase is a homodimeric protein enzyme of 86 kilodaltons. Each monomer contains five cysteine residues, two zinc atoms, and one magnesium atom crucial to its catalytic function, and it is optimally active at alkaline pH environments. ALP has the physiological role of dephosphorylating compounds.

In humans, it is found in many forms depending on its origin within the body – it plays an integral role in metabolism within the liver and development within the skeleton. Due to its widespread prevalence in these areas, its concentration in the bloodstream is used by diagnosticians as a biomarker in helping determine diagnoses such as hepatitis or osteomalacia.

The level of alkaline phosphatase in the blood is checked through the ALP test, which is often part of routine blood tests. The levels of this enzyme in the blood depend on factors such as age, gender, blood type. Blood levels of alkaline phosphatase also increase two to four times during pregnancy. This is a result of additional alkaline phosphatase produced by the placenta. Additionally, abnormal levels of alkaline phosphatase in the blood could indicate issues relating to the liver, gall bladder or bones. Kidney tumors, infections as well as malnutrition has also shown abnormal level of alkaline phosphatase in blood.

Alkaline phosphatase levels in a cell can be measured through a process called "The scoring method". "The scoring method" is a technique used where a sample of the enzyme is extracted from the inside of blood cells and is analyzed and compared for varying enzyme activity. A blood smear is usually taken and undergoes differential centrifugation to isolate leukocytes and staining to categorize each leukocyte into specific "leukocyte alkaline phosphatase indices." This marker is designed to distinguish leukocytes and determine different enzyme activity from each sample's extent of staining.

Alkaline Phosphatase catalyzes the hydrolysis of 4 – nitrophenyl phosphate (4 – NPA) with the formation of free 4 – nitrophenol and in organic phosphate

4 - Nitrophenol phosphate + H2O — $^{ALP/MG}$→4 - Nitrophenol + P1

Reagent composition

R1 – ALP buffer: DEA buffer 1.25mol/l, MgCl 0.6mmol/l, biocides

R2 – ALP substrate: 4 – NPP 50mmol/l, biocides

Reagent preparation

Working reagent – mix 4ml of R1 + 1ml of R2 (stable for 4wks at +2 to 8°)

Samples

Serum or heparinized plasma, or EDTA, oxalates, citrates inhibit enzyme

Procedure

- **Preincubate working reagent, sample and controls to reaction temperatures**
- **Set the photometer to '0' absorbance with distilled water**
- **Pipette in a cuvette**

Reaction temperature	**37°C**
Working reagent	**1ml (ALP)**
Sample control	**20ul**

- **Mix gently by inversion, insert the cuvette in to the cell holder and start stop watch**
- **Incubate for 1min and record the initial absorbance**
- **Repeat the absorbance reading exactly after 1, 2, and 3 minutes**
- **Calculate the difference between absorbances**

• Calculate the mean of the results to obtain the average change in absorbance per minute (ΔA/min)

Calculation: ΔA/min * 2746

ALANINE AMINOTRANSFERASE (ALT)/SERUM GLUTAMIC-PYRUVIC TRANSAMINASE (SGPT)

Purpose: Quantitative estimation of ALT in human serum by optimized UV kinetic (Modified IFCC Method). Measurement of ALT (SGPT) is useful in diagnosis, treatment of hepatobiliary diseases such as cirrhosis, metastatic carcinoma, and viral hepatitis. Increased ALI levels also indicate damage to heart/skeletal muscle. Injury to these tissues results in the release of the ALT enzyme to general circulation, following a myocardial infarction, serum levels of ALT are elevated and reach a peak 48 to 60 hours after onset.

Principle: ALT catalyzes the transfer of an amino group between 2-Oxoglutarate and L-alanine. The pyruvate formed in the first reach reacts with NADH in the presence of lactate dehydrogenase (LD form NAD+ ALT activity is determined by measuring the rat oxidation of NADH at 340 nm. Lactate dehydrogenase is included in the reagent to convert endogenous pyruvate in the sample to lactate during the lag phase prior to measurement. The enzymatic reaction

sequence employed in the assay of alanine aminotransferase is as follows:

2-oxoglutarate + L-alanine---- ALT----> L-glutamate + Pyruvate

Pyruvate + NADH + H^+ ------ LDH---> Lactate + NAD^+

Performance specifications

1. Linearity: This method is linear for ALT concentrations up to 240 IU/L of serum

2. Measurement range: This method has a measurement range of 2- 440 IU/L of ALT activity in serum 3. Sensitivity: The minimum detection limit by this kit is 4 IU/L

4 Precision CV: Within run 4.7-5.8 between RUN 44-10.1

Primary sample:

1. Use only serum as specimen for the test

2. Collect 4 mL of venous blood in a plain vacutainer tube

3. Allow the tube to stand for 30 min and separate the serum by centrifugation at 2500 rpm for 10 min.

4. Do not use lysed serum for testing as it may give very high results

5. Do not use contaminated/turbid samples for testing

6. Process the sample on the same day within 3 hours of collection.

7. If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 2-8°C for up to 7 days.

Type of container and additive: Use plain vacutainer tubes for collecting samples. No additive/preservative is need to be added.

Reagents:

Reagent A: Composition in the test: Tris buffer 100 mM pH 7.15, L-alanine 500 mM, B: 2-oxoglutarate 15 mM, NADH 0.18 mM, LDH 1.2 kU/L store all components in 2–8°C.

Instrument: Semi-autoanalyzer.

Procedure:

1. Switch on the machine and press "FLUSH "button by keeping the tubing in a distilled water for 2 minutes.

2. Press "PROC". Different test procedures will be displayed.

3. Select the test to be processed by entering its number and then press "ENTER" key.

4. Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used.

5. Feed the blank with each batch of and ensure the absorbance of the blank is less than 0.8. If the absorbance of the 'blank is more than 0.8', discard the reagent

6. Then feed the test samples and record the values

7. Check whether the sample is hemolyzed, icteric or lipemic processing. If the sample is lysed collect another sample proceed. If it is icteric or lipemic, dilute

the sample 1 in 10 distilled water and proceed. Multiply the result displayed dilution factor 10.

Assay: Kinetic	**Reagent volume: Reagent A 400 µL**
	Reagent B 100 µL
Wavelength: 340 nm	**Sample volume: 50 uL**
Temperature: 37° C	**Zero setting with distilled water**
Number of readings: 3	**Time: 60 sec**

Interferences: High levels of ascorbic acid (above 40 mg/dL) hemoglobulin above 400 mg/dL and lipemia above 2000 mg/dL triacyl glycerol.

Calculating of results: Calculation: U/L = Delta A/min x factor given

Biological reference range:

Male: 41 IU/L

Female: 31 IU/L.

Alert level values: More than 200 IU/L

Laboratory interpretation: Increase of ALT suggests necrosis of liver, say in hepatocellular jaundice and in myocardial infarction.

Potential sources of variability:

1. Lysed serum specimens may give falsely elevated values as erythrocytes contain fifteen times the ALT activity in serum

2. ALT activity remains stable in serum for up to 7 days if the serum specimen is stored at 2–8°C hence if analysis is not done on the same day within 3 hours of collection, separate the serum and store it at 2-8°C.

3. Do not use if the absorbance of the blank reagent is greater than 0.800 as it indicates deterioration of the reagent 14.4. Pyridoxal phosphate can elevate ALT values by activating the apoenzyme form of the transaminase. Pyridoxal phosphate may be found in diluent water contaminated with microbial growth High levels of pyruvate may also interfere with assay performance.

QUANTITATIVE ESTIMATION OF ALKALINE PHOSPHATASE

Purpose: Quantitative estimation of alkaline phosphatase in human serum by PNPP-DEA kinetic method. Measurement of ALP is useful in the diagnosis and treatment of hepatobiliary diseases of obstructive origin both intrahepatic and extrahepatic and bone diseases such as rickets, osteocalcin and Paget's disease and healing fractures. Elevations also occur in the third trimester of pregnancy and levels are elevated during periods of active bone growth. Marked elevations of serum ALP in the absence of jaundice in the presence of primary source indicates metastasis.

Principle: At PH 9.8 alkaline phosphatase catylyses the hydrolysis of colorless 4-nitrophenyl phosphate to yellow colored 4-nitrophenol and phosphate. 4-nitrophenol absorbs light at 405 nm the rate of increase in absorbance at 405 nm is directly proportional to the enzyme activity. The enzymatic sequence employed in the assay of alkaline phosphatase is follows:

4-nitrophenyl phosphate + H2O ---- ALP-- PH 9.8----> Phosphate + 4-nitrophenol.

Performance specifications:

1. Linearity: This method is linear for A concentrations up to 2800 IU/L of serum

2. Measurement range: This method has a measurement range of 1-2800 IU/L of ALP activity in serum 3. Sensitivity: The minimum detection limit by this kit is 1 IU/L

Primary sample:

1. Use only serum as specimen for the test.

2. Collect 2 mL of venous blood in a plain vacutainer tube. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes.

3. Do not use lysed serum for testing as it may give very high results

4. Do not use contaminated/turbid samples for testing.

5. Process the sample on the same day within 3 hours of collection.

6. If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at 2–8 °C for up to 24 hours only.

Type of container and additive: Use plain vacutainer tubes for collecting samples.

Reagents:

1. Reagent A, Reagent B.

2. Diethanolamine Buffer (pH 9.8) 1M, Magnesium chloride as **acetate) 0.5 mM, 4 nitrophenylphosphate 10 mM.**

3. Stabilizer to arrest autohydrolysis.

4. Discard the reagent if the initial absorbance, read against w at 405 nm, is above 1.000.

The absorbance of working ALP reagent increases slowly storage

5. Do not use the constituted reagent if it has turned yellow.

Instrument: Semi-autoanalyzer

Procedure:

1. Switch on the machine and press "FLUSH "button by keeping the tubing in a container with 2% detergent for 2 minutes followed by distilled water for 2 minutes.

2. Press "PROC". Different test procedures will be displayed

3. Select the test to be processed by entering its number and then press "ENTER" key.

4. Now the assay parameters of the specific test procedure will be displayed. Note down the volume of the reagent and the sample to be used.

5. Feed the blank with each batch and ensure the absorbance of the blank is less than 1.000. If the absorbance of the 'blank is more than 1.000' or if the reagent has turned yellow, discard the reagent 6. Then feed the test samples and record the values.

7. Check whether the sample is hemolyzed, icteric or lipemic before processing. If the sample is lysed, collect another sample and proceed. If it is icteric or lipemic dilute the sample 1 in 10 with distilled water and proceed. Multiply the result displayed by dilution factor 10.

Assay: Kinetic **Reagent volume: 500 uL**

Wavelength: 405 nm **Sample volume: 10 uL**

Temperature: 37°C **Zero setting with distilled water**

Number of readings: 3 **Time: 60 sec**

Light path: 1 cm

Interferences: Fluoride, oxalate, citrate and EDTA inhibit alkaline phosphate activity and should not be used as anticoagulants. Hemolysis interferes due to the high concentration of alkaline phosphatase in red cells.

Calculation of results: Delta A/min x 3300 = U/L ALP

Biological various reference range:

Children up to 1-14 years: <480 U/L at 30°C

Adult: 73-207 U/L at 30°C

Critical/Alert level values: 250 U/L

Laboratory interpretations: High values in obstructive jaundice (above) 213-249 U/L) and moderately high in hepatocellular jaundice and increase in rickets, osteomalacia, hyperparathyroidism and Paget's disease.

Potential sources of variability:

1. Lysed serum specimens may give falsely elevated values

2. ALP activity remains stable in serum for up to 24 hours the serum specimen is stored at 2-8°C. Hence if analysis is not done on the same day within 3 hours of collection separate the serum and store it at 2-8°C

3. Do not use if the absorbance of the blank reagent is 1.000 as it indicates deterioration of the reagent and color of the reconstituted ALP reagent has turned yellow.

QUANTITATIVE ESTIMATION OF ANGIOTENSIN CONVERTING ENZYME

Purpose: To measure the activity of angiotensin converting enzyme in serum. Elevated levels of ACE activity occur in serum of patients with active sarcoidosis, occasionally in premature infants with respiratory distress syndrome; in adults

with tuberculosis, Gaucher's disease, leprosy, and in many lung and liver diseases.

Principle: The following reaction is catalyzed by ACE:

FAPGG -------- > FAP + Glycylglycine

FAPGG is hydrolyzed to furylacryloylphenylalanine (FAP) and glycylglycine. Hydrolysis of FAPGG results in a decrease in absorbance, at 340 nm. The rate of decrease in absorbance is directly proportion to ACE activity in the sample.

Performance specifications:

1. Linearity: This method is linear up to 250 U/L of serum

2. This method has a measurement range of 8-250 IU/L

3. Sensitivity: The minimum detection limit by this kit is 8 IU/L

Primary sample:

1. Use only serum as specimen for the test

2. Collect 2 mL of venous blood in a plain vacutainer tube.

3. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 min

4. Do not use lysed serum for testing as it may give very high results

5. Do not use contaminated/turbid samples for testing

6. Process the sample on the same day within 3 hours of collection

7. If analysis is not done on the same day/within 3 hours of collection, separate the serum and store it at -20°C for up to 24 hours only.

Type of container and additive: Use plain tubes for collecting samples. No additive/preservative is needed to be added

Reagents:

1. Reagent I: Furyl acryloyl phenylalanyi glycylglycine 28 mmol/L

2. Reagent 2: Hepes 40 mmol/L pH 8.4

Sodium chloride 0.185 mol/L

Stabilizers and preservatives

Stable at 2–8°C up to the expiration date on the label.

Reconstitution of working reagent: Reconstitute a vial of reagent 1 with exactly 4.2 mL

of reagent 2. Mix until complete solubilization.

Instrument: Semi-autoanalyzer-Spectrophotometer (to take absorbance at 37°C)

Procedure:

1. The temperature of the reaction mixture should be maintained at 37°C.

2. ACE reagent is reconstituted.

3. ACE reagent is reconstituted with 4.2 mL

4. 1.0 mL ACE reagent is pipetted into test tubes and is brought to temperature of 37°C.

5. 0.1 mL of serum sample is added to the test tube labelled test.

6. Reaction mixture is aspirated. After 1 minute read A1 the initial absorbance read at 340 nm and after exactly 5 minutes from the first reading read A2 absorbance at 340 nm.

7. The absorbance (A) of test and calibrator at 340 nm vs. water as blank. These are initial A1 read and exactly 5 minutes later the absorbance is read A2 absorbance.

8*. Calculations: ACE activity (in U/L) = (A1 - A2) * 2200 or as per* kit.

9. Final value will be calculated and displayed by the analyzer.

One unit of ACE activity is defined as that amount of enzyme that will catalyze formation of one micromole of FAP per minute und conditions of assay.

Interferences: ACE is a metal protein so does not use chelate in sample preparation. Turbid lipemic icteric and lysed sera will interfere

Biological reference range: 67 - 113 U/L (37°C)

Critical/Alert level values: Below and above values of reference range

Laboratory interpretations: Sarcoidosis, asbestosis, silicosis, leprosyan Gaucher's disease will have increased values. Reduced levels are found in lung cancer, tuberculosis and cystic fibrosis.

Potential sources of variability:

1. Lysed serum specimens may give falsely elevated values

2. ACE activity remains stable in serum for up to one week only if the serum specimen is stored at -20 °C hence if analysis is not done on the same day within 3 hours of collection, separate the serum and store it at -20 °C.

3. Discard the vial if dry reagent exhibits caking due to possible moisture penetration, does not dissolve completely or if the solution appears turbid.

4. ACE activity is inhibited by EDTA and by heavy metal ions that may serve to replace the zinc ion of the enzyme. Upon administration of the angiotensin converting enzyme-inhibitory drug, captopril, currently used for treating hypertension, ACE serum activity is markedly reduced but usually returns to normal levels in about 12 hours. Administration of other such drugs may produce a similar response.

QUANTITATIVE ESTIMATION OF LACTATE DEHYDROGENASE

Purpose: LDH is present in almost all the tissues of the body. Its increased activity in serum reflects several pathologic states, 1.c myocardial infarction, liver disorders, pernicious anemia megaloblastic anemia, and progressive muscular dystrophy and cancer.

Principle:

1. Lactate dehydrogenase is a hydrogen transfer enzyme catalyzes the following reaction:

Lactic acid + NAD^+ ------LDH-----> Pyruvic acid + NADH

2. The reaction is reversible but the conditions for the reverse reaction are different than those for the forward (e.g. the pH for the forward reaction is 8.8 to 9.8 and for the reverse reaction is 7.4 to 7.8).

3. LDH activity can be determined colorimetrically using 2,4-dinitrophenylhydrazine (2,4 DNPH) as the chromogen in alkaline medium.

Pyruvic acid produced during the LDH activity, reacts with 2,4-DNPH produces red color product which can be read at 510 nm spectrophometrically.

Performance specifications:

1. Linearity: This method is linear up to 1000 Units/L of serum

2. Measurement range: This method has a measurement range of 100-200 Units/L

3. Sensitivity: Lower limit of detection is 100 Units/L

Primary sample:

1. Use serum sample for the analysis.

2. Collect 2 mL of venous blood from a peripheral vein in a plain vacutainer tube.

3. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes

4. Do not use hemolyzed/contaminated serum for testing

Type of container and additive:

1. Use plain tubes for collecting samples.

2. Do not use hemolyzed/contaminated serum for testing

Reagents:

1. Preparation of substrate

2. Glycine buffer: **Glycine-3.753 g**

NaCl-2.922 g

Water-500 ml

3. 35% Sodium lactate solution: **4 0.1 N NaOH**

5. Buffered substrate: **Glycine buffer - 120 mL**

NaOH solution - 20 mL

Sodium lactate - 10 mL. Adjust the pH to

10 with 0.1 N NaOH.

Store in refrigerator.

6. NAD+: 10 mg in 2.0 mL distilled water

7. Standard: 220 mg sodium pyruvate in 100 mL glycine buffer.

8. Working standard: Dilute 5 mL of stock to 100 mL with glycine buffer (1 u mole pyruvate/mL).

9. 2,4-Dinitrophenylhydrazine: 200 mg DNPH in 1000 mL of 1 N HCl.

10. 0.4 N Sodium hydroxide

11. Serum: 1:5 dilution in saline.

Instrument: Spectrophotometer

Procedure: Standardization protocol

Reagents (ml)	Blank	S1	S2	S3	S4	S5
Buffered substrate	1.0	0.9	0.8	0.7	0.6	0.5
Water	03	0.3	0.3	0.3	0.3	0.3
Std Sodium pyruvate	--	0.1	0.2	0.3	0.4	0.5
Conc. U/L	--	333	666	999	1998	3996

			Incubate at 37°C for 5 min			
2,4-DNPH	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
			Incubate at 37°C for 15 min			
0.4N NaOH	**10**	**10**	**10**	**10**	**10**	**10**
			Incubate at RT for 10 min			
OD at 510 nm						

Reagent	*Blank*	*Control*	*Test*
Buffered substrate (mL)	**--**	**0.1**	**0.1**
NAD^+ (ml)	**--**	**0.2**	**0.2**
		Incubate at 37 °C for 5 minutes	
1.5 Diluted serum (ml)	**--**	**--**	**0.1**

		Keep at 37°C for 15 minutes	
DNPH (ml)	**1.0**	**1.0**	**1.0**
Diluted serum (mL)	**--**	**1.0**	**--**
		Keep at 37°C for 15 minutes	
0.4N NaOH (mL)	**10.0**	**10.0**	**10.0**
		Room temperature for 10 minutes	

Read at 510 nm against water.

Calculation:

LDH activity U/L= (At-Ab)/As X std.conc. X 1/15 X 1000/0.02

Note: At-Test absorbance, Ab-Blank absorbance, As-std absorbance

1/15 = per 15 minutes

1000/0.02 = conversion of aliquot of specimen taken to 1 lite

Reference range: Adults: 70 to 240 U/L; Children: 150 to 590

Potential sources of variability:

1. Use of only clear, unhemolyzed serum separated from erythrocytes as soon as possible. Lysed serum specimens may give falsely elevated values

2. On storage, the working reagent may develop a turbid, which makes the use of reagent blanks necessary with every run 10.3. Repeated freezing and thawing is harmful for the enzyme.

MANUAL METHEMOGLOBIN REDUCTION TEST: SCREENING FOR G6PD DEFICINCY

Value of test: The methemoglobin reduction test is one of the simpler and less expensive tests to screen for G6PD deficiency. Reduced G6PD activity in red cells can cause acute intravascular hemolysis following exposure to oxidant agents or fava beans (favism), neonatal jaundice and less commonly, chronic hemolytic anemia. The severity of clinical symptoms is mainly dependent on the variant of defective G6PD gene inherited.

Principle of test: Hemoglobin is oxidized to methemoglobin (Hi) by sodium nitrite. The redox dye, methylene blue activates the pentose phosphate pathway, resulting in the enzymatic conversion of Hi back to hemoglobin in those red cells with normal G6PD activity. In G6PD deficient cells there is no enzymatic reconversion to hemoglobin.

Reagents:

1-Methylene blue, 0.4 mmol/l Reagent No. 57

2-Sodium nitrite-glucose reagent*

***The reagent must be prepared fresh on the day of use. To make 40 ml:**

Sodium nitrite . . . . . 0.5 g

Glucose . . . . . 2.0 g

Dissolve the chemicals in 40 ml of distilled (deionized) water.

Preparation of reagents in tubes for long term storage

To store the reagents in dried ready to use form:

– Mix equal volumes of methylene blue reagent with sodium nitrite-glucose reagent.

– Dispense in 0.2 ml amounts into small glass tubes.

– Dry the contents of the tubes at room temperature.

– Stopper the tubes and store in the dark at room temperature.

***Note*: In dried form the reagents are stable for up to 6 months.**

Blood sample: EDTA anticoagulated venous blood is suitable. It should not be collected during a hemolytic crisis but when the patient has recovered and reticulocyte numbers have fallen back to normal levels. This is because reticulocytes contain higher levels of G6PD and may mask low G6PD activity in mature red cells. The blood must be tested within 8 hours of being collected. When the patient is anemic, use a plasma reduced blood sample (remove sufficient plasma until the PCV is about 0.40).

Test method

1- Take 3 small glass tubes and label Test, Normal, Deficient.

2- Pipette into each tube as follows:

Tube	Test	Normal control	Deficient control
Sodium nitrite-glucose reagent (fresh)	0.1 ml	–	0.1 ml
Methylene blue reagent	0.1 ml	–	–
Patient's blood	2 ml	2 ml	2 ml

3- Stopper the tubes and mix well (gentle mixing). Incubate all three samples at 35–37℃ for 90 minutes.

4- Take 3 large tubes (15 ml capacity) and label as described in step 1. Pipette 10 ml of distilled (deionized) water into each tube.

5- Transfer 0.1 ml of well mixed sample from the *Test*, *Normal*, and *Deficient* tubes to the large tubes. Mix the contents of each tube.

6- Examine the color of the solution in each tube.

Interpretation of test results:

Color of test solution is similar to the……..Normal G6PD activity

red color of the *Normal* tube

Color of test solution is similar to the……Reduced G6PD activity

brown color of the *Deficient* tube…….. (G6PD deficiency in homozygote)

***Note*: Results from a heterozygote are midway between normal G6PD activity and G6PD deficiency in the homozygote.**

SEMI- AUTOANALYZER DETERMINATION FOR GLUCOSE-6-PHOSPHATE-DEHYDROGENASE

Purpose: Glucose-6-phosphate-dehydrogenase (G6PD) deficiency is one of the most common human enzyme deficiencies, in the world. During G6PD deficiency, the red cells are unable to regenerate reduced Nicotinamide adenine dinucleotide phosphate (NADPH) a reaction that is normally catalyzed by the G6PD enzyme. Since the X chromosome carries the gene for G6PD enzyme, this deficiency mostly affects the males as females are protected by the other normal X chromosome. The two major conditions associated with G6PD deficiency are hemolytic anemias and neonatal jaundice, which may result in neurological complications and death. Screening and detection of G6PD deficiency helps in reducing such episodes, through appropriate selection of treatment, patient counselling and abstinence from disease-precipitating drugs such as anti-malarial like primaquine and other agents and favism.

Principle: G6PD in RBCs is released by a lysing agent present in the reagent. The G6PD released catalyzes the oxidation of Glucose-6 phosphate with the reduction of NADP to NADPH. The rate reduction of $NADP^+$ to NADPH is measured as an increase in absorbance, which is proportional to the G6PD activity in the sample.

G6P + $NADP^+$ ----- G6PD----->6-Phosphogluconic acid +NADPH + H^+

Performance specifications:

Linearity: This method is linear for up to 20 Unit/g of Hb.

Primary sample:

1. Use only whole blood as specimen

2. Collect 2 mL of venous blood in a vacutainer EDTA anticoagulant tube.

3. Do not use lysed blood for testing as it may give very high results

4. Do not use contaminated/turbid samples for testing.

5. Sample materials: Fresh whole blood sample collected in EDTA, heparin or ACD, red cell G6PD in whole blood is reported to be stable for 7 days at 2-8°C, but is unstable in hemolysates freezing is not recommended.

Type of container and additive: Add 2.0 ml of blood in Vacutainer tube and shake well for 30 sec. Reagents:

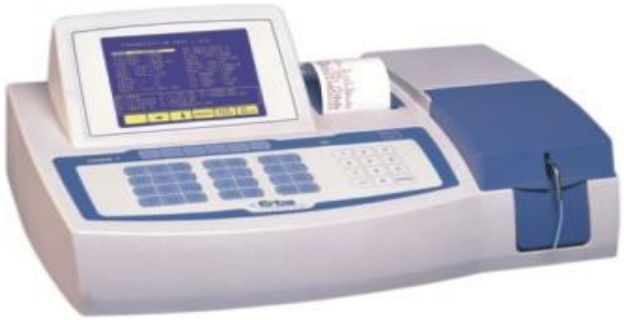
L1: G6PDH reagent 5x 1 ml

L2: Starter reagent 10 ml

Reagent preparation: Make up G6PD reagent (LI) with distilled as per the volume mentioned on the label. This working reacts stable for 6 hours at RT and at least 5 days when store at 2-8°C starter reagent (L2) is ready to use.

Add 10 mL of blood to the EDTA added tube and shake well 30 sec.

Instrument: Semi-autoanalyzer Chema.



Procedure:

1. Wavelength: 340 nm

Temperature: 37°C

Light path: 1 cm

2. Pipette into a clean dry test tube labelled test (T)

3. Addition sequence T (mL)

G6PD working reagent (L1) 1.0

Whole blood 0.01

Mix well and incubate for 5-10 min at RT and add

Starter reagent 2.0

4. Mix well and incubate for 5 min at 37°C and read the initial absorbance A_0 and repeat the absorbance reading after every 1, 2 and 3 minutes.

5. Calculate the mean absorbance change per minute (ΛA/min) If the G6PD activity is very low the absorbance change per minute will also be very low. In such cases, read the initial absorbance A1 and read another absorbance A2 exactly 5 min later. Calculate the mean absorbance change per min. ΛA/min = A2 – A1/5

Reference range: G6PD Activity (U/g Hb): 6.4 to 18.7 at 37°C

Safety precautions:

1. Handle all samples as potentially infectious

2. Handle all reagents with care and avoid contact with eye, mouth and skin

3. Do not perform mouth pipette

4. Discard used reagents and sample as per disposal procedure

Potential sources of variability: Lysed blood specimens may give falsely elevated values.

QUANTITATIVE ESTIMATION OF AMYLASE

Purpose: Quantitative estimation of the activity of amylase in human serum. The clinical significance of the estimation of amylase lies almost entirely in the diagnosis of acute pancreatitis, in which the enzyme level frequently exceeds more than 10 times the normal values. Some other causes include salivary gland disorder, abdominal disturbances affecting the pancreas and intake of drugs.

Principle: Amylase is a hydrolytic enzyme that splits complex carbohydrates such as starch and glycogen to glucose.

Starch ----- Amylase----> glucose

The iodometric method is based on the ability of iodine to form a vivid blue color in combination with starch. The byproduct of amylase action may also form colored substances with iodine but at different wavelengths from the characteristic starch-iodine complex. In this method, iodine color reagent is added to the substrate-sample mixture after an incubation period. The greater the amount of amylase activity, the lighter will be the color.

Performance specifications:

1. Linearity: This method is linear for amylase activity up to 300 Somogyi units/dL in serum.

2. Measurement range: This method has a measurement range of 50-300 Somogyi units/dL of amylase activity in serum.

3. Sensitivity: The minimum detection limit of amylase in serum by this method is 50 Somogyi units/dL.

4. The amylase of serum is activated by chloride ions, so the dilutions of serum must be made in physiological saline.

Primary sample:

1.Collect 4 ml of blood in a plain vacutainer tube for blood collection

2. Do not use lysed serum for testing as it may give very high results

3. Do not use contaminated/turbid samples for testing

4. Process the sample on the same day within 3 hours of collection or store at -20 °C for one week.

Type of container and additive: Use plain vacutainer tube for blood collection

Reagents:

1. Phosphate buffer

Na_2HPO_4 - 1.735g

KH_2PO_4 - 1.009 g (for 1 liter)

Mix 84 mL of A and 16 mL of B solution (pH 7.4)

2. Starch solution: 5g/liter

3. Prepare the buffer and starch solution fresh every time a sample is processed or at intervals of not more than 2 to 3 days.

4. Buffered substrate 0.4 mg/mL. Weigh 20 mg starch in 50 mL phosphate buffer.

5. Iodine reagent stock: Dissolve 30 g of potassium iodide with 250 mL water, weigh 13 g iodine in a closed container and transfer quantitatively to a liter volumetric flask with the iodide solution Shake well to dissolve and make to the mark with water Standardize against thiosulphate. The iodine concentration should be 47.5 to 52.5 mmole/liter. Adjust if necessary.

6. Working iodine standard solution: Prepare from reagent 3 by diluting 1 to 10 with water.

7. Sodium chloride solution: 0.9 g in 100 mL water.

Instrument: DU-640 Beckman spectrophotometer.

Procedure: Dilute the serum/plasma 1: 10 with saline

	Test	Control
Buffered substrate (mL)	1.0	1.0
Incubate 37°C for 5 minutes		
Serum (1:10) (ml)	0.1	--
Incubate at 37°C for 15 minutes		
Iodine, working standard (ml)	0.4	0.4
Water (mL)	8.5	8.6

Then measure the color at 660 nm using water as a blank

Calculation:

Amylase activity= (Absorbance of control - Absorbance of test) 800 / Absorbance of control

1 Somogyi unit of amylase activity = 5 mg starch hydrolyzed under aforesaid conditions (Enzymatic reaction for 15 minutes at 37 C at pH 7.4).

Amount of starch present in the reaction mixture = 0.4 mg

0.4/ 5= amylase units

Enzyme activity factor (amylase activity/dL serum)

= 0.4/ 5 X 100/ 0.1 X 10*

***Dilution factor**

Reference range: 60-180 Somogyi units/dL (or) 95 -290 IU//L

Critical/Alert level value: Not applicable

Potential sources of variability:

1. Lysed serum specimens may give falsely elevated values

2. Amylase activity remains stable in serum for up to one week, on refrigeration for two months

3. With the exception of heparin, all common anticoagulants inhibit amylase activity because they chelate Ca (IT) Citrate, EDTA, and oxalate inhibit it by 15%

4. The amylase of serum is activated by chloride ions, so the dilutions of serum must be made in physiological saline

5. Misleading increases in the activities of amylase and pancreatic amylase in the serum of a patient with macroamylasemia.

DETERMINATION OF ORNITHINE AMINOTRANSFERASE ACTIVITY

Purpose: To determine ornithine aminotransferase enzyme activity in cultured lymphocytes.

Principle: OAT is a pyridoxal phosphate requiring mitochondrial transaminase that catalyzes the reversible interconversion of ornithine and alpha ketoglutarate to pyrolline-5-carboxylate and glutamate. The pyrolline-5-carboxylate released is estimated using ortho amino benzaldehyde by the method of Katsunuma et al using ornithine as substrate.

Primary sample:

1. Use only heparinized blood to culture lymphocytes.

2. Collect 4 mL of venous blood in sterile heparin vacutainer tube.

3. The sample should be processed the same day.

4. Type of container and additives: Collect 4 mL of heparin vacutainer tube collecting sample.

Reagents:

Reagents for lymphocyte culture

1. Lymphoprep-commercially available from sigma

2. Phosphate buffered saline (PBS) - pH 72-74

Disodium hydrogen phosphate (Na_2HPO_4) - 14.8g

Potassium dihydrogen phosphate (KH_2PO_4) - 4.7

Sodium chloride (NaCl) - 68.0g

Distilled water - 1000 ml

3. F_{12} medium with fetal calf serum (FCS)--To get from culture lab.

4. Phytohemagglutinin (PHA)-commercially available

5. Tris-HCI Buffer (pH 8.0) - 121 g/L

6. Ornithine 20 mM (mol wt -132) - 26 mg/10m

7. α-ketoglutarate (α-KG) 10 mM (mol wt - 146) - 14 mg/10m

8. Pyridoxal phosphate 10 mM (mol wt - 247) - 24 mg/10 ml

9. 10% TCA.

10. Ortho aminobenzaldehyde (OAB) - 10 mg/1 ml

methanol

11. Pyroll 5 carboxylic acid (mol wt - 377)

Instrument: Spectrophotometer.

Procedure:

Lymphocyte separation - Perform using sterile things

1. Mix 4 mL of heparin blood with equal volume of working PBS.

2. To 10 mL of lymph prep overlay 10 mL of the blood diluted with PBS.

3. Centrifuge at 3000 rpm for 30 minutes.

4. Carefully discard the supernatant.

5. Remove the lymphocyte layer and add to a sterile vial or bottle.

6. Add equal volume of F12 with FCS to the lymphocyte separated and add 10 p L of PHA.

7. Keep for 72 hours at 37°C in CO2 incubator.

8. After 72 hrs, centrifuge the cells and remove the supernatant.

9. Wash the cells in PBS twice.

10. Re-suspend the cells in 1.5 mL in PBS and sonicate the culture at 60-kilo cycles for 10 sec.

11. Perform protein estimation by Lowry method.

12. Use 0.5 mL for OAT assay.

Sl. No.	Reagents	Blank	*T with B6*	*T without B6*
1.	Tris HCl (mL) pH 8.0	3.5	1.0	1.0
2.	Omithine (mL)	--	1.0	1.0
3.	α-KG (mL)	--	1.0	1.0
4.	Pyridoxal (mL)	--	10µL	--
5.	Enzyme (mL)	--	0.5	0.5
		Incubate at 37°C for 30 minutes		
6.	10% TCA (ML)	1.0	1.0	1.0
7.	OAB (mL)	0.1	0.1	0.1

Keep at boiling water bath for 5 minutes cool and read at 440 nm.

Reference range: Compared with the control and interpreted

Potential sources of variability: Lysed blood specimens should not be used for the separation

3.7 mg Pyroll 5 carboxylic acid/100 ml = 100 PM from this stock plot a standard graph from 10 to 50 nM. 0.5 mL of lymphocyte is used for the assay.

Therefore, for 100 mL = 100 x X/0.5

The value obtained is for 1/2 hour so multiply by 2 for 1 hour this gives the unit activity

For specific gravity = Unit activity/mg of protein.

DETERMINATION OF GLUTATHIONE PEROXIDASE (GPx) ACTIVITY

Purpose: Glutathione peroxidase (GPx) is a selenium dependent selenoprotein, an antioxidant enzyme present in our system. During oxidative stress conditions, this enzyme reacts with the oxidant species and reduces their effect. It is used to know the antioxidant status of the patient.

Principle: Glutathione peroxidase, reacts with H_2O_2 in the presence of GSH and converts it into H_2O and thus functions as an antioxidant.

$2H_2O_2$ ------ GPX ----> $2H_2O + O_2$

GSH GSSG

The enzyme activity is expressed as p moles of glutathione utilized minutes/gm hemoglobin.

Performance specifications:

1. Linearity: This method is linear for GP'x concentrations up to 60 µ mole glutathione utilized/g Hb.

2. Measurement range: This method has a measurement range of 5.0-60 µ mole glutathione utilized/g Hb.

3. Sensitivity: The minimum detection limit by this method 5.0 µ mole glutathione utilized/g Hb.

Primary sample:

1. Use only EDTA red blood cells as specimen for the test

2. Collect 2.0 mL of venous blood in EDTA Vacutainer tube

3. Do not use hemolyzed sample,

4. Process the sample immediately and if not done, store the at 4°C until processed, for a maximum of 24 hours.

Type of container and additive:

Collect 2 mL of EDTA vacutainer tube for blood collection,

Reagents:

1. Sodium phosphate buffer, 0.32 M, pH 70:

Na_2HPO_4 - 4.23 g in 100 mL

NaH_2 PO_4 $2H_2O$ -4.1 g in 100 mL

2. EDTA (0.8 mm) : 30 mg in 100 mL

3. Sodium azide (2.0 mm) : 12.2 mg in 100 mL.

4. Test glutathione (4 mm) : 122 mg in 10 mL

5. Hydrogen peroxide (2.5 mm) : 1:100 dilution again
3 mL made up to 100 mL.

6. Trichloroacetic acid (10%) : 10 g in 100 mL.

7. Phosphate solution (0.3 M) : 5.34 g - disodium
hydrogen phosphate
100 mL-distilled water.

Crystals developed during storage at 4°Care dissolved by heating.

8. DTNB reagent: 20 mg 5,5-dithiobis-(2 nitrobenzoic acid in 100 mL of one percent sodium citrate solution. This solution is stable for 10 weeks at 4°C.

Instrument: Spectrophotometer

Procedure:

1. 10 mL of EDTA blood is taken and the erythrocytes were washed thrice with saline after removal of plasma.

2. The buffy coats along with the upper layer of erythrocytes are discarded with each saline wash to remove leukocytes. Add 1.0 mL of hemolysine solution.

3. The estimation is done as follows:

Reagent	*Blank*	*Standards*					*Test*	*Control*
		S1	S2	S3	S4	S5	--	--
Buffer (µL)	400	400	400	400	400	400	400	400
Sodium azide (lµ)	200	200	200	200	200	200	200	200
EDTA (µL)	200	200	200	200	200	200	200	200
H2O2(µL)	200	200	200	200	200	200	200	200
Standard (µL)	--	25	50	75	100	125	--	--
Concentration (µg)	--	5	10	15	20	25	--	--
Test GSH solution (µL)	--	--	--	--	--	--	200	200
10% TCA (mL)	--	--	--	--	--	--	--	1.0
Hemolysate (mL)	--	--	--	--	--	--	0.1	0.1
Distilled water (mL)	0.7	0.675	0.65	0.625	0.600	0.575	0.7	0.7

				Keep at room temperature for 10 min				
10%TCA (mL)	--	--	--	--	--	--	**1.0**	--
				Centrifuge and take the supernatant				
Phosphate solution (mL)	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
DTNB (mL)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
				Read the absorbance at 412 nm				

Calculation: TOD - COD X Std. Conc. X 20,000/ SOD X 307 x 10 x Hb = µmole glutathione utilized/g Hb

Reference range: 27.6 – 48.4 µmole glutathione utilized/g Hb

Safety precautions:

1. Handle all samples as potentially infectious

2. Handle all reagents with care and avoid contact with eye, mouth and skin

3. Ensure the reagent and specimens are at room temperature before use.

4. Do not perform mouth pipetting

5. Discard used reagents and sample as per disposal procedure.

Potential sources of variability:

1.Below and above the reference range,

2. Lysed RBC samples may give falsely decreased values as enzymes are released from the erythrocytes.

3. Hemolysate has to be preserved in -20°C if the assay is not being performed immediately.

QUANTITATIVE ESTIMATION OF SUPEROXIDE DISMUTASE

Purpose: Quantitative estimation of Superoxide Dismutase. Measurement of SOD is useful in the diagnosis of antioxidant status.

Principle: Superoxide anion, $O_2^{--\cdot}$ is an intermediate in the autoxidation of epinephrine. The ability of super oxide dismutase to inhibit the autoxidation of epinephrine at pH 9.8 provides the basis of the assay for the enzyme. SOD catalyzes the following reaction:

$O_2^{--\cdot} + O_2^{--\cdot} + 2\ H^+$ ------- SOD -----> $O_2 + H_2O_2$

Performance specifications:

1. Linearity: This method is linear for up to 6000 U/g Hb

2. Measurement range: This method has a measurement range of 500-6000 U/g Hb of SOD activity in RBC.

3. Sensitivity: The minimum detection limit is 500 U/g Hb

Primary sample:

1. Use only heparin vacutainer tube for blood collection

2. Do not use hemolyzed sample.

3. Process the sample immediately and if not done store the RBC at 4°C until processed, for a maximum of 24 hours.

Type of container and additive: Collect 4 mL of heparin tube for blood collection.

Reagents:

1. 50 mM of Carbonate-bicarbonate buffer, pH 9.8

Na_2CO_3-529 mg; $NaHCO_3$ - 420 mg; EDTA-1 mg made up to 100 mL with distilled water.

2. Epinephrine: 1.8 mM solution, prepared freshly - 1 mg in 1 ml dissolved by adding dilute HCl.

3. Absolute ethanol.

4. Chloroform.

Instrument: Spectrophotometer (Kinetic assay mode)

Procedure:

1. Preparation of the Enzyme: The extraction of the enzyme is carried out according to the method of Bartosz et al (1978).

2. Take 1 mL of heparinized blood. Wash with saline three times after removing the plasma

3. The buffy coats along with the upper layer of erythrocytes are discarded with each saline wash to remove leukocytes.

4. Add 1 ml of cold distilled water to the remaining RBCS. Cyclomix well to get the hemolysate

5. To the 0.5 ml of the hemolysate add 3.5 mL cold distilled water.

6. Following this, add 1.0 ml of chilled ethanol and 0.6 ml. of ice-cold chloroform

7. Shake the mixture well for a few minutes at and then centrifuge at 5000 rpm for 15 minutes

8. Take the supernatant for the enzyme assay as follows:

9. Assay:

	Blank	Control	Test
Water (mL)	1.2	0.8	0.75
Carbonate buffer (mL)	1.8	1.8	1.8
Sample (mL)	--	--	0.05
Epinephrine (mL)	--	0.4	0.4

10. As soon as the epinephrine is added, immediately the increase in absorbance at 480 nm is measured in a spectrophotometer by kinetic assay for interval of 15 seconds and total of 240 seconds. 11. Autoxidation of epinephrine to adrenochrome is performed in a control tube without the enzyme. One unit of enzyme activity is defined as the quantity of enzyme required to produce 50% inhibition in epinephrine autoxidation.

Calculation:

T-(165 seconds - 105 seconds)

C-(165 seconds - 105 seconds)

C-T/C/2X 45000 = Units/min/g Hb

Reference range: 2600-4963 Units/min/g Hb.

Safety precautions:

1. Handle all samples as potentially infectious

2. Handle all reagents with care and avoid contact with eve, mouth and skin

3. Ensure the reagents and specimens are at room temperature before use

4. Do not perform mouth pipetting 10.5. Discard used reagents and sample as per disposal procedure

Potential sources of variability:

1. Lysed RBC samples may give falsely decreased values an enzyme is released from the erythrocytes. 2. Do not use if the lysate has been stored in the fridge even for 24 hours

3. Hemolysate has to be preserved in -20°C if the assay is not being performed

ENZYME IMMUNOASSAY FOR CREATINE KINASE-MB TEST SYSTEM

Intended Use: The Quantitative Determination of Circulating Creatinine Kinase (MB-Isoform) Concentrations in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence.

SUMMARY AND EXPLANATION OF THE TEST

Creatinine kinase (CK) is an enzyme found primarily in muscle and brain tissue, which exists as three dimeric isoenzymes - CKMM (CK-3), CK-MB (CK-2), and CK-BB (CK-1) - built from subunits designated M and B. The CK-MB isoenzyme, which has a molecular mass of approximately 87,000 Daltons, accounts for 5 to 50% of total CK activity in myocardium. In skeletal muscle, by contrast, it normally accounts for just 1% or less, CK-MM being the dominant form, though the percentage can be as high as 10% in conditions reflecting skeletal muscle injury and regeneration (e.g. severe exercise, muscular dystrophy, polymyositis).

Serial measurement of biochemical markers is now accepted universally as an important determinant in ruling in or ruling out acute myocardial infarction. CK-MB is one of the most important myocardial markers (in spite of not being cardiac-specific), with well-established roles in confirming acute myocardial infarction (AMI) and in monitoring reperfusion during thrombolytic therapy following AMI.

In AMI, plasma CK-MB typically raises some 3 to 8 hours after the onset of chest pains, peaks within 9 to 30 hours, and returns to baseline levels within 48 to 72 hours.7 The pattern of serial CK-MB determinations is more informative than a single determination.

One CK-MB measurement, even when taken at an appropriate time, cannot definitively confirm or rule out the occurrence of AMI. High levels might reflect skeletal injury rather than myocardial damage. A value within the reference range might be significant if it represents an increase from the patient's baseline levels. Accordingly, it has been recommended that CK-MB be measured on admission to the emergency room and at regulated intervals thereafter. The model described by the Heart Emergency Room (ER) Program13 documented that serial testing for CK-MB isoenzyme mass on presentation and 3, 6 and 9 hours later in patients with symptoms suggestive of acute ischemic coronary syndrome presenting with a non-diagnostic or equivalent electrocardiogram was more effective (100% sensitivity with 100% negative predictive value) than continuous serial electrocardiograms, electrocardiography and graded exercise testing.

In this method, CK-MB calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies directed against distinct and different epitopes of CK-MB are added and the reactants mixed. Reaction between the various CK-MB antibodies and native CK- MB forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-CK-MB antibody bound conjugate is separated from the unbound enzyme-CK-MB conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce light.

The employment of several serum references of known CK-MB levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with Creatine Kinase concentration.

PRINCIPLE

Immunoenzymometric assay:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-CK- MB antibody.

Upon mixing biotin labeled monoclonal antibody, the enzyme- labeled antibody and a serum containing the native antigen reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A. CK-MB Calibrators

Six (6) vials of serum references for CK-MB antigen manufactured at levels of 0(A), 5(B), 25(C), 100(D), 200(E), and 400(F) ng/ml. Reconstitute each vial with

1.0ml of distilled or deionized water. The reconstituted calibrators are stable for 7 days at 2-8°C. In order to store for a longer period of time, aliquot the reconstituted calibrators in cryo vials and store at -10°C. *DO NOT FREEZE THAW MORE THAN ONCE*. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using gravimetric protein weight from a >99% purified preparation as measured with PAGE.

B. CK-MB Tracer Reagent

One (1) vial containing enzyme labeled affinity purified antibody and biotin labeled monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Light Reaction Wells

One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate

One (1) vial containing a surfactant dissolved in buffered saline. A preservative has been added. Store at 2-8°C.

E. Signal Reagent A

One (1) vial containing Luminol solubilized in buffer. Store at 2-8°C.

F. Signal Reagent B

One (1) vial containing hydrogen peroxide (H_2O_2) dissolved in buffer. Store at 2-8°C.

Materials Required but Not Provided:

1.Pipette(s) capable of delivering 0.025ml (25µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%

3.Microplate washer or a squeeze bottle (optional).

4.Microplate Luminometer

5.Container(s) for mixing of reagents (see below).

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate cover for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Storage container for storage of wash buffer.

11.Distilled or deionized water.

12.Quality Control Materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or gel barrier. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.

2.Working Signal Reagent Solution - Store at 2 - 8°C.

Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (a slight excess of solution is made). Discard the unused portion if not used within 36 hours after mixing. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-25°C).

*****Test procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025ml (25µl) of the appropriate calibrators, controls and samples into the assigned wells.

3.Add 0.100ml (100µl) of the Tracer Reagent to each well. It is very important to dispense all reagents close to the bottom of the microwell.

Note: Use a multichannel pipette to quickly dispense Enzyme Reagent to avoid drift if the dispensing is to take more than a few minutes.

4.Swirl the microplate gently for 20-30 seconds to mix. Cover with a plastic wrap.

5.Incubate for 15 minutes at room temperature (20-25°C).

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat four (4) additional times.

8.Add 0.100ml (100µl) of working signal reagent to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells

DO NOT SHAKE THE PLATE AFTER SIGNAL ADDITION

9.Incubate at room temperature in the dark for five (5) minutes.

10.Read the relative light units in each well with a chemiluminescence microplate reader for 0.5-1.0 seconds. The results should be read within thirty (30) minutes of adding the working signal reagent.

NOTE: Always add reagents in the same order to minimize reaction time differences between wells.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of CK-MB in unknown specimens.

1.Record the RLUs obtained from the printout of the microplate luminometer

2.Plot the light intensity for each duplicate serum reference versus the corresponding CK-MB concentration in ng/ml on linear graph paper.

3.Draw the best-fit curve through the plotted points.

4.To determine the concentration of CK-MB for an unknown, locate the average RLUs of the unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

EXPECTED VALUES

CK-MB values are consistently higher in plasma than in serum; thus, serum is preferred. Compared with fasting values in non- obese non-diabetic individuals, CK-MB levels are higher in obese non-diabetic subjects and lower in trained athletes.

Each laboratory is advised to establish its own ranges for normal and abnormal populations. These ranges are always dependent upon locale, population, laboratory, technique and specificity of the method.

Based on the clinical data gathered from published literature the following ranges have been assigned. These ranges should be used as guidelines only:

TABLE 1

Expected Value Range for CK-MB CLIA Test System

Adult (Normal)	**2.0 – 5.2 ng/ml**

SECTION 1: BIOCHEMISTRY

C- HORMONES

ENZYME IMMUNOASSAY FOR CALCITONIN HORMONE TEST SYSTEM

Intended Use: The Quantitative Determination of Calcitonin Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Calcitonin is a 32 amino acid alpha helix produced by the follicular cells of the thyroid gland. A cleavage product of procalcitonin (PCT), calcitonin is a product of the CALC1 gene in humans and provides support in regulating calcium homeostasis, lowering serum calcium concentrations and preventing hypercalcemia. Calcitonin is characterized by an N-terminal disulfide bridge, which contributes to its biological activity, and a C-terminal proline residue.

Calcitonin plays a role in calcium metabolism, with osteoclasts the most significant homeostatic targets. Calcitonin binds to CT receptors (CTRs) on osteoclasts, halting calcium resorption via prevention of cell differentiation and motility. CTR receptors are also found in the kidneys and hypothalamus, providing an excretion route for excess serum calcium. Calcitonin modulates calcium absorption via CTR receptors on renal tubules, preventing excess calcium uptake. CTR receptors belong to the family of G- protein coupled receptors, utilizing cAMP messengers to amplify and transduce signals initiated by calcitonin-CTR binding.

Calcitonin has emerged as a therapeutic avenue for hypercalcemia patients, utilized as a biomarker PCT for its rapid biomarker for medullary carcinoma of the thyroid (MCT), providing a facile and direct measurement of carcinogenic activity. Calcitonin levels are typically low in normal populations, and elevated levels suggest the presence of hypercalcemia or potential loss of thyroid function.

Medullary thyroid carcinoma is typically associated with elevated levels of calcitonin. Parafollicular C cells containing mutations in the RET gene will display elevated expression of calcitonin and the presence of nodules in the lymph nodes, potentially disrupting calcium homeostasis.

PRINCIPLE

Sandwich Equilibrium Method:

The calcitonin immunoassay is an adapted two-site sandwich ELISA. In this assay, standards and patient samples are simultaneously incubated with the enzyme labeled detection antibody and a biotin coupled capture antibody on a coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, concentration. Concentrations of calcitonin present in the controls and patient samples are determined directly from this curve.

The essential reagents required for a sandwich equilibrium assay include high affinity and specificity antibodies (signal and capture), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the calibrator, control or patient sample is added to the wells coated with anti-calcitonin antibody. Calcitonin from the sample binds to the anti-calcitonin (MoAb) on the wells. Subsequently an enzyme labeled anti-calcitonin is added to the wells. Calcitonin from the sample forms a sandwich between the two antibodies. Excess enzyme and sample are removed via a wash step.

The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

A suitable substrate is added to the wells to generate color in varying intensity depending upon the concentration of calcitonin in the wells. The intensity of the color in the sample can be visually compared to the known calibrators to obtain qualitative results or the color development can be read with the help of a microplate spectrophotometer to obtain semi-semi-quantitative results.

REAGENTS

Materials Provided:

A.Calcitonin Calibrators

Six (6) vials of references for Calcitonin at levels of 0(A), 10(B), 40(C), 150(D), 400(E) and 1000(F) pg/ml. Store at 2- 8°C. *Reconstitute each vial with 1ml of distilled or deionized water.* The reconstituted calibrators are stable for 1 hour at 2 8°C. A preservative has been added. For longer periods after reconstitution,

aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

B.Calcitonin Control M

One (1) vial of reference control for Calcitonin. Store at 2-8 °C. *Reconstitute each vial with 1ml of distilled or deionized water.* The reconstituted control should be assayed immediately after reconstitution. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months.Freeze and thawed cycles should be minimized to one time only.

C.Calcitonin Enzyme Reagent

One (1) vial containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store at 2-8°C.

D.PCT Antibody Coated Plate

One 96-well microplate coated with procalcitonin antibody, packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

H.Substrate Reagent

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (0.5M H_2O_2) in buffer. Store at 2-8°C.

G.Stop Solution

One (1) vial containing a strong acid (0.5M H SO). Store at 2-8C

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not expose reagents to heat, sun, or strong light. Opened reagents are stable for sixty (60) days when stored at 2-8°C, unless otherwise specified. Kit and component stability are identified on label.

Note 3: The above components are for a single 96-well microplate

Materials Required but Not Provided:

1.Pipette capable of delivering 0.050ml (50µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl), and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Microplate washers or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

5.Absorbent paper for blotting the microplate wells.

6.Plastic wrap or microplate covers for incubation steps.

7.Vacuum aspirator (optional) for wash steps.

8.Timer.

9.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8 °C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml (100µl) of the specimen is required.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3.Add 0.050 ml (50 µl) of the Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4.Swirl the microplate gently for 20-30 seconds to mix (500 – 600 rpm) and cover.

5.Incubate 60 minutes (1 hour) at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100 ml (100 µl) of Substrate Reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for twenty (20) minutes.

10.Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. The results should be read within fifteen(15) minutes of adding the stop solution.

Note 1: **For re-assaying specimens with concentrations greater than 1000 pg/ml, dilution should be performed.**

Note 2: **Do not use reagents that are contaminated or have bacterial growth.**

Note 3: **Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous**

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Calcitonin in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader

2. Plot the absorbance for each duplicate calibrator versus the corresponding calcitonin concentration in pg/ml on linear graph paper. Connect the points with a best-fit curve.

To determine the concentration of calcitonin for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXPECTED RANGES OF VALUES

Calcitonin levels were measured in thirty-one (31) apparently normal individuals. The values obtained ranged from 0.292 to 118.643 pg/ml. Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows the normal or Gaussian distribution as shown in histograms. The geometric mean ± 2 standard deviations of the mean were calculated to be 4.49 to 41.83 pg/ml.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal"

persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR CORTISOL TEST SYSTEM

Intended Use: The Quantitative Determination of Total Cortisol Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence

SUMMARY AND EXPLANATION OF THE TEST

Cortisol (hydrocortisone, compound (F) is the most potent glucocorticoid produced by the human adrenal cortex. As with other adrenal steroids, cortisol is synthesized from cholesterol, through a series of enzymatically mediated steps, by the adrenal cortex. The first and rate-limiting step in adrenal steroidogenesis, conversion of cholesterol to pregnenolone, is stimulated by pituitary adrenocorticotropic hormone (ACTH) which is, in turn, regulated by hypothalamic corticotropin releasing factor (CRF). ACTH and CRF secretion are inhibited by high cortisol levels. In plasma, the major portion of cortisol is bound with high affinity to corticosteroid-binding globulin (CBG, transcortin), with most of the remainder loosely bound to albumin. Physiologically effective in anti-inflammatory activity and blood pressure maintenance, cortisol is also involved in gluconeogenesis. Cortisol acts through specific intracellular receptors and has effects in numerous other physiologic systems, including immune function, glucose-counter regulation, vascular tone, substrate utilization and bone metabolism. Cortisol is excreted primarily in urine in an unbound (free) form.

Cortisol production has an ACTH-dependent circadian rhythm with peak levels in the early morning and a nadir at night. The factors controlling this circadian rhythm are not completely defined. The circadian rhythm of ACTH/cortisol secretion matures gradually during early infancy, and is disrupted in a number of physical and psychological conditions. Furthermore, increased amounts of ACTH and cortisol are secreted independently of the circadian rhythm in response to physical and psychological stress.

Elevated cortisol levels and lack of diurnal variation have been identified in patients with Cushing's disease (ACTH hyper secretion). Elevated circulating

cortisol levels have also been identified in patients with adrenal tumors. Low cortisol levels are found in primary adrenal insufficiency (e.g. adrenal hypoplasia, congenital adrenal hyperplasia, Addison's disease) and in ACTH deficiency. Due to the normal circadian variation of cortisol levels, distinguishing normal and abnormally low cortisol levels can be difficult. Therefore, various tests to evaluate the pituitary-adrenal (ACTH-cortisol) axis, including insulin-induced hypoglycemia, short- and long-term ACTH stimulation, CRF stimulation and artificial blockage of cortisol synthesis with metronome have been performed. Cortisol response characteristics for each of these procedures have been reported.

The Cortisol CLIA Kit uses a specific monoclonal anti- cortisol antibody, and does not require prior sample extraction of serum or plasma. Cross-reactivity to other naturally-occurring steroid is low. The employment of several serum references of known cortisol concentration permits construction of a graph of activity and con- centration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with cortisol concentration.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for a chemiluminescence immunoassay include antibody, tracer-antigen conjugate and native antigen.

Upon mixing biotinylated antibody, tracer-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the tracer-antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effect the separation of the antibody bound fraction after decantation or aspiration.

The tracer activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen con- centration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A.Cortisol Calibrators

Six (6) vials of serum reference for Cortisol at concentrations of 0 (A), 1.0 (B), 4.0 (C), 10.0 (D), 20.0 (E) and 50.0 (F) µg/dl. Store at 2-8°C. A preservative has been added.

Cortisol Tracer Reagent

One (1) ready to use vial containing Cortisol (Analog)- horseradish peroxidase (HRP) conjugate in a protein stabilizing matrix with red dye, preservative and binding protein inhibitors. Store at 2-8°C.

B.Cortisol Biotin Reagent

One (1) vial containing anti-cortisol biotinylated mIgG conjugate in buffer, dye and preservative. Store at 2-8°C.

C.Light Reaction Wells

One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E.Signal Reagent A

One (1) vial containing luminol in a buffer. Store at 2-8°C.

F.Signal Reagent B – 7.0 ml/vial

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Materials Required [But Not Provided]:

1.Pipette capable of delivering 0.025 ml (25µl), 0.050 ml (50µl) 100 ml (0.100 µl) 1.0ml (1000 µl volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.50ml (50µl), 0.100 ml (0.100 µl) 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Microplate washer or a squeeze bottle (optional).

4.Microplate Luminometer

5.Test tubes for signal A and B.

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate covers for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8oC for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20oC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C.

2. Working Signal Reagent Solution - Store at 2 - 8°C. Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). Discard the unused portion if not used within 36 hours after mixing. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

*****Test procedure should be performed by a skilled individual or trained professional*****

1.Format the reaction wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25µL) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.050 ml (50µl) of the ready to use Cortisol Tracer Reagent to all wells.

4.Swirl the microplate gently for 20-30 seconds to mix.

5.Add 0.050 ml (50µl) of Cortisol Biotin Reagent to all wells.

6.Swirl the microplate gently for 20-30 seconds to mix.

7.Cover and incubate for 45 minutes at room temperature.

8.Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9.Add 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.

10.Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells. Do not shake the plate after signal addition.

11.Incubate at room temperature for five (5) minutes in the dark.

12.Read the relative light units in each well with a Chemiluminescence microplate reader for 0.5- 1.0 seconds. The results should be read within 30 minutes after adding substrate

Note: Dilute the samples suspected of concentrations higher than 50 µg/dl 1:5 and 1:10 with cortisol '0' µg/dl patient serum.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of cortisol in unknown specimens.

1.Record the RLUs obtained from the printout of the microplate reader

2.Plot the RLUs for each duplicate serum reference versus the corresponding cortisol concentration in µg/dl on linear graph paper.

3.Draw the best-fit curve through the plotted points.

To determine the concentration of cortisol for an unknown, locate the average RLU's for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/dl) from the horizontal

axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU's (19241) of the unknown intersects the calibration curve at (18.9) cortisol concentration

EXPECTED RANGES OF VALUES

A study of normal adult population was undertaken to determine expected values for the Cortisol CLIA Test System. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1.

TABLE I

Expected Values for the cortisol CLIA Test System (in µg/dl)

	Male (75 specimens)	Female* (83 specimens)
Mean X	13.2	13.5
Std.	4.2	4.2

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR DEHYDROEPIANDROSTERONE TEST SYSTEM

Intended Use: The Quantitative Determination of Dehydroepiandrosterone Sulfate Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Dehydroepiandrosterone (DHEA) is a C19 steroid secreted by the adrenal cortex, and is a precursor in testosterone and estrogen biosynthesis. Due to the presence of a 17-oxo [rather than hydroxyl] group, DHEA possesses relatively weak androgenic activity, which has been estimated at ~10% that of testosterone.

The physiologic role of DHEA is not well-defined. Since DHEA has a relatively low affinity constant for sex hormone binding globulin (SHBG), the bioactivity at the cell level maybe more significant than other androgenic steroids that have much higher affinity to SHBG. Abnormal levels have been reported in obesity and schizophrenia. Excessive DHEA secretion can cause acne, hirsutism and virilization. DHEA measurement is important in the investigation of adrenal androgen production for adrenal hyperplasia and tumors.

DHEA has a fast clearance turnover rate compared to its sulfated conjugate. This leads to marked difference in circulation concentration compared to the sulfate derivative, which has much longer half-life. DHEA levels do show circadian rhythm that reflects the secretion of ACTH and also varies during the menstrual cycle.

Measurement of serum DHEA is a useful marker of adrenal androgen synthesis. Abnormally low levels have been reported in hypoadrenalism, while elevated levels occur in several conditions; including virilizing adrenal adenoma and carcinoma, 21-hydroxylase and 3β-hydroxysteroid dehydrogenase deficiencies, and some cases of female hirsutism.[2] Since very little DHEA is produced by the gonads, measurement of DHEA may aid in the localization of the androgen source in virilizing conditions.

The DHEA kit uses a specific anti-DHEA antibody, and does not require prior sample extraction of serum or plasma. Cross- reactivity to other naturally occurring and structurally related steroids is low.

The employment of several serum references of known DHEA concentration permits construction of a graph of activity and con- centration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with DHEA concentration.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen.

Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effect the separation of the antibody bound fraction after decantation or aspiration.

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen con- centration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained

REAGENTS

Materials Provided:

A.DHEA Calibrators

Six (6) vials of serum reference for DHEA at concentrations of 0 (A), 0.5 (B), 2.0 (C), 5.0 (D), 10.0 (E) and 30.0 (F) in ng/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 3.47. For example: 1ng/ml x 3.47 = 3.47 µM/L

B.DHEA Enzyme Reagent

One (1) vial of DHEA (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with red dye. Store at 2-8°C.

C.DHEA Biotin Reagent

One (1) vial of reagent contains anti-DHEA biotinylated purified rabbit IgG conjugate in buffer, blue dye and preservative. Store at 2-8°C.

D.Streptavidin Coated Plate

One 96-well microplate coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E.Wash Solution Concentrate

One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F.Substrate Solution

One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G.Stop Solution

One (1) vial contains a strong acid (0.5M H SO). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and componenet stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1. Pipette capable of delivering 0.025 & 0.050ml (25 & 50µl) with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.

3.Adjustable volume (200-1000µl) dispenser(s) for conjugate.

4.Microplate washer or a squeeze bottle (optional).

5.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate cover for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum or heparanised plasma in type and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop veni-puncture tube with or without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin (for plasma). Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20oC for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20 - 27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.05ml (50µl) of the DHEA Enzyme Reagent to all wells.

4.Swirl the microplate gently for 20-30 seconds to mix.

5.Add 0.050ml (50µl) of Anti- DHEA Biotin Reagent to all wells.

6.Swirl the microplate gently for 20-30 seconds to mix.

7.Cover and incubate for 60 minutes at room temperature.

8.Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

10.Add 0.100ml (100µl) of substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11.Incubate at room temperature for twenty (20) minutes.

12.Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

13.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 30ng/ml 1:5 with DHEA '0' ng/ml calibrator.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of DHEA in unknown specimens.

1.Record the absorbance obtained from the printout of the microplate reader

2.Plot the absorbance for each duplicate serum reference versus

the corresponding DHEA concentration in ug/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3.Connect the points with a best-fit curve.

4.To determine the concentration of DHEA for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ug/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance in the patient sample (1.68) intersects the dose response curve at (2.36ng/ml) DHEA concentration.

Note: Computer data reduction software designed for ELISA Assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal "adult population, the expected ranges for the DHEA ELISA Test System are detailed in Table 1.

TABLE I

Expected Values for DHEA ELISA Test System

	(ng/ml)
Male	1.8 – 12.5
Female	1.3- 9.8

ENZYME IMMUNOASSAY FOR ADRENOCORTICOTROPIC HOROMONE TEST SYSTEM

Intended Use: The Quantitative Determination of Adrenocorticotropic Hormone Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

SUMMARY AND EXPLANATION OF THE TEST

Adrenocorticotropic hormone (ACTH) is a hormone produced in the anterior, or front, pituitary gland in the brain. The function of ACTH is to regulate the levels of steroid hormones that are released from the adrenal glands including cortisol, aldosterone, and androgen precursors. ACTH is secreted in response to a variety of severe stressors such as pain or emotional stress and ultimately results in analgesic, anti-inflammatory, and tissue regeneration effects.

An overactive pituitary gland can result in increased ACTH levels leading to excess cortisol production (hypercortisolism), also known as Cushing's syndrome. Conversely, hypopituitarism characterized by reduced ACTH levels can lead to adrenocortical insufficiency. Addison's disease, or primary adrenal insufficiency, can be diagnosed when ACTH levels are high, but there is insufficient cortisol produced by the adrenal gland.

Monitoring ACTH levels is a key aspect of mediating symptoms in patients with adrenal abnormalities.

PRINCIPLE

Sandwich Equilibrium Method:

ACTH immunoassay is an adapted two-site sandwich Chemiluminescence immunoassay CLIA. In this assay, standards and patient samples are simultaneously incubated with the tracer labelled detection antibody and a biotin coupled capture antibody on a coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the tracer bound to the solid phase is incubated with the with the signal reagent containing luminol. A dose response curve of RLU unit vs. concentration is generated using results obtained from the calibrators. Concentrations of ACTH present in the controls and patient samples are determined directly from a curve with the help of a Microplate Luminometer to obtain semi-quantitative results.

The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A. ACTH Calibrators (Dried)

Six (6) vials of references for ACTH at approximate* concentration range of 0, 20, 100, 250, 750 and 2000 pg/ml. Store at 2-8°C. *Reconstitute each vial with 1ml of distilled or deionized water*. The reconstituted calibrators are stable for 1 hour at 2 8°C. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

B. ACTH Control M (Dried)

One (1) vial of ACTH control containing Calcitonin. Store at 2-8°C. *Reconstitute with 1ml of distilled or deionized water*. The reconstituted control should be assayed immediately after reconstitution. A preservative has been added. For longer periods after reconstitution, aliquot into smaller portions and freeze (<-20°C) for up to 3 months. Freeze and thawed cycles should be minimized to one time only.

C. ACTH Tracer Reagent

One (1) vial contains anti-ACTH-HRP (horseradish peroxidase) conjugated antibody in a protein-based buffer and a non-mercury preservative. Store at 2-8°C.

D. ACTH Light Reaction Wells

One 96-well white microplate coated with anti-ACTH antibody and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C

F. Signal Reagent A

One (1) vial containing luminol in buffer. Store at 2-8°C.

G. Signal Reagent B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C. .

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not expose reagents to heat, sun, or strong light.

Opened reagents are stable for sixty (60) days when stored at 2 8°C, unless otherwise specified. Kit and component stability are identified on label.

Note 3: The above components are for a single 96-well microplate.

Materials Required but Not Provided:

1. Pipette capable of delivering 0.050ml (50µl) volumes with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl), and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3. Microplate washers or a squeeze bottle (optional).

4. Microplate Luminometer

5. Absorbent paper for blotting the microplate wells.

6. Plastic wrap or microplate covers for incubation steps.

7. Vacuum aspirator (optional) for wash steps.

8. Timer.

9. Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8 °C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.10 ml (100 µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

2. Working Signal Reagent Solution - Store at 2 - 30°C.

Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). Discard the unused portion if not used within 36 hours after mixing. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Pipette 0.050 ml (50 µl) of the ACTH tracer to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4. Swirl the microplate gently for 20-30 seconds to mix (500-600 rpm) and gently cover.

5. Incubate 45 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.

8. Add 0.100 ml (100 µl) of working signal reagent to all wells.

(See Reagent Preparation Section) Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE PLATE AFTER SIGNAL ADDITION

9. Read the relative light units (RLUs) in each well for 0.2 – 1.0 seconds. The results should be read within thirty (30) minutes of adding the signal reagent solution.

Note 1: Do not use the working signal reagent solution if older than 36 hours.

Note 2: Do not use reagents that are contaminated or have bacterial growth.

Note 3: Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.

Note 4: It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the microwells at an angle while touching the side of the well.

Note 5: For reassaying specimens with concentrations greater than 2000 pg/ml, dilution should be performed.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of ACTH in unknown specimens.

1. Record the RLU obtained from the printout of the microplate reader.

2. Plot the RLU for each duplicate serum reference versus the corresponding ACTH Calibrators concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of ACTH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

Note: Computer data reduction software designed for CLIA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXPECTED RANGES OF VALUES

ACTH levels were measured in three hundred and fifty-four (354) apparently normal individuals. The values obtained ranged from 7.2 – 63.3 pg/ml.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR ALDOSTERONE TEST SYSTEM

Intended Use: The Quantitative Determination of Aldosterone Concentration in Human Serum or Plasma by Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Aldosterone is a steroid that is synthesized in the zona glomerulosa of the adrenal cortex. Like all steroids, aldosterone is derived from cholesterol through a series of enzymatic reactions.1 It is considered the main mineralocorticoid

hormone and acts in response to elevated potassium levels or lowered sodium levels in the blood. Aldosterone is the final product of the renin-angiotensin-aldosterone system (RAAS) and is essential in mediating blood-pressure and extracellular volume homeostasis. Increased blood aldosterone levels with normal to reduced blood renin abundance are increasingly associated with many cases of hypertension and congestive heart failure. Additionally, aldosterone has been recognized to have adverse effects on endothelial, renal, and central nervous system tissues.

Aldosterone is a key hormone involved in sodium conservation throughout the body. When aldosterone is released, it acts on the mineralocorticoid receptor (MR) which in turn activates specific amiloride-sensitive sodium channels (ENaC) to increase potassium excretion by the kidneys while sodium excretion is decreased. This results in a decrease of blood potassium while increasing sodium levels.

PRINCIPLE

Delayed Competitive Enzyme Immunoassay:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody.

REAGENTS

Materials Provided with the kit

A. Aldosterone Calibrators

Six (6) vials of serum reference for aldosterone at concentrations of 0 (A), 25 (B), 125 (C), 250 (D), 500 (E), 1500 (F) in pg/ml. Store at 2-8°C. *Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted calibrators are stable for 30 days at 2-8°C. A preservative has been added. Concentrations can be expressed in ng/dl by dividing by 10.

B. Aldosterone Control (Lyophilized)

One (1) vial of human serum-based matrix containing Aldosterone at an established range. Store at 2-8°C. *Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted controls are stable for 30 days at 2-8°C. A preservative has been added.

C. Aldosterone Enzyme Reagent

One (1) vial containing Aldosterone (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with dye. Store at 2-8°C.

D. Aldosterone Biotin Reagent

One (1) vial containing biotinylated anti-aldosterone IgG conjugate in buffer, dye and preservative. Store at 2-8°C.

E. Streptavidin Coated Plate

One 96-well microplate coated with streptavidin and packaged in an aluminium bag with a drying agent. Store at 2-8°C.

F. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

G. Substrate Reagent

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution

One (1) vial containing a strong acid (0.5M H_2SO_4). Store at 2-8°C.

Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required:

1. Pipette capable of delivering 0.025ml (25µl) and 0.050ml (50µl) with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3. Adjustable volume 0.050ml-1.0ml (50µl-1000µl) dispenser(s) for conjugate.

4. Microplate washer or a squeeze bottle (optional).

5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6. Absorbent Paper for blotting the microplate wells.

7. Plastic wrap or microplate cover for incubation steps.

8. Vacuum aspirator (optional) for wash steps.

9. Timer.

10.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparinized plasma in type, and taken with the usual precautions in the collection of venepuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venepuncture tube(s) or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality

control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminium bag, seal and store at 2-8°C.

2. Pipette 0.025 ml (25 µl) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.050 ml (50µl) of the Aldosterone Biotin Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Cover and incubate for 15 minutes at room temperature.

6. Add 0.050 ml (50µl) of Aldosterone Enzyme Reagent to all wells.

Add directly on top of the reagents dispensed in the wells.

7. Swirl the microplate gently for 20-30 seconds to mix.

8. Cover and incubate for 45 minutes at room temperature.

9. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

10.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

11.Add 0.100 ml (100µl) of substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

12.Incubate at room temperature for twenty (20) minutes.

13.Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

14.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 1500pg/ml 1:5 and 1:10 with Aldosterone '0' pg/ml calibrator.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of aldosterone in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader.

2. Plot the absorbance for each duplicate serum reference versus the corresponding aldosterone concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Connect the points with a best-fit curve.

4. To determine the concentration of aldosterone for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of

the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

Note: Computer data reduction software designed for ELISA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 pg/ml should be > 1.8.

2. Four out of six quality control pools should be within the established ranges.

Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.

2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.

3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.

4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

5. The addition of substrate solution initiates a kinetic reaction terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.

6. Plate readers measure vertically. Do not touch bottom of wells.

7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

8. Use components from the same lot. No intermixing of reagents from different batches.

9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from IFU may yield inaccurate results.

10.All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.

11.It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population the expected ranges for the Aldosterone ELISA Test System are detailed in Table 1.

Expected Values for the Aldosterone Test System

Age	Posture Unspecified	Supine	Upright
0-6 days	50-1020 pg/ml		
1-3 weeks	60-1790 pg/ml		
1-11 months	70-990 pg/ml		
1-2 years	70-930 pg/ml		
3-10 years	40-440 pg/ml		
11-14 years	40-310 pg/ml		
15 years and older	Less than or equal to 310 pg/ml	Less than or equal to 160 pg/ml	40-310 pg/ml

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal"

persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR β-HUMAN CHRONIC GONADOTROPIN, FOLLICALE STIMULATING HORMONE, LUTEINIZING HORMONE, PROLACTIN HORMONE SEQUENTIAL: FERTILITY PANAL TEST SYSTEM

Intended Use: The Quantitative Determination of HCG, PRL, LH and FSH Concentration in Human Serum and Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Human chorionic gonadotropin (hCG) concentration increases dramatically in blood and urine during normal pregnancy. hCG is secreted by placental tissue, beginning with the primitive trophoblast, almost from the time of implantation, and serves to support the corpus luteum during the early weeks of pregnancy. hCG or hCG similar glycoproteins can also be produced by a wide variety of trophoblastic and nontrophoblastic tumors. The measurement of hCG, by assay systems with suitable sensitivity and specificity has proven great value in the detection of pregnancy and the diagnosis *of early* pregnancy disorders. hCG is detectable as early as 10 days after ovulation, reaching 100 mIU/ml by the first missed period. A peak of 50,000 to 100,000mIU/ml is attained by the third month, then a gradual decline is observed.[2,3]

Prolactin hormone (PRL), secreted from the lactotrophs of the anterior pituitary, is a protein consisting of a single polypeptide chain containing approximately 200 amino acids. The primary biological action of the hormone is on the mammary gland where it is involved in the growth of the gland and in the induction and maintenance of milk production. There is evidence to suggest that prolactin may be involved in steroidogenesis in the gonad, acting synergistically with luteinizing hormone (LH). High levels of prolactin appear to inhibit steroidogenesis as well as inhibiting LH and follicle stimulating hormone (FSH) synthesis at the pituitary gland.[1,2] The clinical usefulness of the measurement of prolactin hormone (PRL) in ascertaining the diagnosis of hyperprolactinemia and

for the subsequent monitoring the effectiveness of the treatment has been well established.

Luteinizing hormone (LH) is a glycoprotein consisting of two subunits with a molecular mass of 30,000 daltons. The α-subunit is similar to other pituitary hormones [follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG)] while the β-subunit is unique. The β-subunit confers the biological activity to the molecule. The α-subunit consists of 89 amino acid residues while the β- subunit contains 129 amino acids. The carbohydrate content is between 15% and 30%.

Follicle Stimulating hormone (FSH) is a glycoprotein consisting of two subunits with an approximate molecular mass of 35,500 daltons. The α-subunit is similar to other pituitary hormones [luteinizing stimulating hormone (LH), thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG)] while the β-subunit is unique. The β-subunit confers the biological activity to the molecule. Stimulation by gonadotropin-releasing hormone (GnRH) causes release of FSH, as well as LH, from the pituitary and is transported by the blood to their sites of action, the testes or ovary.

The clinical usefulness of the measurement of luteinizing hormone (LH) in ascertaining the homeostasis of fertility regulation via the hypothalamic - pituitary - gonadal axis has been well established. In addition, the advent of *in vitro* fertilization (IVF) technology to overcome infertility associated problems has provided the impetus for rapid improvement in LH assay methodology from the technically demanding bioassay[3] to the procedurally simple and rapid immunoenzymometric assays.

In men, FSH acts on the Sertoli cells of the testis, stimulating the synthesis of inhibin, which appears to specifically inhibit further FSH secretion, and androgen-binding protein. Thus, it indirectly supports spermatogenesis.

In women, FSH acts on the granulosa cells of the ovary, stimulating steroidogensis. All ovulatory menstrual cycles have a characteristic pattern of FSH, as well as LH, secretion. The menstrual cycle is divided into a follicular phase and a luteal phase by the midcycle surge of the gonadotropins (LH and FSH). As the follicular phase progresses, FSH concentration decreases. Near the time ovulation occur, about midcycle, FSH peaks (lesser in magnitude than LH) to its highest level.

In this method, hCG/PRL//LH/FSH (referred to as antigens, in the Product Insert) combination calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled

antibodies (directed against distinct and different epitopes of the hormones) are added and the reactants mixed. Reaction between the various antibodies (specific to the respective hormones) and native hormones forms a sandwich complex that binds with the streptavidin coated to the well.

In the PRL procedure, a sequential method of antibody addition is followed. That is, the biotinylated antibody is introduced first, and after an appropriate reaction period, the plate is washed. Then an enzyme liked antibody, directed against a different epitope is added and the plate is processed as the other antigens.

After the completion of the required incubation period(s), the antigen antibody enzyme bound conjugate is separated from the unbound enzyme antigen conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to-produce color.

The employment of several serum references of known hormone levels permits construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with the specific hormone concentration.

PRINCIPLE

Immunoenzymometric assay:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated specific monoclonal antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. For PRL assay, the sequence of addition is separated into two steps. That is, the biotinylated antibody binds to the prolactin antigen and is simultaneously deposited to the well surface. The second incubation initiates the binding of the enzyme linked antibody to the antigen bound through the biotinylated antibody on the well.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the

antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

MATERIALS

Reagents for 2 X 96 well Microplate

A.Combi-Cal™ FSH/LH/hCG/sPRL Calibrators

Six (6) vials of references for antigens at levels indicated below. A preservative has been added. The calibrators, human serum based, were calibrated using a reference preparation indicated in the chart.

Antigen	hCG (mIU/ml)	hLH (mIU/ml)	PRL (ng/ml)	FSH (mIU/ml)
A	0	0	0	0
B	25	5	10	5

B. hCG Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for hCG in buffer, blue dye, and preservative. Store at 2-8°C.

C.PRL Biotin Reagent

One (1) vial containing biotin labeled antibody specific for PRL in buffer, green dye, and preservative. Store at 2-8°C.

D.PRL Enzyme Reagent

One (1) vial containing enzyme labeled antibody specific for PRL in buffer, red dye, and preservative. Store at 2-8°C.

E. LH Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for hLH in buffer, yellow dye, and preservative. Store at 2-8°C.

F.FSH Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for FSH in buffer, green dye, and preservative. Store at 2-8°C.

G.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

H.Substrate Solution A

Two (2) vials containing tetramethylbenzidine (TMB) in acetate buffer. Store at 2-8°C.

J.Substrate Solution B

Two (2) vials containing hydrogen peroxide (H_2O_2) in acetate buffer. Store at 2-8°C.

I.Streptavidin Coated Microwells

Two (2) 96-well microplates coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

K.Stop Solution

Two (2) vials containing a strong acid (1N HCL). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.

Materials Required but Not Provided:

1.Pipette(s) capable of delivering 0.025ml (25µl) and 0.050ml (50µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5% (optional).

3.Microplate washers or a squeeze bottle (optional).

4.Microplate Luminometer.

5.Container(s) for mixing of reagents (see below).

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate cover for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Storage container for storage of wash buffer.

11.Distilled or deionized water.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe disposal of kit components must be according to local regulatory and statutory requirement.

PECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type and the usual precautions in the collection of venipuncture samples should be observed. The blood should be collected in a plain redcap venipuncture tube without additives. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml (100µl) of the specimen is required for LH and FSH. For prolactin and hCG, 0.050ml (50µl) of sample is needed.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature until expiration date printed on concentrate label.

2.Working Substrate Solution – Stable for one year

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled solution 'B.' Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE (HCG, LH & FSH)

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (*20 - 27° C*).

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2A. <u>For HCG</u>: Pipette 0.025ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.

2B. <u>For LH and FSH</u>: Pipette 0.050ml (50µl) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.100ml (100 µl) of the appropriate enzyme reagent to each well. It is very important to use the right 'Enzyme Reagent' for each assay for correct results.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 60 minutes at room temperature for LH and/or FSH or 20 minutes for hCG.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3)

washes. An automatic or manual plate washer can be used. Follow the manufacturer' sinstruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

7.Add 0.100ml (100µl) of substrate solution to all wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION.

8.Incubate at room temperature for fifteen (15) minutes.

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix by rotation so that a uniform yellow color is obtained

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620- 630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: It is very important to dispense all reagents in the center of the coated well. Always add reagents in the same order to minimize reaction time differences between wells.

TEST PROCEDURE (PROLACTIN)

1.Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 100µl of the PRL Biotin Reagent to each well. It is very important to use the right 'Enzyme Reagent' for each assay for accurate results.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 30 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the

manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100ml (100µl) of the Prolactin Enzyme Reagent to each well.

9.Incubate 30 minutes at room temperature.

10.Follow steps 6 through 10 as outlined in the procedure for HCG, LH & FSH above.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of each corresponding hormone in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader

2. Plot the absorbance for each duplicate serum reference versus the corresponding antigen concentration in appropriate units on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of corresponding hormone for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in relative units) from the horizontal axis of the graph.

`Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction.

EXPECTED VALUES (HCG)

A study of non-pregnant females and adult males was undertaken to determine expected values for hCG in Fertility Panel ELISA Test System. The mean (X) values, standard deviations (σ) and expected ranges (±2 S.D.) are presented in Table 1.

TABLE I
Expected Values for the hCG

Number	**125**
Mean (x)	**2.9**
Standard Deviation (σ)	**1.4**
Expected Ranges (±2σ)	**0.1-5.7**

Expected levels for hCG during normal pregnancy[3] are listed in Table 2.

TABLE 2
Expected Values for hCG levels (3rd IS 75/537) during normal pregnancy (in mIU/ml)

1st week	**10-30**
2nd week	**30-100**
3rd week	**100-1000**
4th week	**1000-10,000**
2nd & 3rd month	**30,000-100,000**
2nd trimester	**10,000-30,000**
3rd trimester	**5,000-15,000**

EXPECTED VALUES (LH, FSH & PRL)

A study of an apparent normal adult population was undertaken to determine expected values for LH and FSH in the Fertility Panel ELISA Test System. The expected values are presented in Table 3.

TABLE 3
Expected Values for the LH, FSH & PRL

	LH	PRL	FSH
Women		Adult	
Follicular Phase	0.8-10.5	1.2-19.2	3.0-12.0
Midcycle	18.4-61.3		8.0-22.0
Luteal Phase	0.8-10.5		2.0-12.0
Postmenopausal	8.2 -40.8		35 - 151
Men	0.7-7.4	1.8- 17.0	1.0-14.0

SEROLOGY OF HUMAN CHRONIC GONADOTROPIN: URINE PREGNANCY TESTS

From the earliest stage of development (9 days old), the placenta produces hormones, either on its own or in conjunction with the fetus. The very young placental trophoblast produces appreciable amounts of a hormone, human

chorionic gonadotropin (HCG) that is excreted in the urine. Human chorionic gonadotropin is not found in the urine of normal, young, nonpregnant woman.

As with all glylcoprotein hormones (LH, FSH, TSH), hCG is composed of two sub units, alpha and beta. The alpha sub unit is common to all glycoproteins and the beta sub unit confers unique specificity to the hormone. Because hCG is a glycoprotein hormone that is unique to the developing placenta (and some tumors), pregnancy tests are based on the detection of hCG in serum or urine. Its small size permits it to pass directly into the urine from the circulation.

Early in pregnancy, concentration of hCG in maternal serum rises quickly, with a doubling time of roughly two days during the first few weeks as the trphoblastic tissues increase in size. Within 10 to 12 weeks, hCG values with peak at 150,000 to 200,000 mIU/ml and then gradually fall to normal plateau values of 10,000 to 50,000 mIU /ml in the second and 3rd trimesters.

A later- term pregnancy in which there is a sudden drop in hCG from the plateau may indicate threatened abortion. Ectopic pregnancies have much lower hCG values and do not go to term. Molar pregnancies and other trophoblastic malignancies can have very high values of hCG, considerably beyond those encountered in normal pregnancy.

Monoclonal based assay which uses two different antibodies, one against the α-subunit and one against the β sub unit in a sandwich that capture the whole hCG molecule on a solid phase is being developed. Detection or quantitation of hCG is then generally accomplished by a color indicator reaction mediated by an enzyme (e.g., alkaline phosphatase) linked to the second antibody.

Serology of HCG in Urine

The amount of HCG excreted in the urine is almost the same as that found in the blood. HCG can be detected in the urine of pregnant women 26 to 36 days after the first day of the last menstrual period or 8 to 10 days after conception. Pregnancy test should be negative 3 to 4 days after delivery. If the measured value in urine is negative, but clinical examination indicates possible pregnancy, the test should be repeated in two days. Urine pregnancy test may be negative even though serum tested at the same time is positive because the serum assay is more sensitive, being optimized for the protein matrix found in serum.

Urine pregnancy tests

Laboratory pregnancy tests are based on the detection of rapidly rising levels of hCG in urine. Immunologic pregnancy tests are done in one of two ways. They

differ in the carrier for the external source of hCG, which is either a latex particle or red blood cells. The presence of hCG, is usually measured in urine because a urine sample is so easy to obtain. Urine pregnancy testing kits can be divided in to:

• Rapid latex slide tests of the inhibition (indirect) or the direct type

• Haemagglutinaton inhibition

Rapid latex slide tests

A. Inhibition (indirect) latex slide test

In this type of test, two reagents are supplied, an antiserum containing anti hCG antibody, and a latex reagent consisting of polystyrene particles sensitized (coated) with hCG, positive and negative controls etc.

• In the inhibition test, urine is first mixed with the antiserum, and the latex reagent is added.

- If hCG is present in the urine, it will combine with the anti hCG antibody. This will leave no antibody free to combine with the latex hCG and therefore, there will be no agglutination of the latex particles.

- If there is no hCG in the urine, the antibody will be free to combine with the latex hCG and cause agglutination of the latex particles.

- In this test, therefore, no agglutination indicates a positive test and agglutination indicates negative test.

B. Direct latex slide test

Are more sensitive than inhibition tests. In this test, the latex reagent consists of particles coated with the anti- hCG antibody. This reagent is mixed directly with the urine.

- If hCG is present in the urine, it will combine with the antibodies and cause agglutination of the latex particles.

- If no hCG is present in the urine, there will be no agglutination of the latex particles.

- In this test, therefore, agglutination of the particles indicates positive test and no agglutination indicates a negative test.

Note: the direct test is read in the opposite way to the inhibition (indirect) test.

C. Inhibition tube haemagglutination

In this type of test, the principle is the same as in the latex slide test except that the hCG is coated on red cells, not on polystyrene particles. The urine is reacted with anti hCG antiserum in the small tube provided, and red cells coated with hCG are added. The contents of the tube are mixed and then left at room temperature (20-28 OC) for 1-2 hours to allow time for the red cells to settle.

- If the urine contains hCG, it will combine with the antibody. This will leave no antibody to react with the hCG on the red cells. The non-agglutinated cells will settle and be seen as a red ring in the bottom of the tube.

- If the urine contains no hCG, the anti hCG antibody will react with the hCG on the red cells and cause their agglutination (haemagglatination). The agglutinates will settle and be seen covering evenly the bottom of the tube.

- In the inhibition (indirect) haemagglutinatan tube test, therefore, a red ring of non-agglutinated cells in the bottom of the tube indicates a positive test and a covering of agglutinated cells indicates a negative test.

N.B. Generally, urine for hCG is reported as positive for hCG if it is positive and negative for hCG if it is negative. There are different ICT tests developed for pregnancy

D. Semiquantitative test

If required, the amount of hCG in specimen can be measured semiquantitatively by preparing serial dilutions of the specimen in physiological saline and testing each dilution.

• Most manufactures of slide and tube tests provide details of how to perform a semiqntitative technique. A more accurate result is obtained by using a tube technique.

• Quantitative analysis of hCG aids in making a differential diagnosis of a viable pregnancy versus a nonviable pregnancy, twins or multiple gestations, or developing hydatidiform mole.

Factors that affect urine pregnancy test

The time in the pregnancy when the test is carried out. Interfering substance (drugs , red cell etc) & sensitivity / specificity of the assay. Negative or inconclusive results may occur if the concentration of HCG in the urine is below that which the test is capable of detecting reliably.

The presence of excessive amounts of protein or blood in the urine may cause false positive results. The presence of detergent can cause false positive or false negative result. Therefore, every material that will be used in this test should be free from detergent. Turbid specimens (due to amorphous debris or epithelial cells) may give inconclusive results. Such specimens should be filtered or centrifuged. Bacterial contamination of the urine may cause unreliable results. Heavily contaminated urine is unsuitable for testing.

Important- always read carefully the manufacturer's information leaflet.

Urine specimen

An early morning specimen is preferable because this is the most concentrated and will therefore, contain the highest level of HCG. If the specimen cannot be tested immediately, it should be refrigerated at 40c, but for not longer than 48 hours. Specimens preserved with boric acid are also suitable for testing. When tested, the urine (and test reagents) should beat room temperature.

URINE PREGNANCY TEST STRIP

There are two types of tests for pregnancy testing: one uses blood sample and other uses urine. Both tests detect the presence of beta subunit of a hormone called human chorionic gonadotropin (hCG). The Urine pregnancy test (UPT) is an easy and rapid chromatographic immunoassay for the qualitative detection of human chorionic gonadotropin (hCG) in urine for the early detection of pregnancy.

Human Chorionic Gonadotropin (hCG)

Human chorionic gonadotropin (hCG) is a hormone produced by the developing placenta shortly after fertilisation. In normal pregnancy, hCG can be detected in urine as early as 7 to 10 days after conception. Levels of hCG rise rapidly, frequently exceeding 100mIU/mL by the first missed menstrual period, and peaking at 100,000-200,000 mIU/mL about 10-12 weeks into pregnancy. The appearance of hcG soon after conception and it's subsequent rise in concentration during early gestational growth make it excellent marker for the early detection of pregnancy.

Principle of Urine Pregnancy Test

Most of the urine pregnancy test kits are based on lateral-flow technology. Most of them qualitatively detect the presence of hCG in urine specimen at the

sensitivity of 25 mIU/mL. The test uses two lines to indicate results. The test line utilizes a combination of antibodies including a monoclonal hCG antibody to selectively detect elevated levels of hCG. The control line is composed of goat polyclonal antibodies and colloidal gold particles.

The assay is conducted by adding a urine sample to the sample well of the test device and observing the formation of colored lines. The sample migrates via capillary action along the membrane to react with the colored conjugate.

Positive samples react with the specific antibody-hCG-colored conjugate to form a colored line at the test line region of the membrane. Absence of this colored line suggests a negative result. A colored line will always appear in the control line region if the tests has been performed properly.

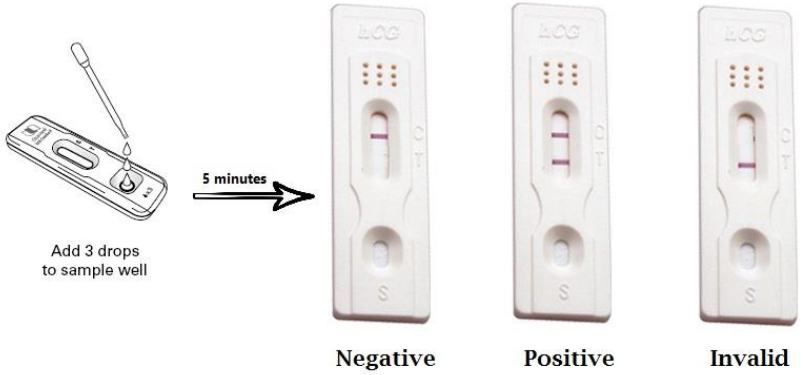
Specimen Collection

Collect urine into a clean, dry container. The first morning urine specimen is preferred since it generally contains the highest concentration of HCG; however, urine specimens collected at any time of the day may be used. Refrigerate specimens at 2° to 8°C (36° to 46°F) for up to 72 hours, if the testing is not performed immediately. If samples are refrigerated, bring them to room temperature before testing.

Procedure

1. **Allow the Pregnancy Test Strip and urine sample to reach room temperature (15-30°C) before opening the foil pouch.**
2. **Remove the Pregnancy Test Strip from the pouch and use it as soon as possible.**
3. **Place the test device on clean and level surface. Hold the dropper vertically and transfer 3 full drops to the specimen well and start the timer. Avoid air bubble formation.**
4. **Wait for the colored line(s) to appear. Read the result after 5 minutes. Do not read the result after 15 minutes.**

Results and Interpretation



POSITIVE: Two coloured lines appear. One line should be in the Control region (C) and another line should be in the Test region (T). This means there is a strong possibility that patient is pregnant.

NEGATIVE: One coloured line in the Control region (C). No apparent colored line appears in the Test region (T). This means patient is either not pregnant or has tested too early.

INVALID: The result is invalid if the Control Line (C) fails to appear. Insufficient volume of urine or incorrect procedure are the most likely reasons for an invalid result.

Limitations

1. **In cases where very high levels of HCG are present (>500,000 mIU/ml) a false negative result can occur due to a "Prozone" effect. If pregnancy is still suspected, simply dilute specimen 1:1 with deionized water and retest.**
2. **If a urine sample is too dilute (ie: low specific gravity) it may not contain a representative level of HCG. If pregnancy is still suspected, a first morning urine sample should be obtained and retested 48 hours later.**
3. **A number of conditions other than pregnancy, including trophoblastic disease and certain non-trpohoblastic neoplasms including testicular tumors, prostate cancer, breast cancer and lung cancer cause elevated levels of hCG. Therefore, the presence of hCG in urine should not be used to diagnose pregnancy unless these conditions have been ruled out.**
4. **Drugs containing hCG may interfere with the test, and produce misleading results.**

ENZYME IMMUNOASSAY FOR ALPHA-FETOPROTEIN, β-HUMAN CHRONIC GONADOTROPIN, UNCOJUGATED ESTRIOL: TRIPLE SCREEN PANAL TEST SYSTEM

Intended Use: The Quantitative Determination of Alpha- Fetoprotein (AFP), β-Human Chorionic Gonadotropin (hCG) and Unconjugated Estriol (uE3) Concentrations in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Monitoring of hCG, AFP and uE3 concentrations, at regular intervals, is considered to be very important to determine the fetal well-being. The collective information provided by these three assays (*Triple Screen)* provides the clinician with the comprehensive picture of the development of a healthy fetus and the health of the mother. Any anomaly seen during the first trimester can be corrected, unless it is caused by some genetic abnormality. This kit provides the clinician with a single tool to monitor all three analytes, using 0.125ml (125µl) of patient serum (0.050ml (50µl) for AFP, 0.050ml (50µl) for uE3 and 0.025ml (25µl) for hCG), in a single 75 minutes combination assay.

Alpha-Fetoprotein (AFP) is a glycoprotein with a molecular weight of 70 kDA. AFP is normally produced during fetal development by the hepatocytes, yolk sac and to a lesser extent by the gastrointestinal tract. Serum concentrations reach the highest level at twelve weeks of gestation. This peak level gradually decreases to less than 25ng/ml after one year of postpartum. Thereafter, the levels reduce further to less than 10ng/ml. The presence of abnormally high AFP concentrations in pregnant women is considered a risk marker for open neural tube defects (ONTDs).

Elevated levels of AFP are found in patients with primary hepatoma and yolk sac-derived germ tumors. AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma.

Human chorionic gonadotropin (hCG) concentration increases dramatically in blood and urine during normal pregnancy. hCG is secreted by placental tissue, beginning with the primitive trophoblast, almost from the time of implantation, and serves to support the corpus luteum during the early weeks of pregnancy. HCG or hCG similar glycoproteins can also be produced by a wide variety of trophoblastic and nontrophoblastic tumors. The measurement of hCG, by assay systems with suitable sensitivity and specificity has proven great value in the detection of pregnancy and the diagnosis *of early* pregnancy disorders.

According to the literature, serum and urine concentrations of biologically active (non-nicked) hCG is detectable as early as 10 days after ovulation, reaching 100mIU/ml by the first missed period. It rises exponentially in the first trimester, doubling almost every 48 hours to a peak (50,000 to 200,000mIU/ml) by the end of the first trimester. Then, a gradual decline is observed reaching approximately one fifth of the peak and remains at this level until term.

Unconjugated estriol in the serum of pregnant women originates almost exclusively from precursors in the fetus, via the placenta.[3] The clinical evidence shows that in uncomplicated pregnancies, the production of estriol increases steadily throughout the last trimester; however, in pregnancies complicated by placental insufficiency, the synthesis of estriol decreases rapidly. For many years, the most commonly used method for monitoring estriol synthesis (as an index to fetal stress) has been to measure estriol and estriol conjugates in a 24-hour urine sample. However, changes in renal clearance and diurnal variations can make the results of these determinations suspect. In recent years, investigators have found the determinations of unconjugated estriol in plasma during pregnancy as an alternative to the urinary assay to be a better marker of fetal stress. Abnormally low levels of estriol in a pregnant woman may indicate a problem with the development in the child. Levels of estriol in non-pregnant women do not change much after menopause, and levels are not significantly different from levels in men.

The Triple Screen Panel ELISA test system measures not only AFP, but hCG and uE3 as well. The test is more accurate and screens for additional genetic disorders. Generally speaking, the combination test will identify $\geq$ 60% of the babies with Down Syndrome and 80-90% of the babies with neural tube defects. This option had not been available, especially in developing countries, with conventional testing like ultrasound alone.

In this method, the combination calibrator (containing different levels of AFP, HCG and E3), patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of AFP and HCG) are added and the reactants mixed. Reaction between the various analyte specific antibodies and native analyte forms a sandwich complex that binds with the streptavidin coated to the well. In the case of uE3, an E3 analog coupled with HRP (Enzyme) is added followed by specific biotinylated E3 antibody. A competition occurs between labeled E3 and the native E3 for a limited number of sites on the antibody.

After the completion of the required incubation period, the excess enzyme labeled antibody or analog is washed off via a wash step. Addition of a suitable

substrate produces color, In HCG and AFP the intensity of the color is directly proportional to the concentration while in E3 it is inversely proportional to the concentration of the analyte.

The employment of several serum references of known levels of hCG, AFP and E3 permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's concentration can be interpolated.

PRINCIPLE

Immunoenzymometric assay (for hCG - AFP):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen, biotinylated (AFP/HCG) antibody.

After adding biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

In this method, the combination calibrator (containing different levels of AFP, HCG and E3), patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of AFP and HCG) are added and the reactants mixed. Reaction between the various analyte specific antibodies and native analyte forms a sandwich complex that binds with the streptavidin coated to the well. In the case of uE3, an E3 analog coupled with HRP (Enzyme) is added followed by specific biotinylated E3 antibody. A competition occurs between labeled E3 and the native E3 for a limited number of sites on the antibody.

After the completion of the required incubation period, the excess enzyme labeled antibody or analog is washed off via a wash step. Addition of a suitable substrate produces color, In HCG and AFP the intensity of the color is directly proportional to the concentration while in E3 it is inversely proportional to the concentration of the analyte.

The employment of several serum references of known levels of hCG, AFP and E3 permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's concentration can be interpolated.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration for AFP and hCG. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Competitive Enzyme Immunoassay for uE3:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen.

Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effect the separation of the antibody bound fraction after decantation or aspiration.

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided: (Reagents for 2x96 well Microplate)

A.Combi-Cal™ AFP/uE3/hCG-

Reconstitute each vial with 1ml of distilled or deionized water. The reconstituted calibrators are stable for one (1) year at 2-8°C.

Cal	AFP[1]	hCG[2]	uE3[3]
A	0	0	0
B	10	10	0.5

C	**25**	**25**	**1.0**
D	**75**	**50**	**2.5**
E	**150**	**100**	**10**
F	**400**	**250**	**20**
Units	**ng/ml**	**mIU/ml**	**ng/ml**

[1]AFP calibrated against WHO 1st IRP 72/225

[2]hCG calibrated against WHO 3rd IS 75/537

[3]uE3 prepared gravimetrically from 99+% pure preparations uE3 calibrators can be expressed in molar concentrations (nM/L) by multiplying by 3.45.

For example: 1ng/ml x 3.45 = 3.45 nM/L

A.AFP Enzyme Reagent

One (1) vial contains enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, yellow dye, and preservative. Store at 2-8°C.

B.hCG Enzyme Reagent

One (1) vial contains enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, blue dye, and preservative. Store at 2-8°C.

C.uE3 Enzyme Reagent

One (1) vial contains Estriol (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with red dye. Store at 2-8°C.

D.uE3 Biotin Reagent

One (1) vial contains biotin labeled specific biotinylated affinity purified rabbit IgG in buffer, blue dye, and preservative. Store at 2-8°C.

E.Streptavidin Coated Microplate –

One 96-well microplate coated with streptavidin and packaged preservative has been added. Store at 2-8°C.

F.Sample Diluent

One (1) vial contains normal human serum free of hCG stabilized with preservatives.

J.Substrate A

One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C. See "Reagent Preparation."

H.Substrate B

One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C. See "Reagent Preparation."

I.Stop Solution

One (1) vial contains a strong acid (1N HCl). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a 192 well kit; see table on last page for 96 well kit.

Materials Required but Not Provided:

1.Pipette(s) capable of delivering 0.025, 0.050 & 0.100ml (25, 50 & 100µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.

3.Microplate washers or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

5.Absorbent Paper for blotting the microplate wells.

6.Plastic wrap or microplate cover for incubation steps.

7.Vacuum aspirator (optional) for wash steps.

8.Timer.

9.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparanised plasma in type, and taken with the usual precautions in the collection of venipuncture samples. The blood should be collected in a redtop (with or without gel additives) venipuncture tube or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at -20°C or cooler for up to 30 days, in smaller aliquots. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required for all three (3) parameters.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. The diluted wash buffer can be stored at 2-30°C for up to 60 days.

2.Patient Sample Preparation: For hCG patient samples* (first trimester), dilutions should be made as follows:

Place 0.5ml (500µl) of Sample Diluent into a test tube and add 0.025ml (25µl) of patient sample. Vortex to mix. (Dilution 1:21). Remove 0.025ml (25µl) of (1:21) dilution and dispense into another test tube containing 1.0ml (1000µl) of Sample

Diluent (1/41) (Final Dilution 1:861). Assay the 1:861 dilutions and multiply the results by the dilution factor 861.

*** If hCG from normal populations is to be run, no dilutions are required, unless the patient's hCG is suspected to be greater than 250mIU/ml.**

3. Working Substrate Solution – Stable for one (1) year

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2- 8°C for up to 60 days.

Note: Do not use the working substrate if it looks blue.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20 - 27°C).

*****Test procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen (as is and dilutions) to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025ml (25µl) of the appropriate serum reference, con- trol and specimens (diluted for hCG) into the assigned well.

(For AFP and hCG):

3a. Add 0.100ml (100µl) of the AFP Enzyme Reagent or hCG Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

(For uE3):

3b. Add 0.050ml (50µl) of the U-Estriol Enzyme Reagent to all wells. Swirl the plate gently for 20-30 seconds to mix the contents.

3c. Add 0.050ml (50µl) of the U-Estriol Antibody biotin reagent to all the wells.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 60 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of assayed analytes in unknown specimens.

1. **Record the absorbance obtained from the printout of the microplate reader**

2. **Plot the absorbance for each duplicate serum reference calibrator versus the corresponding analyte concentration in corresponding units on linear graph paper (do not average the duplicates of the serum references before plotting).**

3. **Draw the best-fit curve through the plotted points**

4. **To determine the concentration of analyte for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration in relative units (ng/ml for AFP and uE3 and mIU/ml for hCG*) from the**

horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

***The Figures and Examples are for example only. Do not use it for calculating your results**

*** While regular monitoring of pregnancy hCG levels rise exponentially and thus exceed the upper limits of the *Dose Response Curve* (DRC). It is essential to dilute these samples to obtain valid results. (Please see *'Patient Sample Preparation'* under section 'Reagent Preparation).**

EXPECTED RANGES OF VALUES

Values for AFP, hCG and uE3 for a normal, healthy population and pregnant women, during gestation cycle, are given in Table 1 & 2. The values depicted below represent limited in-house studies in concordance with published literature.

TABLE 1

(Normal Values HCG during pregnancy)

HCG	**Normal Male/Female**	**≤ 5.7 mIU/ml**
	During Normal gestation (mIU/ml)	
	1st Week	**10 – 30**
	2nd Week	**30 – 100**
	3rd Week	**100 – 1000**
	4th Week	**1,000 – 10,000**
	2nd & 3 Month	**30,000 – 350,000**
	2nd Trimester	**10,000 – 30,000**
	3rd Trimester	**5,000 – 15,000**

TABLE 2

Median Values during Gestation.

Gestation (Week)	AFP (ng/ml)	hCG (IU/ml)	uE3 (ng/ml)
15	40.14	40.88	0.68
16	42.91	33.87	0.87
17	52.34	28.71	1.17
18	61.50	26.74	1.51
19	75.57	18.76	1.91
20	83.31	19.24	2.02
21	90.46	23.46	2.78

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR ANDROSTENEDIONE TEST SYSTEM

Intended Use: The Quantitative Determination of Androstenedione Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Androstenedione (ANST) is a steroid hormone (molecular weight of 290.4 daltons) that circulates in blood bound to sex hormone binding protein (SHBG). Its affinity to SHBG, is less than testosterone or dihydrotestosterone but greater than estrogens.

Androstenedione levels are found to have high diurnal variability. The highest concentrations are found in the morning. At puberty, androstenedione levels rise but fall after menopause. Higher levels are measured during pregnancy.

Androstenedione is secreted predominately by the adrenal glands partially regulated by adrenocorticotrophic hormone (ACTH). In addition to ACTH stimulation, ANST is produced in the testes and ovaries from adrenal secreted dehydoepiandrosterone sulfate (DHEAS). ANST is a critical sex steroid precursor.

High level in women can cause symptoms of hyperandrogenism. Increased concentrations are found in women with hirsutism (mostly in common with other androgens). Men are normally asymptomatic, but through peripheral conversion to estrogens can occasionally results in symptoms of mild estrogen excess. Most elevated levels of ANST are idiopathic. However, androgen producing tumors or gonadal tumors produces pronounced higher levels of ANST.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubilized binding sites.

After sufficient reaction is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the

antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided

A.ANST Calibrators

Six (6) vials of serum reference for androstenedione at concentrations of 0 (A), 0.1(B), 0.3 (C), 1.0 (D), 3.0 (E), 10.0 (F) in ng/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (nmol/L) by multiplying by 3.492. For example: 1ng/ml x 3.49 = 3.49 nmol/L

B.ANST Enzyme Reagent

One (1) vial contains ANST (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix with dye. Store at 2-8°C.

C.ANST Antibody Coated Plate

One 96-well microplate coated with Androstenedione-specific rabbit IgG and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D.Wash Solution Concentrate

One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E.Substrate Reagent

One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H 2 O 2) in buffer. Store at 2-8°C.

F.Stop Solution

One (1) vial containis strong acid (0.5M H2 SO 4). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1.Pipette capable of delivering 0.025ml (25µl) and 0.100ml (100µl) with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Adjustable volume 0.050ml-1.0ml (50-1000µl) dispenser(s) for conjugate.

4.Microplate washer or a squeeze bottle (optional).

5.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate cover for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparanised plasma in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored

at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25 µL) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.100 ml (100µl) of the ANST Enzyme Reagent to all wells.

4.Swirl the microplate gently for 20-30 seconds to mix.

5.Cover and incubate for 60 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100 ml (100µl) of substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for twenty (20) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 1000ng/ml 1:5 and 1:10 with Androstenedione '0' ng/ml calibrator or male patient serum pools with a known low value for Androstenedione.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Androstenedione in unknown specimens.

1.Record the absorbance obtained from the printout of the microplate reader

Plot the absorbance for each duplicate serum reference versus the corresponding androstenedione concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

2.Connect the points with a best-fit curve.

To determine the concentration of Androstenedione for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.964) intersects the dose response curve at 1.14ng/ml Androstenedione concentration

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population and females during gestation, the expected ranges for the Androstenedione ELISA Test System are detailed in Table 1.

TABLE 1

Expected Values for the Androstenedione Test System

Adults	
Females	0.3-2.0 ng/ml
Males	0.4-1.5 ng/ml

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the manufacturer only until an in- house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR ANTI-MULLERIAN HORMONE TEST SYSTEM

Intended Use: The Quantitative Determination of AMH Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Anti-Müllerian Hormone (AMH) is a disulfide-linked homodimeric 140kDa glycoprotein from the trans-forming growth factor-β (TGF-β) superfamily of growth and differentiation factors. It is primarily produced by the gonads in both males and females.

In fetal males, AMH is produced by the Sertoli cells and induces regression of the Müllerian duct and therefore promotes development of the male reproductive tract. Infant males have very high levels of AMH (>30 ng/ml) that slowly decreases until post-pubescence where it remains at a low level (<10 ng/ml).

In females, AMH begins to be produced near the time of birth with levels increasing until puberty. After puberty, blood AMH levels decrease until menopause where it becomes nearly undetectable (<0.1 ng/ml). AMH

concentration in female blood has repeatedly been linked to ovarian reserve, thereby giving an indication to patients' remaining reproductive lifespans.[4] Additionally, high levels of AMH (>4.7 ng/ml, 80% CI) in females are an indication of polycystic ovarian syndrome (PCOS).

When AMH levels drop below 1.0 ng/ml in females, they are considered to have low ovarian reserves. Patients in these ranges are advised to not delay family planning or to undergo infertility treatments such as in vitro fertilization.

The AMH test kit is a highly sensitive assay that can be used to measure blood AMH levels in order to monitor progress of patients' infertility treatments and approximate the onset of menopause.

PRINCIPLE

Sandwich Equilibrium Method:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of x-AMH antibody coated on the well.

Upon mixing the enzyme-labeled x-AMH antibody (separate epitope) and serum containing the native antigen, a reaction results between the native antigen and the antibodies without competition or steric hindrance to form a sandwich complex.

After sufficient time results, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A. AMH Calibrators (Lyophilized)

Six (6) vials of references for AMH at levels of 0(A), 0.2(B), 0.5(C), 1.0(D), 5.0(E) and 15.0(F) ng/ml. Store at 2-8 °C. *Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted calibrators are stable for 10 days at 2-8 °C.

To store for a longer period, aliquot the reconstituted calibrators into cryo vials and store at -20 °C. *DO NOT FREEZE/ THAW MORE THAN TWICE.* A preservative has been added.

B. AMH Controls (Lyophilized)

Two (2) vials of reference controls for AMH. Store at 2-8 °C. *Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted controls are stable for 10 days at 2-8 °C. To store for a longer period, aliquot the reconstituted calibrators into cryo vials and store at -20°C. *DO NOT FREEZE/ THAW MORE THAN TWICE.* A preservative has been added.

C. AMH Enzyme Reagent

One (1) vial contains anti-AMH conjugate reagent. Store at 2-8 °C.

D. AMH Antibody Coated Plate

One 96-well microplate coated with x-AMH antibody. Store at 2-8 °C.

E. Wash Solution Concentrate

One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8 °C. *See Reagent Preparation section.*

F. Substrate Reagent

One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8 °C.

G. Stop Solution

One (1) vial contains a strong acid (0.5 M H_2SO_4). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not expose reagents to heat, sun, or strong light.

Note 3: The above components are for one 96-well microplate

Materials Required but Not Provided:

1. Pipette capable of delivering 0.050ml (50µl) and 0.100ml (100µl) volumes with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3. Microplate washers or a squeeze bottle (optional).

4. Microplate reader with 450nm and 620nm wavelength absorbance capability.

5. Absorbent paper for blotting the microplate wells.

6. Plastic wrap or microplate cover for incubation steps.

7. Vacuum aspirator (optional) for wash steps.

8. Timer.

9. Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants for serum or EDTA/heparin containing tubes for plasma. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

If the specimen(s) cannot be assayed immediately after blood withdrawal, the sample(s) may be stored at temperatures of 2-8 °C for up to seven (7) days or -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing (a maximum of two freeze/thaw cycles prior to use). When assayed in duplicate, 0.100 ml (100 µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

Note: Do not use reagents that are contaminated or have bacterial growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8 °C.

2. Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.050 ml (50 µl) of the AMH Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4. Swirl the microplate gently for 20-30 seconds, cover and incubate for 60 minutes at room temperature.

5. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

6. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

7. Add 0.100 ml (100 µl) of TMB Substrate to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION

8. Incubate at room temperature for twenty (20) minutes.

9. Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

10.Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: For re-assaying specimens with concentrations greater than 15 ng/ml, dilution should be performed in human serum or plasma with low AMH values and multiplied accordingly.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of AMH in unknown specimens.

1. Plot the absorbance for each duplicate serum reference versus the corresponding AMH concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

2. Draw the best-fit curve through the plotted points.

3. To determine the concentration of AMH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.299) intersects the dose response curve at 1.33 ng/ml AMH concentration.

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	Abs (A)		Value (ng/ml)
Cal A	A1	0.005	0.005	0
	B1	0.005		
Cal B	C1	0.041	0.043	0.20
	D1	0.045		
Cal C	E1	0.109	0.112	0.50
	F1	0.115		
Cal D	G1	0.215	0.225	1.00
	H1	0.234		
Cal E	A2	1.047	1.068	5.00
	B2	1.097		
Cal F	C2	2.570	2.635	15.00
	D2	2.678		
Patient	E2	0.300	0.299	1.33
	F2	0.298		

Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. Maximum Absorbance (Calibrator 'F') ≥1.5

2. Four out of six quality control pools should be within the established ranges.

Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.

2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.

3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.

4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.

5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.

6. Plate readers measure vertically. Do not touch the bottom of the wells.

7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.

8. Use components from the same lot. No intermixing of reagents from different batches.

9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.

10.All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.

11.It is important to calibrate all the equipment e.g. pipettes, readers, washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

Interpretation

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.

2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.

3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

4. If computer-controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

EXPECTED RANGES OF VALUES

AMH levels were measured by the AMH Test System in apparently normal females of different age groups. The values obtained are given in Table 2.

Table 2

Female Reference Ranges for the AMH Test System

Age Group	N	Mean (ng/ml)	Min. (ng/ml)	Max. (ng/ml)
20-29	24	5.95	1.77	12.41
30-39	80	2.83	0.11	12.67
40-49	38	1.33	0.02	9.77

ENZYME IMMUNOASSAY FOR ESTRONE TEST SYSTEM

Intended Use: The Quantitative Determination of Estrone Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Chemiluminescence

SUMMARY AND EXPLANATION OF THE TEST

Estrone, also known as E1, is a steroid hormone (molecular weight of 270.4 daltons), which circulates predominantly protein- bound. In addition to estrone,

other natural steroidal estrogens include estradiol, estriol and their metabolites. Natural estrogens are hormones secreted principally by the ovarian follicles and also by the adrenals, corpus luteum, and placenta in females. In males, estrogens are primarily secreted by the testes. Exogenous estrogens (natural or synthetic) elicit, to varying degrees, all the pharmacologic responses usually produced by endogenous estrogens.

Estrogenic hormones are secreted at varying rates during the menstrual cycle throughout the period of ovarian activity. During pregnancy, the placenta becomes the main source of estrogens. Estrone in young females acts as a minor estrogen with approximately ten (10) times less potency than estradiol.[1] At menopause, ovarian secretion of estrogens declines at varying rates. However, since estrone can also be biosynthesized by adipose tissue via conversion of androstenedione, estrone becomes the primary estrogen in postmenopausal women.[1] Additionally, orally ingested estrogen is metabolized to estrone by the liver through the first-pass effect, which increases endogenous levels of estrone.[1]

In general, males have low levels of serum estrone in comparison to females. Very high levels of estrone in postmenopausal women have been linked to a higher risk of ER-positive breast cancer while younger females with polycystic ovarian syndrome (PCOS) may exhibit high estrone.[2-4]

Estrone determinations have proved of value in a variety of contexts, including the assessment of breast-cancer risk in postmenopausal women and gynecomastia in males. Its principal uses have been in the differential diagnosis of amenorrhea and in the monitoring of ovulation induction.

The kit uses a specific anti-estrone antibody, and does not require prior sample extraction of serum or plasma. Cross-reactivity to other naturally occurring and structurally related steroids is low.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen. Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubilized binding sites.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided

A.Estrone Calibrators

Six (6) vials of serum reference for estradiol at concentrations of 0 (A), 15 (B), 30 (C), 100 (D), 300 (E), 1000 (F) in pg/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (pmol/L) by multiplying by 3.70. For example: 1pg/ml x 3.70= 3.70 pmol/L

B.Estrone Tracer Reagent

One (1) vial of Estrone (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix red with dye. Store at 2-8°C.

C.Estrone Light Reaction Wells

One 96-well white microplate coated with estrone-specific rabbit IgG and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E.Signal Reagent A

One (1) vial containing luminol in a buffer. Store at 2-8°C.

F.Signal Reagent B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1.Pipette capable of delivering 0.025, 0.050, and 0.100ml (25, 50, 100µl) with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.050ml (50µl), 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Test tubes for Signal Reagent (See Reagent Preparation)

5.Microplate washer or a squeeze bottle (optional).

6.Microplate Luminometer

7.Absorbent Paper for blotting the microplate wells.

8.Plastic wrap or microplate cover for incubation steps.

9.Vacuum aspirator (optional) for wash steps.

10Timer.

11.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or heparanised plasma in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored

at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of wash solution concentrate to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

2.Working Signal Reagent Solution - Store at 2 - 8°C. Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1 ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). Discard the unused portion if not used within 36 hours after mixing. If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

Note: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25 µL) of the appropriate serum reference, control or specimen into the assigned well.

3.Add 0.100 ml (100µl) of the Estrone Tracer Reagent to all wells.

4.Swirl the microplate gently for 20-30 seconds to mix.

5.Cover and incubate for 45 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.

8.Add 0.100 ml (100µl) of working signal reagent solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

9.Incubate at room temperature for five (5) minutes in the dark.

10.Read the relative light units in each well with a chemiluminescence microplate reader for 0.5-1.0 seconds. The results should be read within 30 minutes after adding the working Signal Reagent.

Note: Dilute the samples suspected of concentrations higher than 1000pg/ml 1:5 and 1:10 with estrone '0' pg/ml calibrator or male patient serum pools with a known low value for estrone.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of estrone in unknown specimens.

1.Record the RLU obtained from the printout of the microplate reader.

2.Plot the RLU for each duplicate serum reference versus the corresponding estrone concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3.Connect the points with a best-fit curve.

To determine the concentration of estrone for an unknown, locate the average RLU of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average RLU (41089) intersects the dose response curve at 63.2pg/ml estrone concentration.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population and females during gestation the expected ranges for the Estrone CLIA Test System are detailed in Table 1.

TABLE 1
Expected Values for the Estrone Test System

	Median	Range
Females	-	-
Age 20-49	20	6-400
Age 50-69	10	ND-26
Age 70+	19	ND-104
Male Adults	21	ND-54

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in- house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR FREE TESTOSTERONE ELISA TEST SYSTEM

Indications for Use: The device is an Enzyme Immunoassay (EIA) for the quantitative measurement of free testosterone in human serum. Measurement of free testosterone is used in the diagnosis and treatment of disorders involving the male sex hormones (androgens), including primary and secondary hypogonadism, impotence in males and in females; hirsutism (excessive hair) and virilization (masculinization) due to tumors, polycystic ovaries and adrogenital syndromes.

SUMMARY AND EXPLANATION OF THE TEST

Testosterone, (17β-Hydroxy-4-androstene-3-one), a C steroid, is secreted primarily by the testes, with only a small amount derived from peripheral conversion of 4- Androstene-3, 17-dione (ASD).[2] In adult women, it has been estimated that over 50% of serum testosterone is derived from peripheral

conversion of ASD secreted by the adrenal and ovary, with the remainder from direct secretion of testosterone by these glands.

In the male, testosterone is mainly synthesized in the interstitial Leydig cells and the testis, and is regulated by the interstitial cell stimulating hormone (ICSH), or luteinizing hormone (LH) of the anterior pituitary (the female equivalent of ICSH). Testosterone is responsible for the development of secondary sex characteristics, such as the accessory sex organs, the prostate, seminal vesicles and the growth of facial, pubic and auxiliary hair. Testosterone measurements have been very helpful in evaluating hypogonadal states. Increased testosterone levels in males can be found in complete androgen resistance (testicular feminization). Common causes of decreased testosterone levels in males include: hypogonadism, orchidectomy, estrogen therapy, Klinefelter's syndrome, hypopituitarism, and hepatic cirrhosis.

In the female, testosterone levels are normally found to be much lower than those encountered in the healthy male. Testosterone in the female comes from three sources. It is secreted in small quantities by both the adrenal glands and the ovaries, and in healthy women, 50–60% of the daily testosterone production arises from peripheral metabolism of prohormone, chiefly androstenedione. Common causes of increased serum testosterone levels in females include polycystic ovaries (Stein- Leventhal syndrome), ovarian tumors, adrenal tumors and adrenal hyperplasia. Virilization in women is associated with the administration of androgens and endogenous overproduction of testosterone. There appears to be a correlation between serum testosterone levels and the degree of virilization in women, although approximately 25% of women with varying degrees of virilism have serum testosterone levels that fall within the female reference range.

The majority of testosterone is bound to transport proteins: weakly bound to albumin and cortisol binding protein (25-65% females; 45- 85% males) and tightly bound to sex hormone-binding globulin (SHBG) (35-75% females; 14-50% males). A small fraction exist as unbound or free testosterone; however, this form is biologically active. Therefore, the free hormone concentration is a better indicator of biological activity than total testosterone.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity) $AgAb_{C.W.}$ = Antigen-Antibody Complex

$^{Enz}AgAb_{C.W.}$ = Enzyme-antigen Conjugate -Antibody Complex

ka = Rate Constant of Association

k-a = Rate Constant of Disassociation K = ka / k-a = Equilibrium Constant

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS Materials Provided:

A. Free Testosterone Calibrators

Seven (7) vials of serum reference for Free Testosterone at concentrations of 0 (A), 0.2 (B), 1.0 (C), 2.5 (D), 7.5 (E), 20 (F) and 60 (G) in pg/ml. Store at 2-8°C. A preservative has been added. The calibrators can be expressed in molar concentrations (pM/L) by multiplying by 3.47. For example: 1pg/ml x 3.47 = 3.47 pM/L

B. Free Testosterone Controls

Three (3) vials of serum reference for Free Testosterone at low, middle, and high established concentrations (range values listed on labels). A preservative has been added. Store at 2-8 °C.

C. Free Testosterone Enzyme Reagent

One (1) vial of Testosterone (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with dye. Store at 2-8°C.

D. Free Testosterone Coated Plate

One 96-well microplate coated with testosterone antibody and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate A

One (1) vial contains tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B

One (1) vial contains hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution

One (1) vial contains a strong acid (1N HCl). Store at 2-8°C.

I. Product Instructions

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required but Not Provided:

1. Pipette capable of delivering 0.020 & 0.050ml (20µl & 50µl) volumes with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.

3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.

4. Microplate washer or a squeeze bottle (optional).

5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6. Absorbent Paper for blotting the microplate wells.

7. Plastic wrap or microplate cover for incubation steps.

8. Vacuum aspirator (optional) for wash steps.

9. Timer.

10. Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type, and the usual precautions in the collection of venipuncture samples should be observed. The blood should be collected in a plain redtop

results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubilized binding sites.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

2. Working Substrate Solution - Stable for 1 year.

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use reagents that are contaminated or have bacteria growth.

Note 2: Do not use the working substrate if it looks blue.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20- 27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. **Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**

2. **Pipette 0.020ml (20µL) of the appropriate serum reference, control or specimen into the assigned well.**

3. Add 0.100ml (100µl) of the Free Testosterone Enzyme Reagent to all wells.

4. Swirl the microplate gently for 20-30 seconds to mix.

5. Cover and incubate for 60 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.

10. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Free Testosterone in unknown specimens within the analytical measuring range of 0.11-60 pg/ml.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" adult population, the expected ranges for the Free Testosterone ELISA Test System are detailed in Table 1

Population	Range (in pg/ml)
Male / 20-39	9.2-34.6
Male / 40-59	6.1-30.3
Male / ≥60	6.1-27.9
Female / 20-39	0.2-6.1
Female / 40-59	0.3-4.4
Female / ≥60	0.5-3.4

ENZYME IMMUNOASSAY FOR FREE THYROXIN, FREE TRIIODOTHYRONINE, THYROTROPIN: FREE THYRIOID PANAL

Intended Use: The Quantitative Determination of Free Thyroxine; Free Triiodothyronine; Thyrotropin Concentration for a comprehensive thyroid status of a Human Serum or Plasma sample by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Measurements of thyroid hormones (fT3, fT4 and TSH) are generally regarded as invaluable *in-vitro* diagnostic tests for assessing thyroid function.

The Combination Free Thyroid Panel provides the convenience of combination calibrators, universal plate and flexible reagent selection allowing technicians to perform a variety of assay designs. In this method, serum reference, patient specimen, or control is first added to a microplate well. Enzyme-fT4 (fT3) conjugate and biotinylated fT4 or fT3 antibody are added, and the reactants are mixed. In the case of TSH, the biotinylated and enzyme conjugate are added in

one step. A reaction results between the enzyme conjugate, biotinylated conjugate and the native thyroid hormone (fT3, fT4 or TSH) for the antibody combining sites. Immobilization takes place through the reaction of the incorporated biotin and streptavidin coated on the well. After the completion of the required incubation period, the bound enzyme conjugate is separated from the unbound enzyme conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known thyroid hormone concentration(s) permits construction of a graph of activity and concentration. From comparison to the dose response curve(s), an unknown specimen's activity can be correlated with hormone concentration.

PRINCIPLE

Competitive Enzyme Immunoassay

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate, native antigen and a substrate that produces color.

Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites.

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effect the separation of the antibody bound fraction after decantation or aspiration.

The enzyme activity in the antibody-bound fraction, measured by reaction with TMB, is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody

Upon mixing monoclonal biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, a reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Reagents for 2 X 96 well Microplate, provided

A. Free Thyroid Calibrators

human serum calibrators dispensed in vials with the concentrations as listed in the Table. Store at 2-8°C. A preservative has been added

Exact levels are given on the labels on a lot specific basis

Analyte	fT3 (pg/ml)	fT4 (ng/dl)	TSH (µIU/ml)
A	0	0	0
B	1.3	0.5	0.5
C	3.0	1.2	2.5
D	8.0	2.4	10.0
E	12.0	4.2	20.0
F	22.0	7.6	40.0

B. Strept fT4 Enzyme Reagent

One (1) vial of thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. Strept fT3 Enzyme Reagent

One (1) vial of triiodothyronine -horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C

D. TSH Enzyme Reagent

One (1) vial containing enzyme labeled affinity purified polyclonal goat antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

E. Strept fT4 Biotin Reagent

One (1) vial of biotinylated anti-thyroxine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

F. Strept fT3 Biotin Reagent

One (1) vial of biotinylated anti-triiothyronine (sheep) reagent in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

G. Streptavidin Coated Microplate

Two 96-well microplates coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

H. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

I. Substrate Reagent

Two (2) amber bottles contain tetramethylbenzidine (TMB) and hydrogen peroxide (H 2 O 2) in buffer. Store at 2-8°C.

J. Stop Solution

One (1) vial contains a strong acid (0.5M H 2 SO 4). Store at 2-8°C.

Note 1: TSH concentrations were calibrated using a reference preparation, which was assayed against the WHO 2nd, IRP 80/558

Note 2: Do not use reagents beyond the kit expiration date.

Note 3: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Materials Required but Not Provided:

1.Pipette capable of delivering 0.025ml (25µl) and 0.050ml (50µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate dilutions.

4.Microplate washer or a squeeze bottle (optional).

5.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6.Test tubes for dilution of samples if required.

7.Absorbent Paper for blotting the microplate wells.

8.Plastic wrap or microplate cover for incubation steps.

9.Vacuum aspirator (optional) for wash steps.

10.Timer.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.05ml (50µl) of the specimen is required for fT4 and TSH analysis and 0.10ml (100µl) is required for fT3 analysis.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at 2- 30°C for up to 60 days.

Note: Do not use reagents that are contaminated or have bacteria growth

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well for fT4. Pipette 0.050ml (50µl) for fT3. Pipette 0.025ml (25µl) for TSH.

3. Add 0.050 ml (50µl) of Enzyme Reagent fT4 or fT3 to the appropriate wells. For TSH, add 0.100ml (100µl) of TSH Enzyme Reagent and skip steps 4 and 5.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Add 0.050 ml (50µl) of biotinylated x-fT4 or (x-fT3) reagent to the appropriate wells.

6. Swirl the microplate gently for 20-30 seconds to mix and cover.

7. Incubate 60 minutes at room temperature.

8. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

9. Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2)

additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding

air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

10. Add 0.100 ml (100µl) of substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

11. Incubate at room temperature for fifteen (15) minutes.

12. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

13. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: For reassaying specimens with concentrations greater than highest calibrator, dilute 0.0125ml (12.5µl for fT4 and TSH) or 0.025ml (25µl for fT3) of the specimen and 0.0125ml (12.5µl for fT4and TSH) or 0.025ml (25µl for fT3) of the 0 serum reference into the sample well (this maintains a uniform protein con- centration). Multiply the readout value by 2 to obtain the thyroxine concentration.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroid hormones in unknown specimens.

EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values. The mean (R) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1 for fT4 and Table 2 for fT3. A nonparametric method (95% Percentile Estimate) was used for TSH in Table 3.

TABLE I - Expected Values – (fT4) (in ng/dl)

	Adult	Pregnancy
Mean (X)	1.40	1.50
Std. Dev (σ)	0.3	0.37
Expected Ranges (±2σ)	0.8-2.0	0.76-2.24

TABLE 2 - Expected Values – (fT3) (in pg/ml)

	Adult	Pregnancy
Mean (X)	2.80	3.0
Std. Dev (σ)	0.375	0.6
Expected Ranges (±2σ)	1.4-4.2	1.8-4.2

TABLE 3 - Expected Values – (TSH) (in µIU/ml)

Low Normal Range	0.39
High Normal Range	6.16
70% Confidence Intervals for 2.5 Percentile	
Low Range	0.28-0.53
High Range	5.60-6.82

ENZYME IMMUNOASSAY FOR RAPID THYROXINE TEST SYSTEM

Intended Use: The Quantitative Determination of Total Thyroxine Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum thyroxine concentration is generally re- garded as an important *in-vitro* diagnostic test for assessing thyroid function. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the last three decades. This procedural evolution can be traced from the empirical protein bound iodine (PBI) test to the theoretically sophisticated radioimmunoassay.

This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference calibrator, patient specimen, or control is first added to a microplate well. Enzyme-T4 conjugate is added, and then the reactants are mixed. A competition reaction results between the enzyme conjugate and the native thyroxine for a limited number of antibodies combining sites immobilized on the well.

After the completion of the required incubation period, the antibody bound enzyme-thyroxine conjugate is separated from the unbound enzyme-thyroxine conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum reference calibrators of known thyroxine concentration permits construction of a graph of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with thyroxine concentration.

PRINCIPLE

Competitive Enzyme Immunoassay:

The essential reagents required for a solid phase enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen.

Upon mixing immobilized antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of insolubulized binding sites.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum reference calibrators of

known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

A. T4 Calibrators

Six (6) vials containing serum reference for thyroxine at concentrations of 0 (A), 2.0 (B), 5.0 (C), 10.0 (D), 15.0 (E) and 25.0 (F) µg/dl. A preservative has been added. Store at 2-8°C. For SI units: µg/dl x 12.9 = nmol/L

B. Rapid T4 Enzyme Reagent

One (1) vial containing thyroxine-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix. A preservative has been added. Store at 2-8°C.

C. T3/T4 Conjugate Buffer

One (1) vial containing buffer, dye, preservative, and binding protein inhibitors. Store at 2-8°C.

D. T4 Antibody Coated Plate

One 96-well microplate coated with sheep anti-thyroxine serum and packaged in an aluminium bag with a drying agent. Store at 2-8°C.

E. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate A

One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

G. Substrate B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

H. Stop Solution

One (1) vial containing a strong acid (1.0N HCl). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate

Materials Required but Not Provided:

1. Pipette capable of delivering 0.025 and 0.050ml (25 & 50µl) volumes with a precision of better than 1.5%.

2. Dispenser(s) for repetitive deliveries of 0.100 and 0.350ml (100 & 350 µl) volumes with a precision of better than 1.5%.

3. Adjustable volume (20-200µl) and (200-1000µl) dispenser(s) for conjugate and substrate preparation

4. Microplate washer or a squeeze bottle (optional).

5. Microplate Reader with 450nm and 620nm wavelength absorbance capability.

6. Test tubes for preparation of enzyme conjugate.

7. Absorbent Paper for blotting the microplate wells.

8. Plastic wrap or microplate cover for incubation steps.

9. Vacuum aspirator (optional) for wash steps.

10.Timer.

11.Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum or plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample

should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8 oC for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20 oC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.040ml (40µl) of the specimen is required.

REAGENT PREPARATION

1. Working Reagent A = T4-Enzyme Conjugate Solution

Dilute the Rapid T4 Enzyme Reagent 1:11 with Total T3/T4 conjugate buffer in a suitable container. For example, dilute 160µl of reagent with 1.6ml of buffer for 16 wells. (A slight excess of solution is made.) This working reagent should be used within twenty-four hours for maximum performance of the assay. Store at 2-8°C.

General Formula:

Amount of Buffer required = Number of wells ∗ 0.1

Quantity of Rapid T4 Enzyme necessary = # of wells ∗ 0.01

i.e. = 16 x 0.1 = 1.6ml for Total T3/T4 buffer

16 x 0.01 = 0.16ml (160µl) for Rapid T4 Enzyme

2. Wash Buffer

Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.

3. Working Substrate Solution – Stable for one (1) year

Pour the contents of the amber vial labelled Solution 'A' into the clear vial labelled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplate's wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminium bag, seal and store at 2-8°C.

2. Pipette 0.020 ml (20 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3. Add 0.100 ml (100 µl) of Working Reagent A (T4 Enzyme Conjugate Solution) to all wells (see Reagent Preparation Section).

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 30 minutes at room temperature.

6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

7. Add 0.350ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.100 ml (100 µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9. Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050 ml (50 µl) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: For reassaying specimens with concentrations greater than 25 µg/dl, pipet 12.5µl of the specimen and 12.5µl of the 0-serum reference calibrator into the sample well (this maintains a uniform protein concentration). Multiply the readout value by 2 to obtain the thyroxine concentration.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader.

2. Plot the absorbance for each duplicate serum reference calibrator versus the corresponding T4 concentration in µg/dl on linear graph paper (do not average the duplicates of the serum reference calibrators before plotting).

3. Connect the points with a best-fit curve.

4. To determine the concentration of T4 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in µg/dl) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.135) intersects the standard curve at 6.8 µg/dl T4 concentration

EXPECTED RANGES OF VALUES

A study of euthyroid adult population was undertaken to determine expected values for the Rapid T4 AccuBind® ELISA Test System. The mean (X) values, standard deviations (σ) and expected ranges (±2σ) are presented in Table 1.

TABLE 1
Expected Values for the T4 ELISA Test System (in µg/dl)

	Male	Female *
Number of Specimens	42	58
Mean (X)	7.6	8.2
Std. Dev (σ)	1.6	1.7
Expected Ranges (±2 σ)	4.4 – 10.8	4.8 – 11.6

*Normal patients with high TBG levels were **not** excluded except if pregnant.

It is important to keep in mind that establishment of a range of values that can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR PARATHYROID HORMONE, WHOLE AND INTACT TEST SYSTEM

Intended Use: The Quantitative Determination of Intact PTH (2nd Generation) and Whole PTH (3rd Generation) Concentration in Human Serum or Plasma by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Parathyroid hormone (PTH) is a polypeptide composed of 84- amino acids and vital to calcium homeostasis regulating blood serum calcium (Ca^{2+}) in concert with Vitamin D and Calcitonin. Secreted by the parathyroid gland in response to low Ca^{2+}, PTH stimulates calcium release in the bone marrow, production in the intestines and kidney and minimizes urinary excretion. Meanwhile calcitonin has the opposing effect to increase urinary excretion and reduce blood calcium when Ca^{2+} is at elevated levels.

Intact PTH clears quickly from the bloodstream with half-life of less than four minutes. Detecting elevated PTH levels is imperative in monitoring bone metabolism especially in the presence of hypercalcemia, which virtually makes the primary diagnosis of hyperparathyroidism, as the vast majority (>90%) of such patients have elevated PTH. Differentiation from other forms of (non-parathyroid-mediated) hypercalcemia such as malignancy (the second most

common cause), sarcoidosis, and thyroid toxicosis are associated with suppressed levels of parathyroid hormone or PTH in normal range. In cases of hypocalcemia, PTH levels may not be detectable due to total hypoparathyroidism but are found in normal range in hypocalcemia due to partial loss or inhibition of parathyroid function. Clinical significance of parathyroid hormone has increased in conjunction with the etiology of hypocalcemia and hypercalcemia. Initial studies revealed parathyroid hormone is synthesized as a prohormone followed by significant cleavage and modification, with these fragments comprising the majority of circulating parathyroid hormone. However, PTH fragments lack biological activity, and intact PTH (IPTH) spanning residues 1-84 is responsible for calcium regulation. The N-terminus of PTH is necessary in receptor docking, while the C-terminal residues are responsible for PTH receptor activation. Thus, separation of whole parathyroid hormone from fragmented peptides is integral in osteometabolic analysis.

Most of the intact PTH assays are 2nd Generation, which react with the PTH fragment (7-84) that is not biologically active.

PTH 3rd Generation assays do not react with the PTH fragment (7- 84). This fragment is secreted in significant concentrations in uremic patients.

PRINCIPLE

Sandwich Equilibrium Method:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well

through the interaction of x-PTH antibody (C terminal epitope) coated on the well.

Upon mixing the enzyme-labeled antibody (N-terminal epitope) and a serum containing the native antigen, a reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a sandwich complex. After sufficient time results, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A-PTH Calibrators

Six (6) vials of references for PTH at levels of 0(A), 15(B), 75(C), 150(D), 500(E) and 1000(F) pg/ml. Store at 2-8 °C.*Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted calibrators are stable for 24-48 hours at 2-8 °C. To store for a longer period, aliquot the reconstituted calibrators into cryo vials and store at -20 °C. *DO NOT FREEZE/ THAW MORE THAN TWICE.* A preservative has been added.

Note: The calibrators, human serum based, are traceable to the WHO 1st IS standard 95/646.

B-PTH Controls

Two (2) vials of reference controls for PTH. Store at 2-8 °C. *Reconstitute each vial with 1.0ml of distilled or deionized water.* The reconstituted controls are stable for 24-48 hours at 2-8 °C. To store for a longer period, aliquot the reconstituted calibrators into cryo vials and store at -20°C. *DO NOT FREEZE/ THAW MORE THAN TWICE.* A preservative has been added.

C-PTH Enzyme Reagent 2nd Gen

One (1) vial contains anti-PTH conjugate reagent. Store at 2-8°C.

D-PTH Enzyme Reagent 3rd Gen

One (1) vial contains anti-PTH conjugate reagent. Store at 2-8°C.

E-PTH Antibody Coated Plate

One 96-well microplate coated with x-PTH antibody. Store at 2-8 °C.

F-Wash Solution Concentrate (20x)

One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2-8 °C. *See Reagent Preparation section.*

G-Substrate Reagent

One (1) vial contains tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8 °C.

H-Stop Solution

One (1) vial contains a strong acid (H_2SO_4). Store at 2-8 °C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Do not expose reagents to heat, sun, or strong light.

Note 3: The above components are for one 96-well microplate.

Materials Required but Not Provided:

1-Pipette capable of delivering 0.050ml (50µl) and 0.100ml (100µl) volumes with a precision of better than 1.5%.

2-Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3-Microplate washers or a squeeze bottle (optional).

4-Microplate Reader with 450nm and 620nm wavelength absorbance capability.

5-Absorbent Paper for blotting the microplate wells.

6-Plastic wrap or microplate cover for incubation steps.

7-Vacuum aspirator (optional) for wash steps.

8-Timer.

9-Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum or EDTA plasma in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants for serum or EDTA containing tubes for plasma. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

If the specimen(s) cannot be assayed immediately after blood withdrawal, the sample(s) may be stored at temperatures of -20 °C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing (a maximum of two freeze/thaw cycles prior to use). When assayed in duplicate, 0.100 ml (100 µl) of the specimen is required.

REAGENT PREPARATION

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

Note: Do not use reagents that are contaminated or have bacterial growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1-Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8 °C.

2-Pipette 0.050 ml (50 µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

FOR Generation 2

3-Add 0.050 ml (50 µl) of the x-PTH Enzyme Reagent 2nd Gen to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4-Swirl the microplate gently for 20-30 seconds, cover and incubate for 60 minutes at room temperature. OR Generation 3

5-Add 0.050 ml (50 µl) of the x-PTH Enzyme Reagent 3rd Gen to each well. It is very important to dispense all reagents close to the bottom of the coated well.

6-Swirl the microplate gently for 20-30 seconds, cover and incubate for 75 minutes (1hr 15min) at room temperature using a plate shaker at 150-200 rpm or for two(2) hours without shaking.

7-Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

8-Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

9-Add 0.100 ml (100 µl) of Substrate Reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION

10-Incubate at room temperature for twenty (20) minutes.

11-Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

12-Read the absorbance in each well at 450nm (using a reference wavelength of 630nm to minimize well imperfections) in a microplate reader. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: For re-assaying specimens with concentrations greater than 1000 pg/ml, dilution should be performed in human serum or plasma with low PTH values and multiplied accordingly.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of PTH in unknown specimens.

1-Plot the absorbance for each duplicate serum reference versus the corresponding PTH concentration in pg/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

2-Draw the best-fit curve through the plotted points.

3-To determine the concentration of PTH for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml)

from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example for PTH 2nd Generation, the average absorbance (1.800) intersects the dose response curve at 419 pg/ml PTH concentration (See Figure 1). For PTH 3rd Generation, the average absorbance (0.265) intersects the dose response curve at 78.5 pg/ml PTH concentration.

EXPECTED RANGES OF VALUES

Intact PTH levels were measured in fifty-eight (58) apparently normal individuals. The values obtained ranged from 9.0 to 94in histograms. The geometric mean ± 2 standard deviations of the mean were calculated to be 10.4 to 66.5 pg/ml.

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

SECTION 2: CLINICAL PATHOLOGY

FECAL ANALYSIS METHODS

COLLECTING A STOOL SAMPLE

A stool test involves the collection and analysis of fecal matter to diagnose the presence or absence of a medical condition. IN other words a stool analysis is a series of tests done on a stool (feces) sample to help diagnose certain conditions affecting the digestive tract. These conditions can include infection (such as from parasites, viruses, or bacteria), poor nutrient absorption, or cancer.

The stool culture is a test that allows the detection and identification of pathogenic bacteria in the stool. In the laboratory, a small amount of a fresh fecal sample is applied to a variety of nutrient media (thin layers of gelatin like material in sterile covered plastic dishes). These media are selective, each encourages the growth of some bacteria and discourages the growth of others. Once inoculated, the media are incubated.

Fecal specimen may be used for examination the presence of blood in stool. One of the most common stool tests, the fecal occult blood test (refers to blood in the feces that is not visibly apparent, unlike other types of blood in stool such as melena or hematochezia) can be used to diagnose many conditions that cause bleeding in the gastrointestinal system including colorectal cancer or stomach cancer.

Parasitic diseases such as ascariasis, hookworm, strongyloidiasis and whipworm can be diagnosed by examining stools under a microscope for the presence of worm larvae or eggs. Some bacterial diseases can be detected with a stool culture. Toxins from bacteria such as *Clostridium difficile* ('C. diff.') can also be identified. Viruses such as rotavirus can also be found in stools.

A fecal pH test may be used to determine lactose intolerance or the presence of an infection. Steatorrhea can be diagnosed using a Fecal fat test that checks for the malabsorption of fat. Fecal elastase levels are becoming the mainstay of pancreatitis diagnosis.

To collect a stool sample:

- **label the container with your name, date of birth and the date**
- **place something in the toilet to catch the stool, such as a potty or an empty plastic food container, or spread clean newspaper or plastic wrap over the rim of the toilet**
- **make sure the sample doesn't touch the inside of the toilet**
- **use the spoon or spatula that comes with the container to place the sample in a clean screw-top container and screw the lid shut**
- **if you've been given a container, aim to fill around a third of it – that's about the size of a walnut if you're using your own container**
- **put anything you used to collect the sample in a plastic bag, tie it up and put it the bin**
- **wash your hands thoroughly with soap and warm running water**

Storing a stool sample

Stool samples should be handed in as soon as possible, as some can't be analyzed if they've been refrigerated – your doctor will tell you if this is the case.

If you can't hand the stool sample in immediately, you should store it in a fridge, but for no longer than 24 hours. Place the container in a sealed plastic bag first.

Stool samples must be fresh – if they aren't, the bacteria in them can multiply. This means the levels of bacteria in the stool sample won't be the same as the

levels of bacteria in your digestive system. If the levels of bacteria don't match, the test results may not be accurate.

FECAL GROSS EXAMINATION

The gross appearance of the feces provides some clues to possible GI disorders. The consistency, whether formed, hardened, or liquid, and color change from the normal dark brown alert the healthcare provider to abnormalities. Black color may indicate older blood from the upper GI tract, whereas bright red blood is more likely to be from the lower GI tract. Bright red blood in feces is known as hematochezia. A very pale stool (called an acholic stool) often indicates a biliary obstruction. A ribbonlike fecal specimen could be associated with GI tract obstruction. The normal fecal specimen is dark brown; due to the oxidation of urobilinogen in the intestines, the color changes to orange brown urobilin. The presence of blood-streaked mucus or mucus with pus or eosinophils often accompanies bacterial or amebic dysentery. Dysentery is associated with damage to the intestinal wall due to invasion by these organisms.

Appearance of fecal specimens in some diseases

Appearance	***Possible Cause***
Unformed, containing pus	● **Shigellosis**
and mucus mixed with blood	● **E1EC dysentery**
	● ***Campylobacter*** **enteritis**
Unformed with blood and	● **Amoebic dysentery**
mucus (acid pH)	
Unformed or semiformed	● **Schistosomiasis**
often with blood and mucus	

Bloody diarrhea (without pus cells) (hemorrhagic colitis)	**● EHEC 0157 infection**
Watery stools	**● ETEC, EPEC diarrhea** **● Cryptosporidiosis** **● Rotavirus enteritis**
Rice water stools with mucous flakes	**● Cholera**
Unformed or watery and sometimes with blood, mucus, and pus	**● *Salmonella* infection**
Unformed, pale colored, frothy, unpleasant smelling stools that float on water (high fat content) e.g. post-infective tropical malabsorption	**● Giardiasis** **● Other conditions that cause malabsorption,**
Fluid stools (containing lactose with pH below 6)	**● Lactase deficiency**
Unformed or semiformed black stools (positive occult blood)	**● Melaena (gastrointestinal bleeding)** **● Hookworm disease** **● Iron therapy**

MICROSCOPIC EXAMINATION OF FECAL SPECIEMENS

Fecal leukocytes

Fecal leukocytes, especially neutrophils, are commonly associated with dysentery or invasion of the intestinal wall. In amebic infections, eosinophils are also often present. Wet preparations made with methylene blue are used to detect fecal leukocytes or alternatively, dried smears of the stool sample can be stained with Gram stain or Wright stain to examine for leukocytes. The Wright stain improves differentiation of these cells. The presence of even a few leukocytes can be indicative of an invasive condition. Another type of test that can be used to detect fecal leukocytes is a latex agglutination test for lactoferrin, an enzyme found in the granules of granulocytes, also indicative of fecal leukocytes.

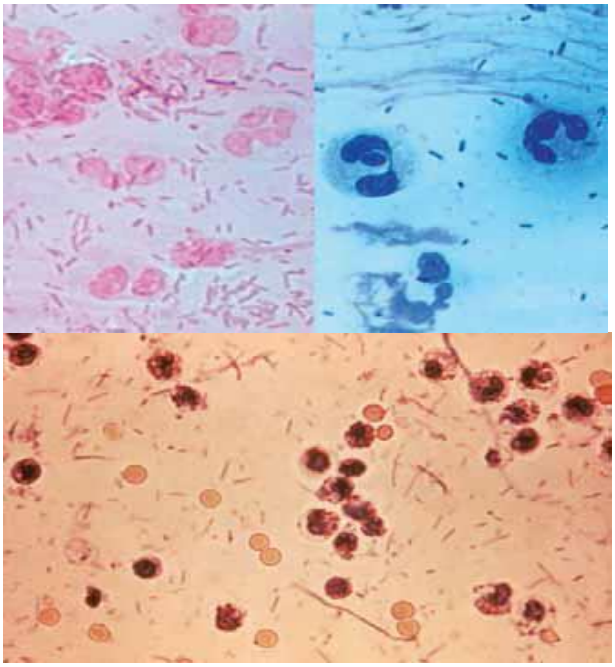


Figure. Fecal leukocytes. A. Gram stain, 1000X, fecal leukocytes. B. Wright stain, 1000X, fecal leukocytes. C. Wet preparation, Shigella dysentery or bacterial dysentery with fecal white blood cells, red blood cells, and bacteria.

Methylene blue preparation to detect fecal leucocytes when the specimen is unformed

– Place a drop of methylene blue stain on a slide. Mix a small amount of specimen with the stain, and cover with a cover glass.

– Examine the preparation for fecal leucocytes using the 40X objective with the condenser iris closed sufficiently to give good contrast.

– Report also the presence of red blood cells (RBC) as these are often present with pus cells in inflammatory invasive diarrheal disease (see following text).

Fecal leucocytes (WBCs): **Look for mononuclear cells and polymorphonuclear cells (pus cells). Mononuclear cells contain a nucleus which is not lobed whereas polymorphonuclear cells contain a nucleus which has two or more lobes. Sometimes the cells are too damaged to be recognized (do not attempt to identify).**

Pus cells are associated with bacteria that cause inflammation of the large intestine. Often red cells are also found. Mononuclear cells are found mainly in typhoid and in some parasitic infections, including amoebic dysentery.

Fecal Fat Testing

Both qualitative and quantitative fecal fat analyses are available.

- ### Qualitative method

Neutral fats are detected qualitatively by staining with Sudan III in 95% ethanol in a wet preparation and microscopic observation for the number and size of fat globules. A normal stool will have less than 60 medium or small-sized orange-red fat globules/high power field. Soaps and fatty acids do not stain directly with the Sudan III stain and the same wet preparation must also have acetic acid and be heated prior to staining and reading microscopically. Normal samples will have less than 100 orange-red fat globules/ high power field and they should not exceed 4 μm.

- ### Quantitative method

If qualitative fecal fat testing is positive, confirmatory quantitative fecal fat analysis is performed. Generally, this is performed in the chemistry department and it is often a reference test. For accurate testing, the patient must maintain a 100 g/day fat intake both before and during specimen collection. Forty-eight- to seventy-two-hour fecal collections are necessary for these tests. Fecal fat analysis can be performed by the Van de Kramer titration, by the acid steatocrit, or by near-infrared spectroscopy.

Meat Fibers Presence

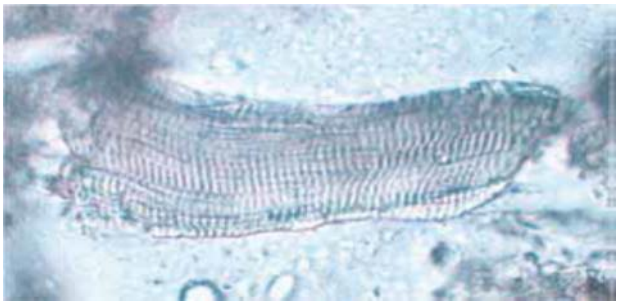


Figure. Undigested meat fiber. Note the clearly defined muscle striations.

Meat fiber examination may be done together with microscopic fecal fat analysis. Eosin in 10% ethanol can be used to assist in identifying these meat fibers with striations in a microscopic wet preparation.

FECAL OCCULT BLOOD TEST

The fecal occult blood test (FOBT) is a simple, inexpensive and most frequently performed chemical screening test on feces. It is done for the detection of blood in the stool that is not visible on gross inspection. When only small amounts of blood being passed in the feces, the blood (or its breakdown products) is not recognized and is referred to as occult (hidden) blood. The amount is usually less than 50 mg of hemoglobin per gram of stool.

CLINICAL SIGNIFICANCE OF FAECAL OCCULT BLOOD TEST

An average, healthy adults usually passes up to 2 ml of blood per 150 gm of stool (2 to 3 mg hemoglobin per gram of stool) into the GI tract daily. Passage of more than 2 ml of blood in the stool in 24 hr. is pathologically significant. The increased amounts are associated with a variety of benign and malignant gastrointestinal diseases, especially colon cancers, blood loss anemia, hookworm infestation, polyps, colitis, diverticulitis, and fissures.

METHODS FOR FAECAL OCCULT BLOOD TEST

There are following methods in clinical use for testing for occult blood in feces.

1- Chemical Methods using guaiac-based reagents prepared in the laboratory, e.g. amino phenazone test, or ready-made reagent in kit tests

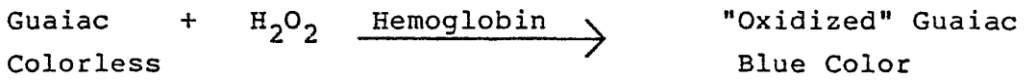
2-Immunochemical methods using a hemoglobin specific cassette or strip test.

3-Other Methods

CHEMICAL METHODS FOR FAECAL OCCULT BLOOD TEST

These are the traditional methods using guaiac-based reagents prepared in the laboratory, e.g. amino phenazone test, or ready-made reagent in kit tests. The conventional tube tests have been replaced by kit method which are very easy to perform and are inexpensive.

Principle: The principle of chemical tests to detect occult blood is based on the fact that hemoglobin and its derivatives react in a similar way to peroxidase enzymes– by catalyzing the transfer of an oxygen atom from the peroxide to a chromogen such as benzidine, o-toluidine, guaiac or amino phenazone. Oxidation of the chromogen is indicated by the production of a blue, blue-green or pink color. A simplified reaction equation is shown below:



Occult-blood-test-principle

Patient Preparation:

Any source of blood will give a positive test. If possible, the patient instructions should follow at least 7 days prior to the test and should continue through the test period. Patient instructions include both drug and diet guidelines.

The test is performed on a paper slide that contains paper squares coated with guaiac, a chemical derived from tree resin. A small portion of stool(fecal) specimen is applied to the paper. A developer solution containing hydrogen peroxide (H_2O_2) is added to the paper. If the blood is present in the specimen, the iron (Fe) in the hemoglobin catalysis the reaction between guaiac in the paper and the H_2O_2. The completed reaction forms a blue color.

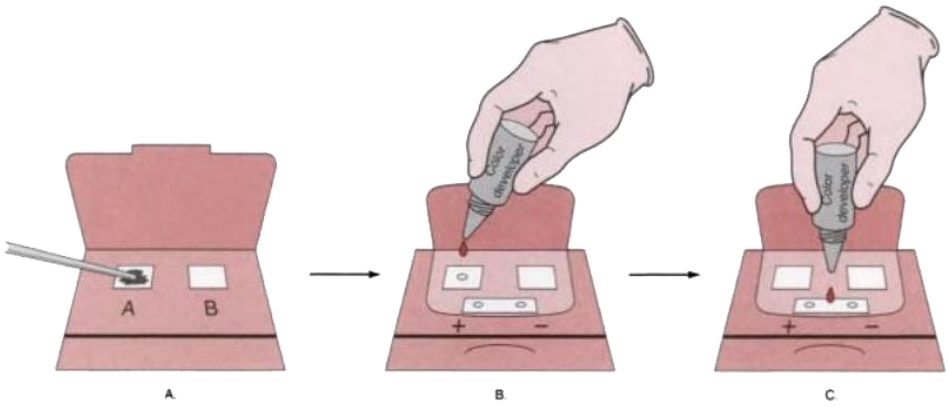


Fig. Fecal-occult-blood-test

IMMUNOCHEMICAL METHODS FOR FAECAL OCCULT BLOOD TEST

Modern fecal occult blood testing is moving to an immunochemical test which is specific for human hemoglobin.

Principle: This test utilizes a qualitative, sandwich dye conjugate immunoassay to selectively identify the globulin component of human hemoglobin in fecal specimens. The immunoassay uses a combination of monoclonal and polyclonal antibodies, utilizes an immunochemical chromatographic method for detection and has a high degree of analytical sensitivity.

Patient Preparation:

Because this test is specific for human blood, no special drug or dietary restrictions are required. However, patients should not collect samples three days before, during or three days after their menstrual period, if they have bleeding hemorrhoids, blood in their urine, open cuts on their hands.

Procedure:

In these types of test, a sample of the patient's stool is placed on a special collection card and returned to the lab. The portion of the collection card containing the patient sample is removed from the collection card, and the sample is mixed with buffer. The buffer solution is then introduced into a test device which contains polyclonal antibodies. The buffer solution will migrate through the test device for a specific amount of time, usually 5 minutes, and a colored line will develop at the "T" if the test sample contains human hemoglobin.

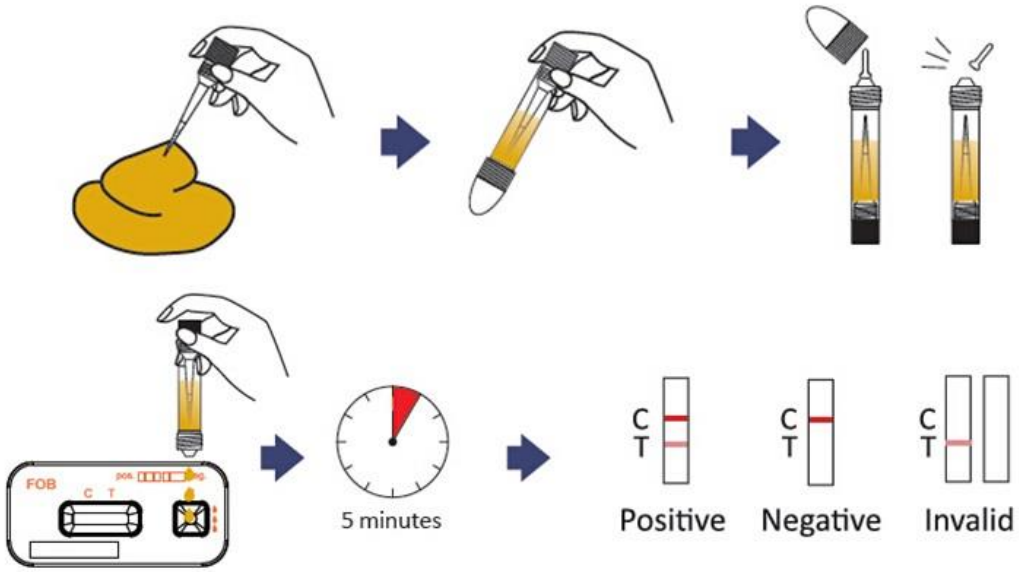


Fig. Immunochemical-fecal-occult-blood-test

OTHER METHODS FOR FAECAL OCCULT BLOOD TEST

Some other methods like over-the-counter (OTC) flushable reagent pad/tissue method are also used which also produces a color change in the presence of blood.

A new, promising, and almost quantitative approach is to measure fecal hemes by the specific fluorescence of their porphyrin derivatives, after extraction of the porphyrins from the specimen to remove interfering substances.

INTERPRETATION OF FECAL OCCULT BLOOD TEST

Positive test result indicates that abnormal bleeding is occurring somewhere in the digestive tract. The commonest causes of positive occult blood tests in tropical and other developing countries are hookworm infection, peptic ulcer, colitis and bleeding from esophageal varices due to cirrhosis of the liver. Other causes include carcinoma in the gastrointestinal tract, erosive gastritis due to alcohol or drugs or inflammatory bowel disease.

FETAL HEMOGLOBIN (APT TEST)

This test determines whether the hemoglobin present is hemoglobin A (maternal) or hemoglobin F (fetal) in origin. The stool or vomitus is mixed with water to yield a pink supernatant. The supernatant is removed and then alkalinized with dilute sodium hydroxide. If the pink color remains after adding the alkali, the blood contains fetal hemoglobin. If the pink color changes to yellow or brown within 2 minutes, the hemoglobin in the sample is maternal hemoglobin.

FECAL CARBOHYDRATES IN MALABSORPTION

Fecal carbohydrate testing is most useful in infant diarrhea to assess fecal diarrhea and inflammatory necrotizing enterocolitis. The copper reduction test (generally performed as the Clinitest is a reagent tablet based on the Benedict's copper reduction reaction, combining reactive ingredients with an integral heat generating system. The test is used to determine the amount of reducing substances (generally glucose) in urine) is used to detect the significant reducing sugars. If this test is positive in the Clinitest, the infant may be tested by other more specific serum tests for carbohydrate tolerance. If carbohydrates are not reabsorbed, the pH of the feces decreases from a normal fairly neutral pH of between 7 and 8 to a pH below 5. Fecal pH is also usually tested using pH paper along with the copper reduction test.

EXAMINATION OF URINE SPECIMENS

COLLECTION AND TRANSPORT OF URINE

Whenever possible, the first urine passed by the patient at the beginning of the day should be sent for examination. This specimen is the most concentrated and therefore the most suitable for culture, microscopy, and biochemical analysis. Midstream urine (MSU) for microbiological examination is collected as follows:

1- Give the patient a sterile, dry, wide-necked, leakproof container and request a 10–20 ml specimen.

***Important*: Explain to the patient the need to collect the urine with as little contamination as possible, i.e. a 'clean-catch' specimen.**

***Female patients*: Wash the hands. Cleanse the area around the urethral opening with clean water, dry the area with a sterile gauze pad, and collect the urine with the labia held apart.**

***Male patients*: Wash the hands before collecting a specimen (middle of the urine flow).**

***Note*: When a patient is in renal failure or a young child, it may not be possible to obtain more than a few milliliters of urine.**

2- Label the container with the date, the name and number of the patient, and the *time* of collection. *As soon as possible*, deliver the specimen with a request form to the laboratory. When immediate delivery to the laboratory is not possible, refrigerate the urine at 4–6 °C. When a delay in delivery of more than 2 hours is anticipated, add boric acid preservative to the urine. Specimens containing boric acid need not be refrigerated.

Deterioration of urine

The following changes occur when unpreserved urine is left at room temperature:

● **Any bacteria in the urine will multiply so that the bacterial count will be unreliable. When the organisms are urease-producing, the ammonia released will increase the pH of the specimen which will result in the destruction of cells and casts. Bacteria will also break down any glucose which may be present.**

● **When white cells, red cells, and casts are present, these will begin to lyze especially in a concentrated specimen.**

● **The concentration of protein in the urine will be altered. When bilirubin is present this may be oxidized to biliverdin which will not be detected. Likewise, urobilinogen will not be detected because it will be oxidized to urobilin.**

Boric acid preservative

At a concentration of 10 g/l (1% w/v), bacteria remain viable without multiplying. White cells, red cells, and casts are also well preserved, and there is no interference in the measurement of urinary protein and glucose. Boric acid has been shown to be inhibitory to some enterococci and *Pseudomonas* strains.

***Important*: Urine for culture must not be preserved with a bactericidal chemical such as thymol, bleach, hydrochloric acid, acetic acid, or chloroform.**

URINE EXAMINATION-COMPLETE AUTOMATED METHOD

Purpose: For qualitative identification and semi-quantitative estimation of color, Ph, specific gravity, protein, glucose, ketone, bilirubin, blood, urobilinogen, nitrites, leukocytes and erythrocytes in urine by automated method using the principle of reflectance photometry. The nature and amount of present in urine reflect ongoing physiological process in health and disease status.

Principle: This instrument has an optical system of different wave lengths and a light emitting diode for each of the test areas detects the color change. When the urine is allowed to react with test areas, a color change occurs in the test area which is detected and measured by the optical system and LED. The specific wave lengths for the evaluation of the reacted test areas are:

- **pH**

This test is based on the double indicator principle that gives a broad range of colors covering the entire urinary pH range. The test strip contains combination of methyl-red (pH range 4.4-6.2.) and bromothymol-blue (Ph range 8.0-9.6) as indicators. The color gradations extend form orang green to blue. Depending upon the pH of urine the color of the face area changes from orange/yellow/green/blue.

- **SPECIFIC GRAVITY**

In the presence of an indicator the polyelectrolyte present in urine give colors ranging from deep blue green in urine of low ionic through green to yellow green in urine of increasing ionic concentration. The test zone of the test strip is provided with an (Polyelectrolyte) and a pH indicator (Bromothymol Blue). The test based on the pKa change of pretreated poly electrolytes in the relation to ionic concentration of urine. The color of the test area ranges from deep blue-green to yellow-orange depending upon the iconic concentration /SG of urine.

- **TOTAL PROTEIN**

It is based on the protein error of a pH indicator. At a constant Ph color change that happens to an indicator is due to protein. The test and area of the reagent strip is impregnated with an indicator, tetrabromophenol blue, buffered to acidic pH 3.0. At this Ph it is yellow in the absence of protein. In the presence of protein, it forms a complex with the dye turning the dye from yellow (no protein) to light green, bluish green, blue (presence of protein) depending upon the concentration of protein in the urine.

- **Glucose**

The test area is impregnated with glucose- oxidase / peroxidase together with potassium iodide and a blue background dye. The oxygen liberated in the final reaction binds with the dye to produce a series of color changes 30 seconds after wetting, the strip with urine. Depending upon the concentration of glucose in urine the color of test area changes from blue (no glucose) to green, yellow, orange or red (presence of glucose).

- **Ketone**

The test area contains sodium nitroprusside and glycine and a buffer. Acetoacetic acid and acetone react with sodium nitroprusside and glycine in an alkaline medium to give violet dye complex (legals test). This test is more sensitive to acetoacetic acid than acetone. It does not react with α hydroxybutyric acid. L-Dopa may give a false positive result.

- **Bilirubin**

The test for bilirubin is based on the coupling of bilirubin with a stable diazonium salt (2-4 dichloroanilinediazonum) in the acid environment of the test area of the strip. The product is a red violet azo dye that effects a color change from buff to tan/tannish purple/pink/ violet.

- **Urobilinogen**

A stable diazonium salt in dimethyl aminobenzaldehyde reacts immediately with urobilinogen in the strong acid environment of the test to effect a change from orange to brown/red azo dye. The intensity of the brown/red color produced is a measure of the concentration of the urobilinogen present.

- **Nitrite**

The sulfanilamide contained in the rest area reacts in presence of an acidic buffer with nitrite to form a diazonium compound. Together with a coupling component, this diazonium compound produce a red azo dye.

- **Leukocyte**

The test is for granulocytic leukocytes. The test area for granulocytic leukocytes contains an indoxylcarboxylic ester and a buffer ester, which is hydrolyzed by granulocytic esterase. The indoxyl ester thus liberated then reacts with a diazonium salt to produce a blue- purple color.

- **Blood (erythrocytes, hemoglobin)**

The test is based on the peroxidase present in hemoglobin and myoglobin catalyze the oxidation of a color indicator 3, 5,3´5´ Tetra methyl Benzenidine by an organic hydroperoxide to a blue green dye that appears green on the original yellow test area of the strip.

PERFORMANCE SPECIACATIONS

- **pH**

• The pH test area permits quantitative differentiation of pH value to one unit within the range of 5-9. pH rending is not affected variation in the urinary butter concentration

• If pooper procedure is not followed and excess urine remain on the strip, a phenomenon Known "running over" may occur, in which acid buffer from the protein reagent area run onto pH area, causing a false lowering in the pH result.

- **Specific Gravity**

• The specific gravity test permits determination of urine specific gravity between 1.000 and 1.030. In general, the specific gravity test correlates within 0.005 with values obtained with the reflective index method.

• Highly buffered alkaline urine may cause low readings relative to the methods.

• Elevated specific gravity readings may be obtained in the presence of moderate quantities (100-750 mg) of protein.

• The chemical nature of the specific gravity test may cause slightly different results from those obtained with the specific gravity methods when elevated amounts of certain urine constituents are present.

- **Protein**

• The test area is sensitive to 15-30 mg albumin/dl urine

• A color matching any color block greater than trace indicates significant proteinuria

• The test area is more sensitive to albumin than to globulin, hemoglobin, Bence-Jones protein, and mucoprotein, therefore a negative result not rule out the presence of these other proteins

• For urine with high specific gravity, the test area may most match the trace color block even though only normal concentrations of protein are present.

- **Glucose**

• The measurement range for glucose is 75-125 mg of glucose/ dl of urine and is specific for glucose.

• Presence of ascorbic acid in urine (most likely to be present in large amounts in the urine of pregnant women and those taking multivitamin medications) will cause a false-positive result.

• This test does not detect other reducing sugars, fructose, galactose, etc. and other non-reducing substances in the urine as it is specific for glucose.

- **Ketones**

• The minimum detection limit of ketones by this method is 5-10 mg/dl for Acetoacetic acid and 40-70 mg/dl for acetone and the maximum detection limit is 100 mg of acetoacetic acid/dl of urine.

• False positive results occur in patients receiving Levodopa or with urine staining MESNA or large amounts of phenylketones

• This test does not detect the ketone body, beta hydroxyl butyric acid

- **Bilirubin**

• The test has a sensitivity of 0.4-0.8 mg/dl of bilirubin in urine and is specific for bilirubin only

• False positive results may obtain with the urine of patients receiving large doses of Chlorpromazine.

• False negative results will be obtained when urine contains large amount of Ascorbic acid and Nitrite and when bilirubin is oxidized to Biliverdin.

- **Urobilinogen**

• This minimum detection limit of urobilinogen is 0.2 EU/dl in urine.

• The absence of urobilinogen cannot be determined with this test.

• The test area will react with interfering substances known to react with Ehrlich's reagent, such as porphobilinogen and *p*-aminosalicylic acid.

• This test is not a reliable method for the detection of porphobilinogen

• Drugs containing azo-dyes (e.g. Azo Gantrisin) may give a masking golden color.

- **Nitrites**

• This test is specific for nitrite and will not react with substances normally excreted in the urine

• This test has sensitivity to sodium nitrite of 0.075 mg/dl. Comparison of the reacted reagent area on a white background may aid in the detection of low levels of nitrite ion, which may otherwise be missed.

• The pink color is not quantitative in relation to the number of bacteria present. Any degree of pink coloration should be interpreted as a positive nitrite test suggestive of 10^5 or more organisms/mL. If the infection is caused by bacteria which do not contain reductase (to convert nitrate to nitrite) the test results will be negative although bacteria are present

• Morning specimen of urine is preferred for Nitrite test as only an incubated sample of urine will contain high bacterial content.

- **Occult Blood**

• The test has a sensitivity to free hemoglobin of 0.015. intact red cells/ul urine. The minimum detection limit for RRC is about 5 RBCs/ul and free Hb equivalent to 10 RBCs/ul urine

• This test is slightly more sensitive to free hemoglobin and myoglobin than to intact erythrocytes.

• False positive results may be obtained in the presence of hypochlorite or when the urine has high bacterial count (as in urinary tract infection) as bacteria contain peroxidase

• The sensitivity of the blood test is reduced in urine with high specific gravity and/or high ascorbic acid content.

- **Leukocytes**

• This test can detect as low as 10-15 WBC/mL, and will not react with erythrocytes or bacteria common in urine.

• Highly colored urine and the presence of the drugs cephalexin and gentamicin interfere with the test results.

• High urinary protein of 500 mg/dl or above diminishes the intensity of the reaction color.

• Elevated glucose concentration or high specific gravity may cause decreased results.

PRIMARY SAMPLE

• Instruct patients to collect a minimum 15 mL of a random sample of urine in a clean dry container (preferably collected from the laboratory).

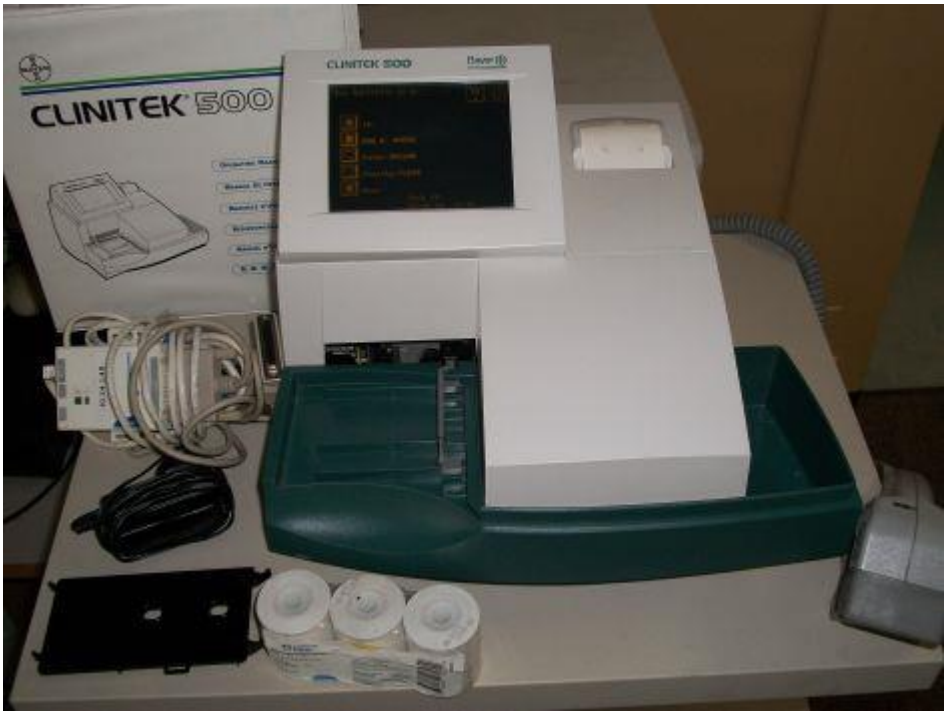
• Instruct patients to void directly into the container and while collecting allow the first portion of urine to escape.

• Use fresh well mixed uncentrifuged urine as specimen for the Process the samples within 1 hour of collection. If delay is an instruct patients to store the sample at 2-8 °C in the refrigerator for a maximum of up to 1 hour.

• Do not accept if insufficient sample is given and samples without an ID number.

Additive/Preservative: No additive or preservative need to the specimen. If delay in transport is anticipated instruct patient to store the sample at 2–8 °C in the refrigerator for a maximum of up to 1 hour.

Instrument: Clinitek 500 Urine analyzer, Bayer.(as shown in Fig.)



Consumables: Multistix 10SG

TEST PROCEDURE

• Ensure the urine sample is at room temperature and mix the urine sample thoroughly before testing.

• Take a test strip from the pack and close it again immediately.

• Briefly, no longer than 1 sec, dip the test strip into the urine making sure that all the test areas are moistened. If the strip is dipped for a longer time reagent from the test areas of the strip will dissolve in the urine.

• When removing the strip, wipe the edge of the strip against the rim of the specimen container to remove excess urine or dab the side edge of the strip on a clean absorbent surface.

• Place the test strip with the pads upwards, onto the tray so that it leading edge is held by the clip at the insertion slot. The retaining bar must be open about 2 mm of strip must be held under the clip.

• Press the start button. An acknowledging beep will sound. The tray will advance slightly, the retaining bar will close and the grey reference pad on the tray will be read.

• Ensure that the retaining bar is locked into place and that the test strip is in the correct position. If the test strip is not correctly located in the middle of the tray, move it gently to the side until it properly aligned, be careful not to move the tray.

• Press the 'Start' button.

• Exactly 55 seconds after you pressed the start button, the first test pad will be measured, followed by the others. After that the tray will return to the start position and the retaining bat will open.

• Remove and dispose of the test strip. Wipe any urine residues from the tray with a lint-free cloth.

• The result will be printed out and the next sample number will be displayed. The next test strip can be dipped, wiped off, placed in the tray and read by pressing the start button.

CAUTIONS AND POTENTIAL SOURCES OF VARIABILITY

• Carry out the examination in a fresh sample within 2 hours of collection, as delay will cause leukocytes to be destroyed, casts to decompose and urea will be broken-down due to bacterial action and make the urine alkaline affect test results

• Perform manual methods only for cross-reference for confirmation or whenever there is a failure of the automated system.

• Observe the following precautions while urine dip sticks:

- Do not touch the edges of the strip where the reagent patch is present

- Tap excess urine from the strip by stroking along the rim of the container after dipping the strip into the sample.

- Do not expose the strip to the atmospheric air when not in use and keep the bottle closed.

- Protect the strips from moisture and excessive heat but not refrigerate.

- Replace the top on the storage container immediately after a strip.

- Darkening of the enzyme-coated area of the strip indicate loss of sensitivity. Hence do not use discolored strips.

- Contamination of glassware with Sodium Hypochlorite and Bleaching powder and detergents like sodium phosphate will oxidize and change the color of chromogen in Dipstick. Hence ensure the glassware is free of these chemicals.

REFERENCE RANGES

• PH Newborn: 5-7 thereafter: 4.5-8 average: 6.

• Specific gravity SG of random urine ranges from 1.003-L000

• Nitrite Normally no detectable amount of nitrite is present in urine.

• Protein Normally no detectable amount of protein is present in urine.

• Ketone Normally, no ketones are present in urine

• Glucose Normally no detectable amount of glucose present in urine.

• Bilirubin Normally, no bilirubin is detectable in urine

• Urobilinogen Present in normal amounts.

• Leucocytes Normal urine yields negative results

• Pathological leucocyte concentration > 20 leukocytes/ml

• Erythrocytes <3 erythrocytes/mL

• Blood Normally no blood is present in urine. Blood may in the urine of menstruating

females.

INTERPRETATION OF RESULTS

• pH-Compare the color that develops on the reagent given on the reagent bottle (after 30 seconds) by the manufacturer of the urine strip.

- **> 7 --- alkaline.**
- **< 7 --- acidic**

• Specific Gravity-Compare the color that develops on the reagent strip with that given on the reagent bottle (after 45 seconds) by the manufacturer of the urine strip.

• Glucose-Negative: no change in color

- **Trace- light green and slightly cloudy.**
- **1+: green (0.5%)**
- **2+: yellow (1.0%)**
- **3+: orange (1.5%)**
- **4+: Brick red (2.0%)**

• Protein – Negative, Trace, 1+, 2+, 3+, 4+

• Ketone bodies

Compare the color developed on the test area with the corresponding color chart provided on the bottle by the manufacturer of the strip at exactly the time specified (after 40 seconds) and grade the result accordingly. Depending on the intensity of the color grade the result as trace (+), (++), (+++), and (++++). Normal urine (in healthy individuals) does not contain any of the ketone bodies. Repeat all positive test results by Ketodiastix strip method.

• Urobilinogen, leukocyte, Nitrite, occult blood-Compare reagent area to corresponding color chart on the bottle label at the specified time. Hold strip close to color blocks and match carefully

SAFETY PRECAUTIONS

• Handle reagents with care and avoid contact with eye, mouth and skin

• Handle all samples as potentially infectious

• Discard used reagents and sample as per disposal procedure

URINE EXAMINATION-MANUAL METHOD

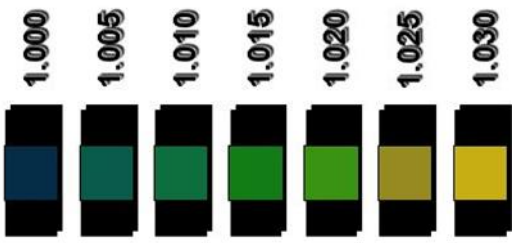
Urine specific gravity test

Specific gravity (concentration), in the context of clinical pathology, is a urinalysis parameter commonly used in the evaluation of kidney function and can aid in the diagnosis of various renal diseases.

One of the main roles of the kidneys in humans and other mammals is to aid in the clearance of various water-soluble molecules, including toxins, toxicants, and metabolic waste. The body excretes some of these waste molecules via urination, and the role of the kidney is to concentrate the urine, such that waste molecules can be excreted with minimal loss of water and nutrients. The concentration of the excreted molecules determines the urine's specific gravity. In adult humans, normal specific gravity values range from 1.010 to 1.030.

Procedure

1. Test SG with the use of a multiple-test *dipstick* that has a separate reagent area for SG. An indicator changes color in relation to ionic concentration, and this result is translated into a value for SG.



Specific gravity color chart.

2. Determine SG with a *refractometer* or total solids meter. The refractive index is the ratio of the velocity of light in air to the velocity of light in the test solution. A drop of urine is placed on a clear glass plate of the refractometer, and another plate is pressed on top of the urine sample. The path of light is deviated when it enters the solution, and the degree of deviation (refraction) is directly proportional to the density of the solution.

3. The *urinometer* (hydrometer) is the least accurate method. It consists of a bulb-shaped instrument that contains a scale calibrated in SG readings. Urine (10 to 20 mL) is transferred into a small test tube–like cylinder, and the urinometer is floated in the urine. The SG is read off the urinometer at the

meniscus level of the urine. This method is becoming obsolete owing to the ease of dipstick testing.

Urine appearance

Urine is normally clear straw-yellow in color. More concentrated urine may appear dark yellow. The presence of blood cells or excess salts may make the urine appear cloudy. Pigments from bile substances may make the urine appear deep yellow or brown. Urine can occasionally appear colorless.

Report the appearance as:

The following are examples of color change causes and not a complete listing.

- **Nearly colorless: Excessive fluid intake for conditions; untreated diabetes mellitus, diabetes insipidus, and certain types of nephritis.**
- **Yellow: Distinctly yellow urine may indicate excessive riboflavin (vitamin B_2) intake.**
- **Yellow-amber: Normal.**
- **Yellow-cloudy: excessive crystals (crystalluria) and/or excessive pus (pyuria).**
- **Orange: Insufficient fluid intake for conditions; intake of orange substances; intake of Phenazopyridine for urinary symptoms.**

- **Red: Leakage of red blood cells or of hemoglobin from such cells; intake of red substances.**
- **Dark:**
    - **Reddish-orange: Intake of certain medications or other substances.**
    - **Rusty-yellow to reddish-brown: Intake of certain medications or other substances.**
    - **Dark brown: Intake of certain medications or other substances; damaged muscle (myoglobinuria due to Rhabdomyolysis) from extreme exercise or other widespread damage, possibly medication related; altered blood; bilirubinuria; intake of phenolic substances; inadequate porphyrin metabolism; melanin from melanocytic tumors.**
    - **Brown black to black: Intake of substances or medications; altered blood; a problem with homogentisic acid metabolism (alkaptonuria), which can also cause dark whites of the eyes and dark-colored internal organs and tissues (ochronotic); Lysol (a product that contains phenols) poisoning; melanin from melanocytic tumors). Paraphenylenediamine is a highly toxic ingredient of hair dye formulations that can cause acute kidney injury and result in black urine.**
    - **Purple due to Purple urine bag syndrome.**
- **Magenta to purple-red: Presence of phenolphthalein, a stimulant laxative previously found in Ex-Lax.**
- **Green, or dark with a greenish hue: Jaundice (bilirubinuria); problem with bile metabolism. Recent surgery requiring high doses of Propofol infusion. The use of a medication (Uribel) that is similar to phenazopyridine for the relief of urinary symptoms.**

Other colors: Various substances ingested in food or drink, particularly up to 48 hours prior to the presence of colored urine.

Urine odor

Normal odor – aromatic or acetone

Abnormal odor – aromatic odor of ketone bodies

Although not routinely reported urine odor may be a significant observation:

-Ketones smell sweet or fruity.

-A specimen contaminated with bacteria may have a pungent smell from the ammonia that is produced.

-The excretion of urine that smells like maple syrup is an indication of a congenital metabolic disorder which has been appropriately named "maple syrup urine disease."

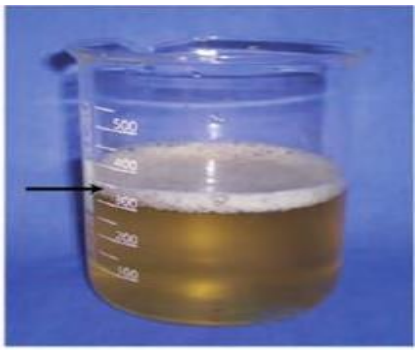
- A "musty or mousy" odor of an infant's urine may indicate phenylketonuria.

-A "sweaty feet" odor is found in isovaleric acidemia or in individuals who have excessive amounts of butyric or hexanoic acid.

-Hypermethioninemia has been associated with a "rancid butter" or "fishy" odor.

-Prolonged presence of any strong unusual odor may be associated with inherited disorders

Urine foam



Shake the sample and observe

- **If the amount of foam produced is in excess and slow to disappear = proteinuria**
- **If the color of the foam great – yellow or brown = bile pigments**
- **If the color of the foam red to brown = hemoglobinuria**

Urine pH

Normal freshly passed urine is slightly acid, with a pH of around 6.0. In certain diseases the pH of the urine may increase or decrease.

Principle:

_ Colored indicator paper is dipped in the urine (or placed in a watch glass and a few drops of urine are added to it).

_ The color changes according to the pH.

_ The paper is then compared with a standard control chart giving the corresponding pH value.

Materials

_ Watch glasses

_ Dropper

_ Forceps

_ Universal indicator paper (for measuring pH from 1 to 10)

_ Indicator paper of limited pH range: for the 5.0–7.0 range and for the 6.0–8.0 range.

The urine specimen must be tested within 1 hour of collection.

Method

1. Place a strip of universal indicator paper in a watch glass. Let a few drops of fresh urine fall from the dropper on to the paper. Alternatively, dip the test paper directly into the urine in the receptacle.

2. Pick the strip of paper up with forceps. Compare the color obtained with those shown on the standard chart. Read off the pH unit given for the color that matches the test paper most closely.

3. According to the result obtained, select a strip of indicator paper for the corresponding limited range. For example, if the pH is 6, use indicator paper for the range 5.0–7.0. If the pH is 7 or more, use indicator paper for the range 6.0–8.0.

4. Repeat the test in another watch glass, using the paper for the corresponding limited range. Read off the pH of the urine on the standard chart, e.g. pH = 6.2 or pH = 7.5.

The pH of urine is normally about 6.0 (range 5.0–7.0). Acid pH values (4.5–5.5) are observed in some forms of diabetes, muscular fatigue and acidosis. Alkaline pH values (7.8– 8.0) are common in patients with infections of the urinary tract and in people on a vegetarian diet.

REAGENT TEST STRIPS

All brands of dipsticks discussed in this chapter use the same two indicators, methyl red and bromothymol blue, and measure a range of pH from 5.0 to 8.5.

Fig. pH color chart.

pH and crystalline deposits

Determination of the pH of urine is useful for the identification of crystalline deposits. Some crystals are deposited only in acid urine, others only in alkaline urine. For example:

— acid urine: oxalates, uric acid;

— alkaline urine: phosphates, carbonates, urates.

Except in very rare diseases, crystalline deposits in urine have no diagnostic significance.

MICROSCOPIC EXAMINATION OF URINE SAMPLE

Preparation and examination of a wet preparation

1- Aseptically transfer about 10 ml of well mixed urine to a labelled conical tube.

2- Centrifuge at 500–1000 g for 5 minutes. Pour the supernatant fluid (by completely inverting the tube) into a second container not the original one. This can be used for biochemical tests to avoid contaminating the original urine which may need to be cultured (depending on the findings of the microscopical examination).

3- Remix the sediment by tapping the bottom of the tube. Transfer one drop of the *well-mixed* sediment to a slide and cover with cover glass.

***Note*: Do not discard the remaining sediment because this may be needed to prepare a Gram smear if WBCs and, or, bacteria are seen in the wet preparation.**

4- Examine the preparation microscopically using the 10X and 40X objective with the condenser iris *closed sufficiently* to give good contrast.

Report the following:

The following may be found in urine:

— erythrocytes

— leukocytes

— epithelial cells

— casts

— fungi

— crystals

— parasite eggs and larvae

— *Trichomonas vaginalis*

— spermatozoa.

Erythrocytes in urine may be:

(a) intact: small yellowish discs, darker at the edges (8mm);

(b) crenate: spiky edges, reduced diameter (5–6mm);

(c) swollen: thin circles, increased diameter (9–10mm).

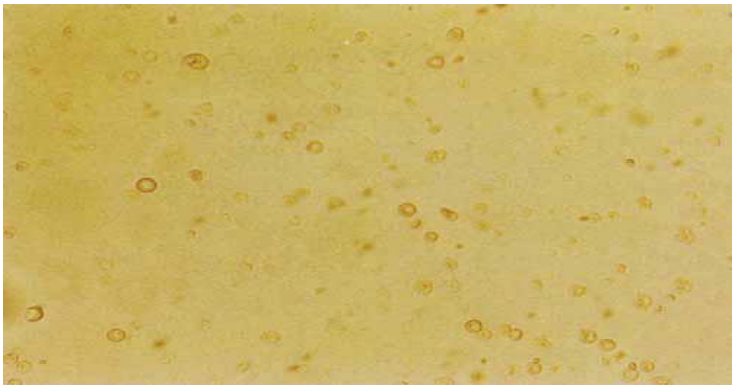


Figure. Red blood cells. The field also contains a white cell and several "ghost" cells (400X).

The shape of the cells often changes during storage of urine and does not have any diagnostic importance. There are normally very few erythrocytes in urine.

***Note*: Erythrocytes may be found in the urine of women if the specimen has been taken during the menstrual period.**

Dipstick test for blood in the urine

The term "occult" means "hidden," and the methods used to test for blood in the urine are capable of detecting even minute amounts not visualized macroscopically. Another reason for this title is that these procedures actually detect the free hemoglobin from lysed red blood cells (RBCs). Those tests which screen for occult blood will detect hematuria, hemoglobinuria, and myoglobinuria.

The dipstick procedure is based on the peroxidase like activity of hemoglobin and myoglobin which catalyzes the oxidation of a chromogen by an organic peroxide. Most dipsticks are capable of detecting intact erythrocytes as well as free hemoglobin and myoglobin.

Method

The dipsticks are placed into the urine and immediately removed. They are then compared with a comparison chart after an appropriate time that is also specified on the chart. The color changes observed on the dipstick will give a semi-quantitative estimation of the amount of substance present. This can be reported as negative, +, ++, +++, ++++ or as an approximate value of the concentration of the substance tested for.

Blood is usually read at 60 seconds, and the color change is from orange to green to dark blue. There are two separate color scales for erythrocytes and hemoglobin. Intact RBCs may display a speckle-pattern reaction in the absence

of free hemoglobin

Fig. Blood color chart.

Table. Reporting the results of microscopic examination of urine for erythrocytes

Number of erythrocytes per microscope field	Result
0–10	few erythrocytes (normal)
10–30	moderate number of erythrocytes
> 30	many erythrocytes

Leukocytes in urine

Leukocytes found in urine may be:

(a) intact: clear granular discs, 10–15mm (the nuclei may be visible);

(b) degenerated: distorted shape, shrunken, less granular;

(c) pus: clumps of numerous degenerated cells. The presence of many leukocytes, especially in clumps, indicates a urinary tract infection.

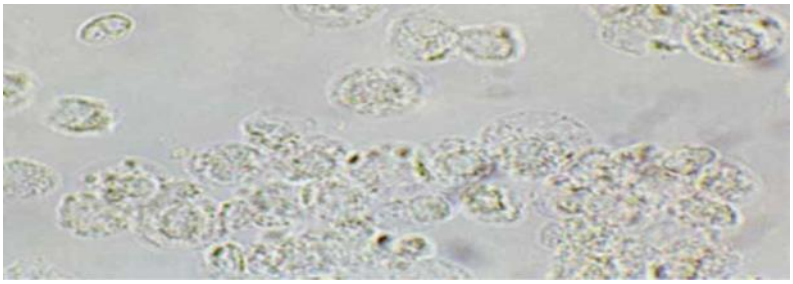


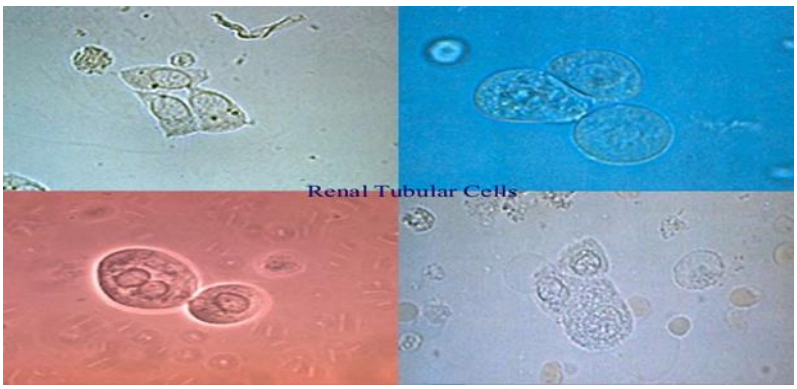
Figure. White blood cells in a hypotonic urine. The nuclei and granules are easily recognized (800X).

How to express the quantity of erythrocytes and leukocytes found in urine deposits

Place one drop of urine deposit on a slide and cover with a coverslip. Using the X40 objective, examine the deposit and count the number of erythrocytes and leukocytes per microscope field. Report the results as described in Tables below.

Table. Reporting the results of microscopic examination of urine for leukocytes

Number of leukocytes per microscope field	Result
0–10	few leukocytes (normal)
10–20	moderate number of leukocytes
20–30	many leukocytes
20–30 (degenerated) in clumps	many leukocytes seen in clumps
> 30 (degenerated) in clumps	full field



Ureteral and renal pelvic cells (Medium-sized oval cells with a distinct nucleus). If many cells are present together with leukocytes and filaments, they may be from the ureter. If a few are present, with no leukocytes, they may be cells from the renal pelvis.

Renal cells are smaller than renal pelvic cells (the size of 1–2 leukocytes) and are very granular. The nucleus is shiny and clearly visible. Renal cells are almost always present with protein in the urine.

Leukocyte esterase (LE): Detecting pus cells

This enzyme is specific for polymorphonuclear neutrophils (pus cells). It detects the enzyme from both active and lyzed WBCs. LE testing is an alternative method of detecting pyuria when it is not possible to examine fresh urine microscopically for white cells or when the urine is not fresh and likely to contain mostly lyzed WBCs. LE can be detected using a reagent strip test.

LEUKOCYTE REAGENT TEST STRIPS

White blood cells can be present in any body fluid depending on a cause for their presence. The most common white blood cell seen in a urine sample is the neutrophil, which is normally present in low numbers. Increased numbers of neutrophils usually indicate the presence of a urinary tract infection; and their presence is indicated by a positive leukocyte esterase test. Screening for urinary tract infections also includes evaluation of pH, protein, and nitrite.

Neutrophils contain enzymes known as esterase. This esterase can be detected by reagent strips that contain an appropriate substrate such as indoxyl carbonic acid ester Leukocyte esterase results are read at 2 minutes. A positive reaction produces a lavender to purple color with a reporting range of values from trace to large. Values reflecting cell numbers from negative to 500 may be reported. False negative strip test results can occur when the urine contains boric acid or excessive amounts of protein (µ500 mg/100 ml) or glucose (µ2 g/100 ml)

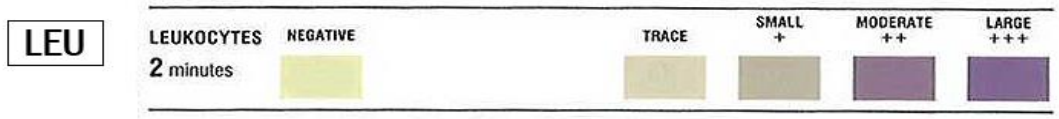
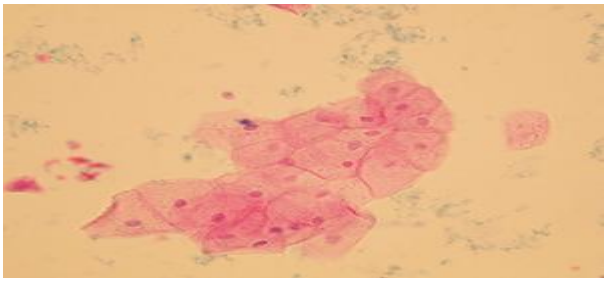


Fig. Leukocyte color chart.

EPITHELIAL CELLS IN URINE



The epithelial cells in the urine may originate from any site in the genitourinary tract from the proximal convoluted tubule to the urethra, or from the vagina. Normally, a few cells from these sites can be found in the urine as a result of the normal sloughing off of old epithelial cells. A marked increase indicates inflammation of that portion of the urinary tract from which the cells are derived. When distinction is possible, three main types of epithelial cells may be recognized: renal tubular, transitional, and squamous.

CASTS FOUND IN URINE

Urine casts are cylindrical, cigar-shaped structures produced by the kidney and present in the urine in certain disease conditions. All casts are composed of a mucoprotein known as Tamm-Horsfall protein which is secreted by the distal loop of Henle, the distal tubule and the collecting ducts at a fairly constant rate. A significant number of urinary casts usually indicates the presence of renal disease.

Formation of Casts

Urinary casts are formed only in the distal convoluted tubule (DCT) or the collecting duct. The proximal convoluted tubule (PCT) and loop of Henle are not locations for cast formation. Casts are the result of solidification of material (protein) in the lumen of the kidney tubules. Once formed, these casts of the tubule are eliminated via the urine and may be seen in the urine sediment. They may contain RBCs, WBCs, renal epithelial cells, fat globules, bacteria, and degenerated forms of any of these structures, which are seen as granules. Aggregates of plasma proteins, including fibrinogen, immune complexes, and globulins, may also be seen as granules within a cast.

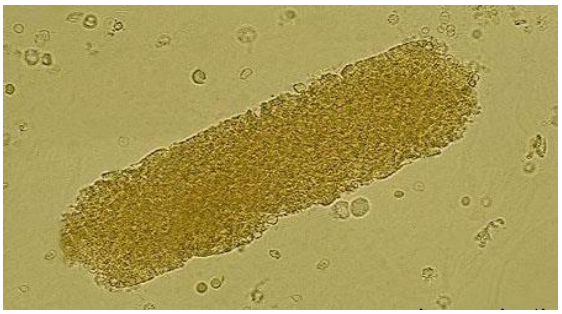
Types of urinary Casts

Urinary casts can be divided into two main categories: Acellular and Cellular Casts.

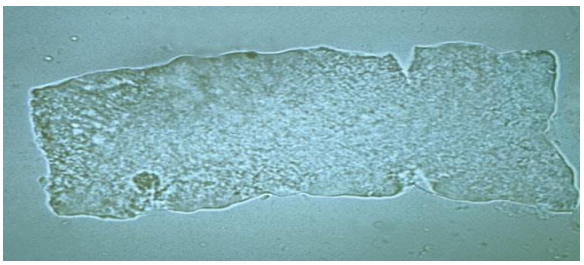
Acellular Casts	Cellular Casts
Hyaline Casts	Red Blood Cell Casts
Granular Casts	White Blood Cell Casts
Waxy Casts	Bacterial Casts
Fatty Casts	Epithelial Cell Casts

Acellular Casts

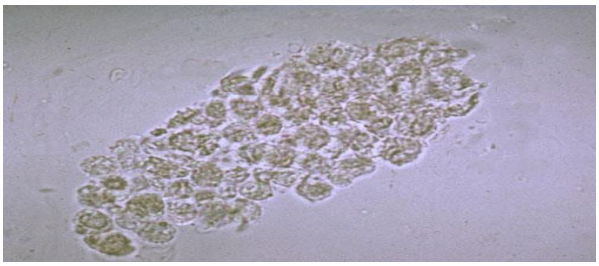
Hyaline casts are the most common type of casts which are composed of solidified Tamm-Horsfall mucoprotein. They have smooth texture and a refractive index very close to that of the surrounding fluid. Generally, hyaline casts have parallel sides with clear margins and blunted ends. Hyaline casts can be seen even in healthy patients. They may be seen in increased numbers during dehydration, exercise or diuretic medicines.



Granular casts result either from the degeneration of cellular casts, or direct aggregation of plasma proteins or immunoglobulin light chains. They have a textured appearance which ranges from fine to coarse in character. Their appearance is generally more cigar-shaped and of a higher refractive index than hyaline casts. They are seen after strenuous exercise, chronic renal diseases, acute tubular necrosis etc.



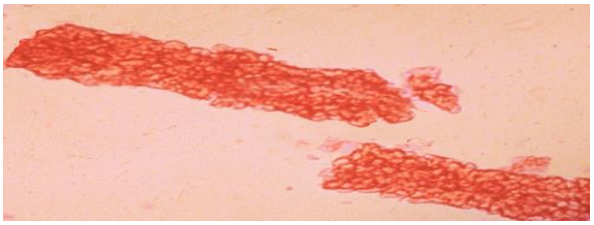
Waxy casts represent the final stage of degeneration of cellular casts. They are more refractile and therefore easier to see compared to hyaline casts. They are usually seen in tubular injury of a more chronic nature than granular or cellular casts like severe chronic renal disease and renal amyloidosis. These casts are also called *renal failure casts*



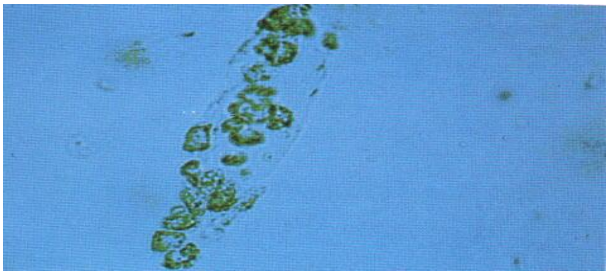
Fatty casts are formed by the breakdown of lipid-rich epithelial cells. These contain lipid droplets within the protein matrix of the cast and are identified by the presence of refractile lipid droplets. They are usually seen in the conditions like tubular degeneration, nephrotic syndrome, hypothyroidism etc.

Cellular Casts

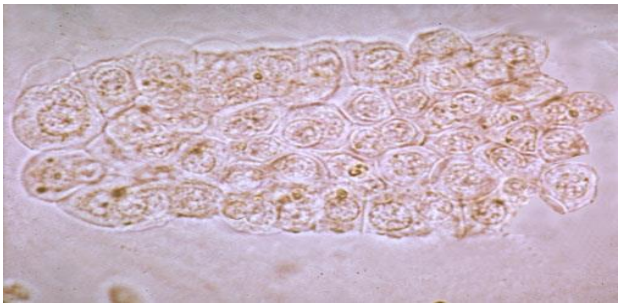
Cellular cast may be composed of any of the cells found in the urine sediment, such as RBC, WBC, or renal tubular epithelial cell. The cellular cast appears to result from a clumping of cells that are incorporated in a protein matrix.



Red blood cells may stick together and form red blood cell casts. Such casts are indicative of glomerulonephritis, with leakage of RBC's from glomeruli, or severe tubular damage.



White blood cells (generally neutrophils) are present within or upon casts. These casts are typical for acute pyelonephritis, but they may also be present with glomerulonephritis. They may also be seen in acute interstitial nephritis, lupus nephritis, and acute papillary necrosis.



Renal Tubular Epithelial Cell Casts

These casts are composed of renal epithelial cells. These casts are seen in conditions such as renal tubular necrosis, viral disease (such as CMV nephritis), and kidney transplant rejection.

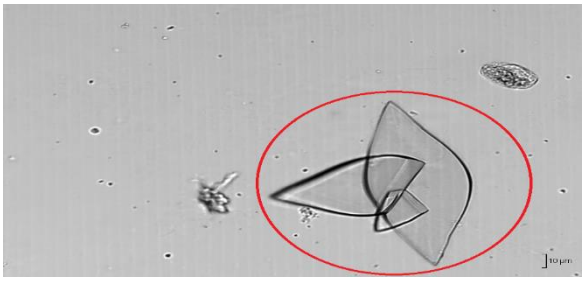
CRYSTAL FOUND IN URINE

Urine contains many dissolved substances(solutes) that the body needs to eliminate as waste chemicals. These solutes can form crystals when the concentration of dissolved substances has increased or when the pH level is increasing in either acid or base. This condition of presence of crystals in the urine is known as crystalluria. Sometimes crystals are found in healthy people and other times they are indicators of organ dysfunction, the presence of urinary tract stones of a like composition (known as urolithiasis), or an infection in the urinary tract.

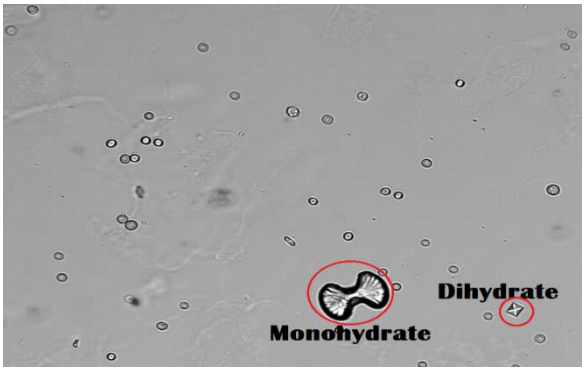
Normal Crystals	Abnormal Crystals
1. Uric acid Crystals	1. Bilirubin Crystals
2. Calcium Oxalate Crystals	2. Cholesterol Crystals
3. Hippuric Crystals	3. Cysteine Crystals
4. Calcium Phosphate Crystals	4. Leucine Crystals
5. Triple Phosphate Crystals	5. Tyrosine Crystals

6. Calcium Carbonate Crystals	**6. Sulfa Crystals**
7. Ammonium Biurate Crystals	**7. Indinavir Crystals**

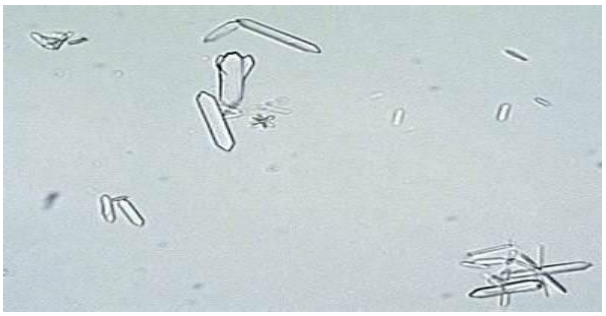
Crystals that are Normal in Urine



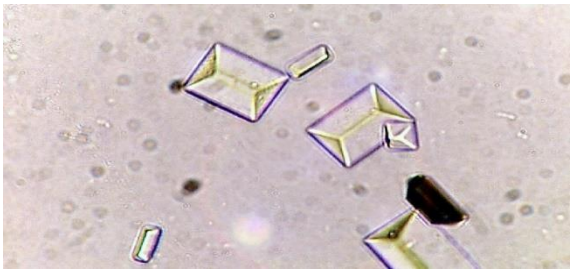
Uric acid crystals are of varying sizes and shapes, found in acidic urine. They resemble rhomboids, parallelograms, and rosettes in shape and are amber in color. Though they are seen in normal urine, are also an indicator of disease processes, such as acute uric acid nephropathy or urate nephrolithiasis.



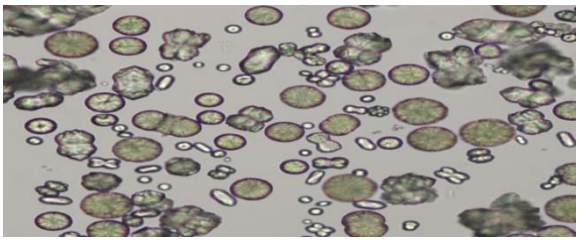
Calcium oxalate crystals are found in individuals with acidic, neutral or alkaline urine. These crystals are colorless when viewed microscopically. There are two forms of the calcium oxalate crystal: the monohydrate and dihydrate form.
The monohydrate calcium oxalate crystal is described as the "picket fence" form. These dumbbells shaped crystals are common in ethylene glycol toxicity. The dihydrate form is octahedral or "envelope" shaped.



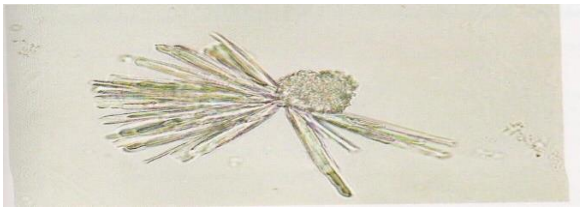
Hippuric acid crystals are found in acid, neutral, or slightly alkaline urine. These colorless crystals are prisms, plates, or needle-like in shape. These crystals are often conglomerated into masses.



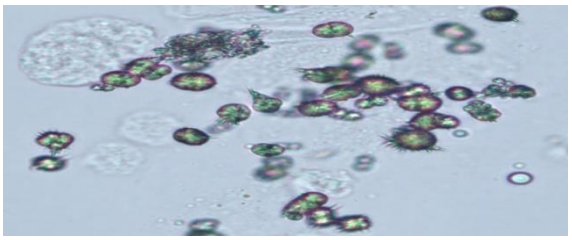
Triple phosphate crystals form in alkaline urine and are composed of magnesium, ammonium and phosphate. These are rectangular in shape or similar with the coffin lid. These are sometimes associated with a bacterial urinary tract infection caused by urea splitting bacteria.



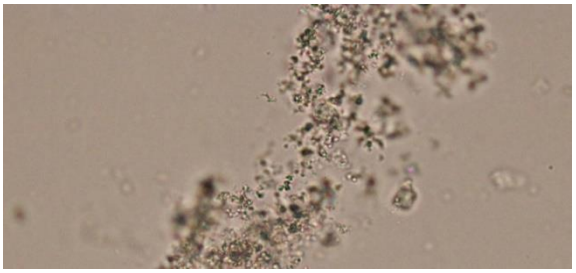
Calcium carbonate crystals are yellow to colorless dumbbells or spheres with radial striations, found in alkaline urine. They are usually large crystals and can be readily observed at low magnification.



Calcium Phosphate Crystals
These are colorless crystals having shape like blunt ended needles or prisms, rosettes. These crystals are found in neutral to alkaline pH.

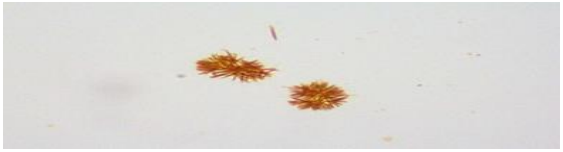


Ammonium urate (or biurets) crystals generally appear as brown or yellow-brown spherical bodies with irregular protrusions resembling “thorny-apples”. These are found in alkaline urine.

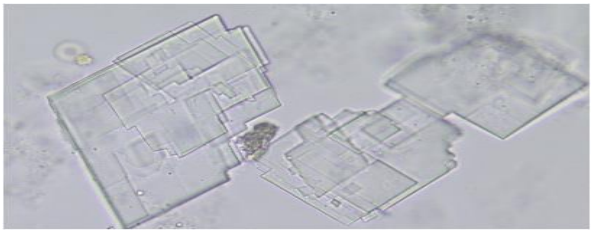


Amorphous urates are found in acid urine. These crystals may appear pink on gross analysis and yellow microscopically. These crystals appear as granules in the urine sediment.
Amorphous phosphates are found in alkaline urine. These granules are colorless microscopically.

Crystals that are abnormal in Urine

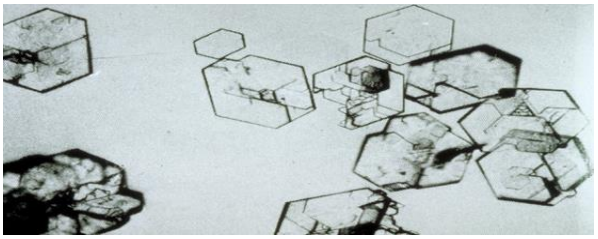


Bilirubin crystals are abnormal crystals in urine. They form from conjugated bilirubin and are needle-like to granular crystals that are yellow in color. They are frequently attached to the surface of cells. Bilirubin crystals are seen in several hepatic disorders.



Cholesterol Crystals

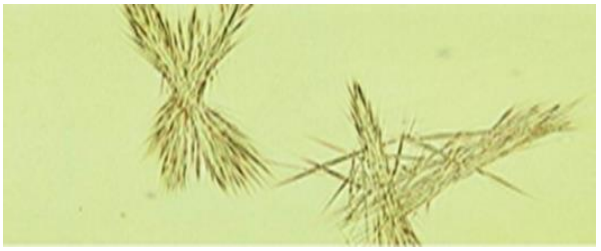
These appear as colorless rectangular plates with a notch in one or more corners and are found in acidic urine. The appearance of cholesterol is associated with the Nephrotic Syndrome.



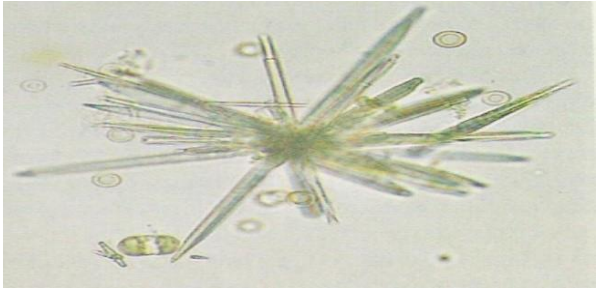
Cystine crystals are flat colorless plates and have a characteristic hexagonal shape with equal or unequal sides. They occur in acidic urine that are associated with an inherited disorder. Presence of cystine crystals represents a proximal tubular defect in amino acid reabsorption.

Leucine Crystals

These are Yellowish-brown spheres with concentric circles with radial striations found in acidic/neutral urine. Leucine crystals may be seen in liver disorders in which amino acid metabolism is impaired.



Tyrosine crystals appear as colorless/yellow pine needles in acidic/neutral urine. Tyrosine crystals may be seen in tyrosinemia and in certain liver disorders in which amino acid metabolism is impaired.



Sulfonamide Crystals

These are flat needles, sheaves of small needles or as spheroids. Often brown in color. The presence of sulfonamide crystals usually indicates administration of the drug and not necessarily a pathological condition. However, their presence is also associated with kidney stone formation.

Yeast cells in urine

These can be differentiated from red cells by their oval shape and some of the yeasts usually show single budding. If in doubt, run a drop of dilute acetic acid under the cover glass. Red cells will be hemolyzed by the acid, but not yeast cells.

***Note*: Glove powder in urine also resembles yeasts. It can be distinguished by adding a drop of iodine (as used in Gram stain). Glove powder granules (starch), turn blue-black.**

Yeast cells are usually reported as few, moderate, or many per HPF. They can be seen in the urine of women with vaginal candidiasis, and occasionally in specimens from diabetics and those with immunosuppression

SCREENING TESTS FOR BILIRUBIN (BILE)

Bilirubin dipstick test

Bilirubin is a yellow compound that occurs in the normal catabolic pathway that breaks down heme in vertebrates.

Bilirubin can be detected in the urine before other clinical symptoms are present or recognizable. The detection of small quantities is very important in the early diagnosis of obstructive and hepatic jaundice. This test is also useful in the differential diagnosis of obstructive (positive) and hemolytic (negative) jaundice.

Most dipsticks are based on the coupling reaction of a diazonium salt with bilirubin in an acid medium. Some dipsticks differ, however, in the diazonium salt that is used and the color that develops. Bilirubin results are read from 30 to 60 seconds, depending on the manufacturer and display a range of colors from buff through various shades of tan or tannish-purple. These colors correspond to levels of bilirubin from negative to large (3+).

Fig. Bilirubin dipstick test chart

ICTOTEST/ TESTS FOR BILIRUBIN

Ictotest is a tablet test that is based on the same diazo reaction as the dipsticks. However, Ictotest is much more sensitive than the dipsticks, being able to detect as little as 0.05 mg/dL. Because of this sensitivity, Ictotest is the recommended procedure when a test for just bilirubin is ordered. It also serves as a good confirmatory test for a positive dipstick.

Procedure

1. Place five drops of urine on one square of the special test mat supplied with Ictotest.

2. Place a tablet in the center of the moistened area.

3. Flow two drops of water onto the tablet so that the water runs off of the tablet and onto the mat.

4. Observe the color of the mat around the tablet at the end of 30 seconds. If a blue or purple color develops, the test is positive.

All other colors including pink or red are negative.

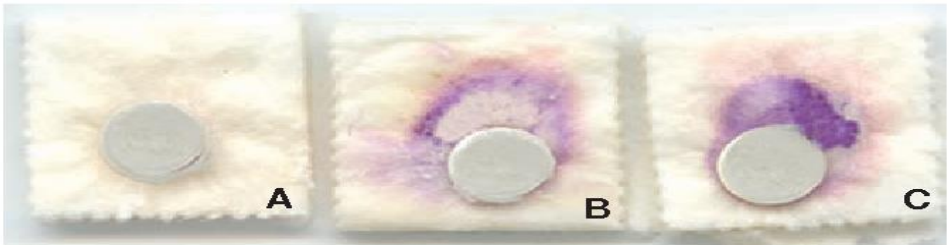


Fig. Ictotest color reactions. (A) negative, (B) moderate, and(C) large.

QUALITATIVE IDENTIFICATION OF UROBILINOGEN IN URINE BY EHRLICH ALDEHYDE TEST

Purpose: Qualitative identification of urobilinogen in urine by Ehrlich aldehyde test. Normal urine indicates the presence of trace amounts of urobilinogen. Increased urobilinogen discharged through the urine is diagnostic of hemolytic jaundice. Absence of urobilinogen indicates hepatic jaundice, obstructive jaundice, or distance in the intestinal micro flora same following antibiotic treatment.

Principle:

urobilinogen reacts with Ehrlich aldehyde reagent (p-dimethyl amino benzaldehyde) in acid medium to form a pink colored compound.

PRIMARY SAMPLE

- **Use urine as specimen for the test.**
- **Collect a minimum 15 mL of random sample of urine**
- **Instruct patients to void urine directly into the container and while collecting allow the first portion of urine to escape.**
- **Use fresh and uncentrifuged samples for the test.**
- **Process the samples within 1 hour of collection. If delay is anticipated, instruct patients to store the samples at 2-8 C to 4 hours till transport to laboratory.**
- **Do not accept the sample if the sample volume is inadequate or samples without an ID number**

CONSUMABLES/REAGENTS

• Ehrlich aldehyde reagent: Conc. HCl - 20 ml; Dist. Water – 80 ml. Mix well and add p-Dimethyl Amino Benzaldehyde -20 g and stir well

• Glass test tubes

PROCEDURE

• Pipette 25 ml of urine into a test tube

• Add 5 drops of Ehrlich's reagent and allow to stand for 5 minutes.

• Note the color change

INTERPRETATION OF RESULTS

• Development of pale pink color -- Presence of urobilinogen in normal amount.

• Development of dark pink cherry red color-- Presence of urobilinogen in increased amounts.

PRECAUTIONS

• Handle all reagents with care and avoid contact with eye, mouth and skin.

•Handle all samples as potentially infectious.

• Discard used reagents and sample as per disposal procedure.

POTENTIAL SOURCES OF VARIABILITY

• Use a fresh sample of urine testing for urobilinogen, since urobilinogen is unstable and gets immediately oxidized to urobilin, which does not answer this test.

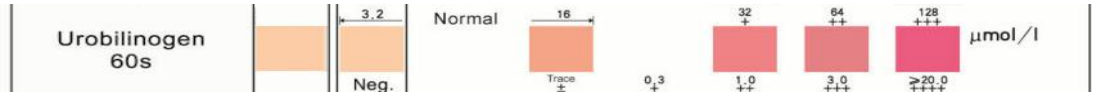
• Samples should be tested within 2 hours of collection.

• Porphobilinogen in urine also answers this test. Hence, this test is considered as a screening test only.

Urobilinogen dipstick test

Urobilinogen is a colorless by-product of bilirubin reduction. It is formed in the intestines by bacterial action on bilirubin.

Urobilinogen results are read at 30 or 60 seconds, depending on the manufacturer and display a range of colors in the pink spectrum from light to dark. Most brands of dipsticks show two blocks on the color chart for normal levels of urobilinogen of 0.1 and 1 mg/dL. The other color blocks range from 2 to 8 or 12, depending on the manufacturer.



Urobilinogen color chart.

DETECTION AND ESTIMATION OF PROTEIN IN URINE

Elevated protein levels are observed in the urine of patients with:

— urinary schistosomiasis

— chronic renal disease

— pyelonephritis

— diabetes mellitus

— systemic disorders (lupus erythematosus)

— multiple myeloma.

However, orthostatic proteinuria, a form of functional proteinuria usually seen in young men, which occurs on standing up and disappears on lying down, has no pathological significance.

Principle: When trichloroacetic acid is added to urine containing protein, a precipitate is formed, which is measured by turbidimetry. This reaction occurs with almost all proteins, including albumin and globulins.

Materials and reagents

_ Spectrophotometer

_ Test-tubes

_ Test-tube rack

_ Centrifuge

_ Mechanical rotator

_ Bovine or human serum albumin

_ Trichloroacetic acid, 5% solution, diluted 1: 4 with distilled water

_ Sodium chloride, 0.85% solution

_ Positive and negative controls

_ Albumin working standard, 0.005% solution (prepared from albumin stock standard, 5.0% solution, diluted 1:100 with sodium chloride, 0.85% solution).

The albumin working standard can be divided into aliquots and stored at -20°C for up to 6 months. If albumin stock standard is not available, commercial serum-based standards containing both albumin and globulin can be used to prepare a working standard solution of the appropriate concentration. As with the albumin standard, the working standard can also be divided into aliquots and stored at –20°C for up to 6 months.

Further details of the method described here are given in the following references: Shahangian S, Brown PI, Ash KO. Turbidimetric measurement of total urinary proteins: a revised method. *American journal of clinical pathology*, 1984, 81:651–654; Tietz NW, ed. *Textbook of clinical chemistry*, 2nd ed. Philadelphia, WB Saunders, 1994.

Method

Collection of specimens

Random, timed or 24-hour urine specimens should be used. No preservatives should be added to the specimens. Specimens that are collected over 24 hours should be stored at 4–8°C during the period of collection, to prevent bacterial growth. Collected specimens should be kept at 4°C until analysis. If analysis is likely to be delayed for more than 24 hours, however, the specimens should be stored at -20°C.

Technique

1. Add 1.6ml of the urine specimen to each of two test-tubes (test and test blank). Repeat the process with the working standard and the control.

2. Add 0.4ml of trichloroacetic acid solution to all of the test-tubes and mix well. Leave to stand at room temperature for 10 minutes.

3. Centrifuge the test blanks at 2000*g* for 10 minutes.

4. Using the spectrophotometer, measure and record the optical density of the tests and blanks at 620nm. The spectrophotometer should be set to zero with distilled water before any measurements are taken. It should also be calibrated, as described below. The analytical range of measurement using this method is 100–1000mg/l.

Calculation

Calculate the concentration of protein in the urine specimen using the following formula:

$OD_T - OD_{TB}$ X C / $OD_R - OD_{RT}$

where:

C = concentration of the calibration solution

OD_R = optical density of the working standard

OD_{RT} = optical density of the working standard test blank

OD_T = optical density of the test specimen

OD_{TB} = optical density of the test specimen blank.

Note:

_ If a serum-based control is used for calibration purposes, an independent material must be used for the purpose of quality control.

_ Because the amount of protein excreted in the urine may vary greatly, any positive results should always be confirmed by repeating the test on one or more separate samples.

_ If this method is used to screen for microproteinuria (which may be correlated with microalbuminuria in the absence of tubular damage, urinary infections and treatment with certain drugs) in high-risk populations such as patients with diabetes, the following modifications should be applied to steps 2 and 4:

2. Leave all the tubes to stand at room temperature for 35 minutes after mixing.

4. Using the spectrophotometer, measure and record the optical density of the tests and blanks at 405nm. The analytical range of this modified method is 25–700mg/l.

Dipstick test for protein

Protein in urine can also be detected using a protein dipstick. The development of any green to blue color indicates the presence of protein. The intensity of the color is proportional to the amount of protein that is present. The results are usually reported as negative to 3+ or 4+and display a range of colors from yellow to blue.

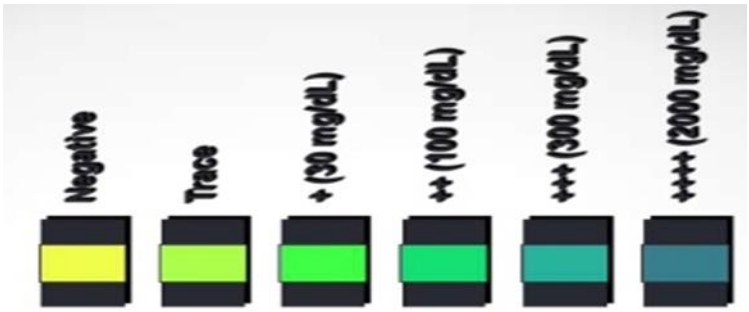


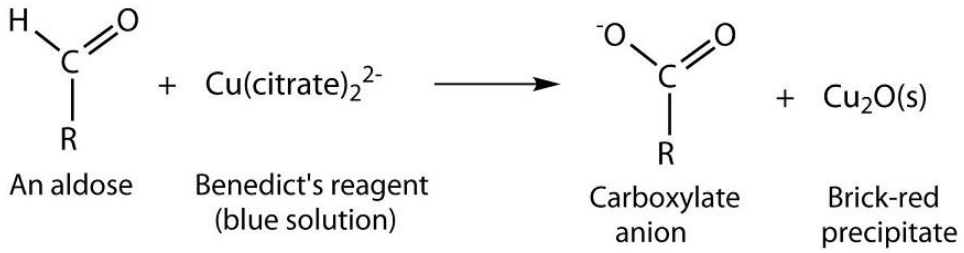
Fig. REAGENT TEST STRIP FOR PROTEIN

DETECTION OF GLUCOSE IN URINE (Benedict method)

Benedict's test is used as a simple test for reducing sugars. A reducing sugar is a carbohydrate possessing either a free aldehyde or free ketone functional group as part of its molecular structure. This includes all monosaccharides (e.g. glucose, fructose, galactose) and many disaccharides, including lactose and maltose.
Benedict's test is most commonly used to test for the presence of glucose in urine. Glucose found to be present in urine is an indication of Diabetes mellitus.

Principle of Benedict's Test

Reducing sugars under alkaline condition tautomerize and form enediols. Enediols are powerful reducing agents. They can reduce cupric ions (Cu^{2+}) to cuprous form (Cu^{+}), which is responsible for the change in color of the reaction mixture. This is the basis of Benedict's test. When the conditions are carefully controlled, the coloration developed and the amount of precipitate formed (Cuprous oxide) depends upon the amount of reducing sugars present.



Composition and Preparation of Benedict's reagent

One liter of Benedict's Solution can be prepared from 100 g of anhydrous sodium carbonate, 173 g of sodium citrate and 17.3 g of copper (II) sulfate pentahydrate.

Constituent	Amount	Functions
Copper sulphate	17.3 gm	Furnishes cupric ions (Cu^{++})
Sodium carbonate	100 gm	Makes medium alkaline
Sodium citrate	173 gm	Complexes with the copper (II) ions so that they do not deteriorate to copper(I) ions during storage
Distilled water	Up to 1000 ml	Solvent

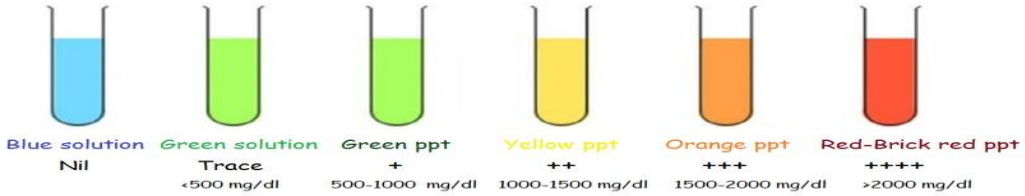
Quality Checking: Benedict's solution is blue in color. In order to check purity of Benedict's solution take 5 ml of Benedict's solution in test tube and heat it. If it does not change color, it means it is pure.

Procedure of Benedict's Test

1. **Pipette 5 ml of Benedict's reagent in a test tube (20x150mm).**
2. **Add 8 drops of urine to the Benedict's reagent.**
3. **Heat carefully on a flame of a gas burner or place in a boiling water for 5-10 minutes.**
4. **Cool under tap water or by placing in a beaker containing tap water.**

5. **Observe the color change and precipitate formation and analyze the test result.**

Result Interpretation of Benedict's Test



The color of the mixture serves as a guide to the amount of sugar in the urine. Remove the tubes and examine the solution in each tube for precipitate and change of color. Report the sugar concentration as follows:

Color	Approximate glucose mg/dl	Indication
Blue solution	Nil	
Green solution	<500 mg/dl	Trace
Green ppt	500-1000 mg/dl	+
Yellow ppt	1000-1500 mg/dl	++
Orange ppt	1500-2000 mg/dl	+++
Red to Brick red ppt	>2000 mg/dl	++++

Factors affecting Benedict's Test

False positive reactions may also be obtained if certain drugs are present, e.g. salicylates, penicillin, streptomycin, isoniazid, and p-aminoalicyclic acid.

Chemicals present in a concentrated urine which may reduce Benedict's reaction include creatinine, urate, and ascorbic acid (reduction is slight).

REAGENT STRIP for GLUCOSE OXIDASE TEST

Reagent strips that are impregnated with the enzyme glucose oxidase detect only glucose. Glucose results are read at 30 or 60 seconds, depending on the manufacturer. The results are reported as negative to 4_ (negative to 2000 mg/dL). The color changes displayed by these values range from blue to brown.

Fig. Glucose color chart.

DETECTION OF KETONE BODIES IN URINE

Normal urine does not contain ketone bodies. Acetone and other ketone bodies may appear in urine:

— in severe or untreated diabetes;

— in certain other conditions (dehydration, vomiting, malnutrition, prolonged starvation and following strenuous exercise).

Principle: When sodium nitroprusside (sodium nitrosyl pentacyanoferrate (III)) is added to urine containing ketone bodies, a purple color is produced.

Materials and reagents

_ Test-tubes

_ Test-tube rack

_ Measuring cylinder, 10ml

_ Dropping pipette

_ Sodium nitroprusside crystals

_ Acetic acid

_ Ammonia.

Method

1. Just before carrying out the test, place a sufficient number of sodium nitroprusside crystals into a test-tube to cover the bottom.

2. Add 5ml of distilled water. Shake well until the crystals are almost dissolved. (Not all the crystals are expected to dissolve as the solution is saturated.)

3. Measure 10ml of urine into another test-tube.

4. Add four drops of acetic acid to the urine, followed by 10 drops of freshly prepared sodium nitroprusside solution. Mix well.

5. Holding the tip of the pipette against the side of the tube, let 20 drops (1 ml) of ammonia solution flow on to the surface of the liquid. Wait for 5 minutes before reading — a positive result may be obvious before this time.

If the result is positive, a purple ring appears on top of the urine. If the result is negative, no color change occurs. Report the result as shown in Table.

Table. Reporting the results of the test for detection of ketone bodies in urine

Color change	Result
None	Negative
Pink ring	+
Red ring	++
Purple ring	+++

Laboratory dipstick tests for ketones

- **Multistix^{R} contains the reagents sodium nitroprusside and an alkaline buffer, which react with dacitic acid in urine to form a maroon color. Ketone results are read at 40 or 60 seconds, depending on the manufacturer. Color change is from buff-pink to maroon and the reaction is reported as either negative, trace, moderate, large or negative to 160 mg/dL.**

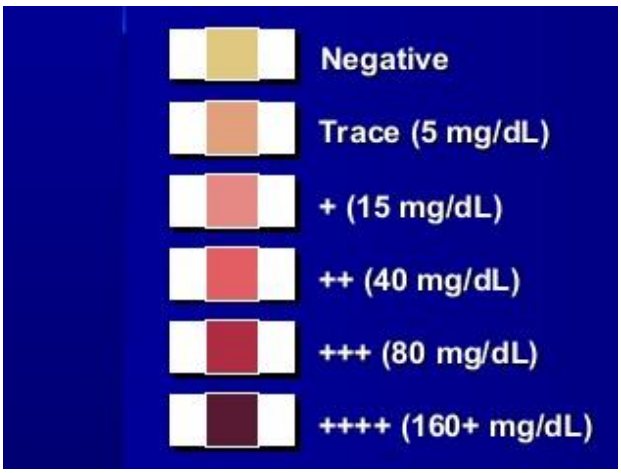


Fig. Urine dipstick for ketones

- **The Acetest^{R} tablet contains sodium nitroprusside, glycine, a strong alkaline buffer (disodium phosphate), and lactose. Acetest can be used to test urine, serum, plasma, or whole blood.Dacitic acid and acetone react with sodium nitroprusside and glycine in an alkaline medium to form a purple color. The lactose in the tablet helps enhance the color. Acetest is about 10 times more sensitive to dacitic acid than to acetone.**

Procedure for Acetest tablet:

1. Place the tablet on a piece of clean, dry white paper.

2. Put one drop of urine, serum, plasma, or whole blood directly on top of the tablet.

3. For urine, compare the color of the tablet with the color chart at 30 seconds.

Results are reported as "small, moderate, or large." For urine, the small color block corresponds to approximately 5–10 mg/dL of dacitic acid, the moderate block is 30–40 mg/dL, and the large block is about 80–100 mg/dL.

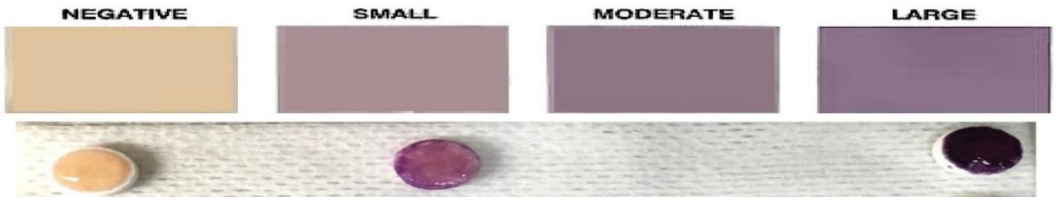


Fig. Acetest reagent tablet color chart.

QUALITATIVE DETECTION OF BILE PIGMENTS BY FOUCHET'S METHOD

PURPOSE: Qualitative detection of bile pigments (bilirubin and biliverdin) by Fouchet's test presence of bile pigments in urine is diagnostic of hepatic jaundice or obstructive jaundice. Presence of bilirubin in urine is detected before the clinical symptoms of jaundice (change of skin color) are recognized.

PRINCIPLE: Barium chloride reacts with sulfate radicals present in urine to form a precipitate of barium sulfate. If bile pigments are present in the urine sample, they adhere to the precipitate and are detected by the oxidation of bilirubin (yellow-colored) to biliverdin (green-colored) on treatment with Ferric Chloride (Fouchet's reagent) in the presence of trichloroacetic acid.

PRIMARY SAMPLE

• Use urine as specimen for the test.

• Instruct patients to collect urine samples in a clean container (preferably collected from the laboratory).

• Instruct patients to void urine directly into the container and while collecting allow the first portion of urine to escape.

• Use fresh and uncentrifuged samples for the test.

• Process the samples within 1 hour of collection. If delay is anticipated instruct patients to store the sample in the refrigerator at 2-8 °C for a maximum of up to 4 hours till transported to the laboratory.

• Do not accept if the sample volume is inadequate/or samples without an ID number

CONSUMABLES/REAGENTS

• Fouchet's reagent prepared by

• Trichloroacetic acid 25 g

• 10% Ferric chloride 10 mL

• Distilled water 50 ml (Make up to 100 mL in a volumetric flask)

• 10 % Barium chloride solution

• Test tubes

PROCEDURE

• Take 2.5 ml of urine into 12x 100 mm test tube

• Add 25 mL of barium chloride and mix well.

• Centrifuge the mixture in a centrifuge at 1500 rpm form 5 minute

• Discard the supernatant and to the deposit add 2 to 4 drops of Fouchet's reagent

INTERPRETATION OF RESULTS

No color change-bile pigments: Nil (negative)

Blue-green color-bile pigments present (positive)

PRECAUTIONS

• Handle all reagents with care and avoid contact with eye, mouth and skin.

• Handle all samples as potentially infectious

• Discard used reagents and sample as per disposal procedure

POTENTIAL SOURCES OF VARIABILITY

• If the urine is not tested fresh, bilirubin present in urine is oxidized to biliverdin which will not answer the test. Hence, the urine has to be tested fresh for bilirubin.

• Also, when urine containing bilirubin is exposed to light it is oxidized to biliverdin and will be negative for when tested for bilirubin

• Drugs like aspirin and pigmented metabolites of certain drug may give an atypical color reaction and mask the normal positive color de the presence of bilirubin

QUALITATIVE DETECTION OF BILE SALTS BY HAY'S TEST

PURPOSE: Qualitative detection of bile salts by Hay's test. This test is useful in the differential diagnosis of jaundice. Presence of Bile salt in urine indicate obstruction to the biliary passages which may be either hepatic (due to gallstones anywhere in the bile passages, carcinoma of head of pancreas and stricture of common bile duct.

PRINCIPLE: Bile salts, when present in urine, lower the surface tension of urine. When sulfur powder is added to the surface of urine, the sulfur particles sink to the test tube. In normal urine the sulfur powder floats on the surface.

PRIMARY SAMPLE

• Use urine as specimen for the test.

• Collect a minimum 15 mL of a random sample of urine.

• Instruct patients to collect urine samples in a clean container (preferably collected from the laboratory).

• Instruct patients to void urine directly into the container and while collecting allow the first portion of urine to escape.

• Use fresh and uncentrifuged samples for the test.

• Process the samples within 1 hour of collection. If delay is anticipated instruct patients to store the sample in the refrigerator at 2 -8°C for a maximum of up to 4 hours till transported to the laboratory

• Do not accept if the sample volume is inadequate/or samples without an ID number.

CONSUMABLES/REAGENTS

• Sulfur powder

• Glass test tubes

PROCEDURE

• Take 10 mL of clear urine in a test tube.

• Sprinkle a little dry sulfur powder on to the surface of the urine.

• Observe the sulfur particles.

INTERPRETATION OF RESULTS

• Sulfur powder sinks to the bottom of the test tube indicates the presence of bile salts

• Sulfur powder do not sink to the bottom of the test tube but floats on the surface of urine indicates the absence of bile salt.

PRECAUTIONS

• Handle all reagents with care and avoid contact with eye, mouth and skin.

• Handle all samples as potentially infectious.

• Discard used reagents and samples as per disposal procedure.

POTENTIAL SOURCES OF VARIABILITY

• Even a slight air current will cause the sulfur powder to sink. Hence ensure that the fan is switched off while performing the test.

QUALITATIVE DETECTION OF FREE HEMOGLOBIN IN URINE BY BENZIDINE TEST

PURPOSE: Qualitative detection of free hemoglobin from lysed RBC and myoglobin in urine by chemical examination (Benzidine test). Presence free hemoglobin indicates hemoglobinuria, myoglobinuria, renal disease like acute infections, toxic damage to kidneys, renal calculi, renal infarction, renal tuberculosis, and trauma to kidneys, Bleeding disorders and excessive anticoagulant therapy.

PRINCIPLE: The peroxidase activity of the heme portion of the hemoglobin molecule present in urine results in the liberation of active nascent oxygen from hydrogen peroxide. The liberated oxygen oxidizes benzidine in acidic medium to form a green-blue colored complex.

PERFORMANCE SPECIFICATIONS

• Do not use alkaline urine as it can cause lysis of RBCs and release of Hb from RBCs.

PRIMARY SAMPLE

• Use urine as specimen for the test.

• Collect a minimum 15 mL of a random sample of urine.

• Instruct patients to collect urine samples in a clean container collected (preferably from the laboratory).

• Instruct patients to void urine directly in to the contains and while collecting allow the first portion of urine to escape

• Use fresh and uncentrifuged samples for the test.

• Process the samples within 1 hour of collection. If delay is anticipated instruct patients to store the sample at 2-8°C in the refrigerator for a maximum of up to 4 hours till transport to the laboratory

• Do not accept if the sample volume is inadequate/or samples without an ID number.

Reagents/Consumables

• Benzidine powder

• Glacial acetic acid

• 3 % w/v Hydrogen peroxide freshly prepared and store in amber colored bottles

• Glass test tubes

PROCEDURE

• Place a pinch of Benzidine powder in a test tube.

• Add 2-3 drops of glacial acetic acid and mix well

• Add 2-3 mL of Hydrogen peroxide solution and mix well.

• Add 05 mL of previously boiled and cooled urine and mix well.

• Observe the color of the mature after 5 minutes.

INTERPRETATION OF RESULTS

• No blue or green color indicates absence of blood/free Hb/free Mb.

• Blue or green color indicates presence of blood/free Hb/free Mb.

PRECAUTIONS

• Handle all reagents with care and avoid contact with eye, mouth and skin.

• Handle all samples as potentially infectious.

• Discard used reagents and sample as per disposal procedure.

POTENTIAL SOURCES OF VARIABILITY

• False positive results may be obtained if the container is contaminated with detergents or bleach

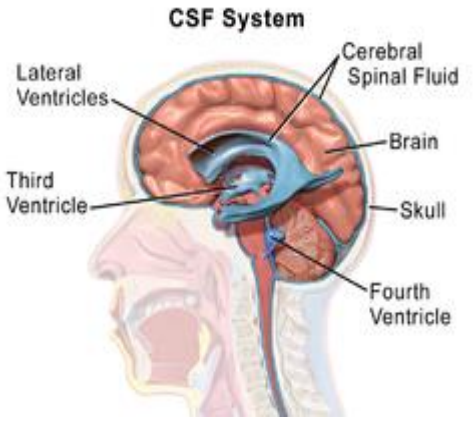
• Positive test results always have to be correlated with the presence of more than 2RBC/HPF on microscopic examination of centrifuged urine sediment.

• Although **normal centrifuged urine sediment does not contain RBCs, the finding of 1-2 RBC per HPF should not be considered as abnormal.**

SECTION 3: OTHER BODY FLUIDS

EXAMINATION OF CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is produced in the brain and serves several functions. This fluid provides physicians with a tool by which to evaluate the central nervous system (CNS). Indications for performing a lumbar puncture and CSF examination include suspicions of encephalitis, meningitis, multiple sclerosis, neurosyphilis, and subarachnoid hemorrhage, among other disorders.



Cerebrospinal fluid (CSF) is a clear, colorless body fluid found in the brain and spinal cord. It is produced by the specialized ependymal cells in the choroid plexuses of the ventricles of the brain, and absorbed in the arachnoid granulations. CSF acts as a cushion or buffer for the brain, providing basic mechanical and immunological protection to the brain inside the skull. CSF also serves a vital function in cerebral autoregulation of cerebral blood flow.

The procedure for obtaining CSF is known as a lumbar puncture.

Lumbar puncture (LP), also known as a spinal tap, is a medical procedure in which a needle is inserted into the spinal canal, most commonly to collect cerebrospinal fluid (CSF) for diagnostic testing. The main reason for a lumbar puncture is to help diagnose diseases of the central nervous system, including the brain and spine. Examples of these conditions include meningitis and subarachnoid hemorrhage.

The Procedure

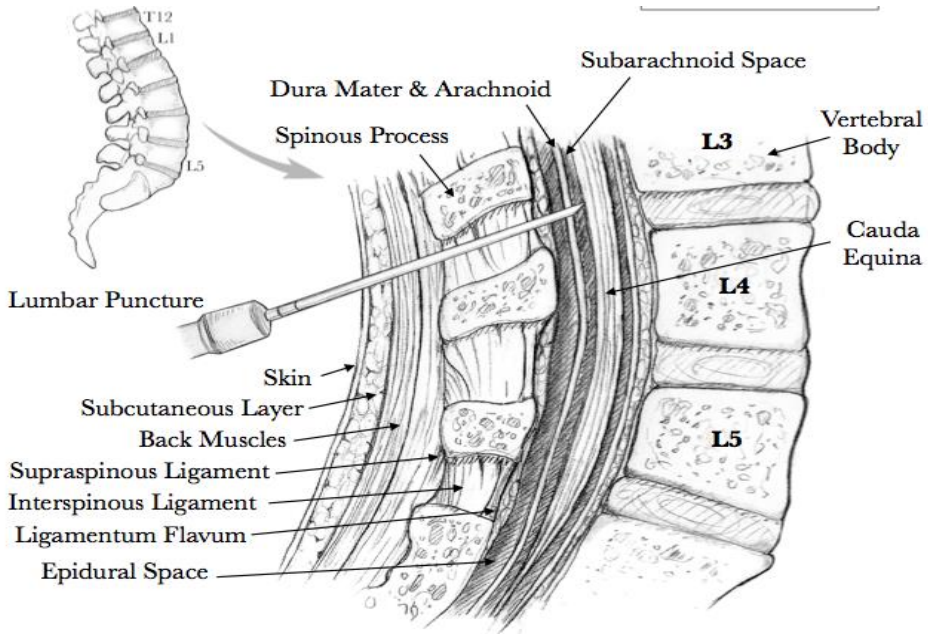
Wearing nonsterile gloves, locate the L3-L4 interspace by palpating the right and left posterior superior iliac crests and moving the fingers medially toward the spine (see the image below). Palpate that interspace (L3-L4), the interspace

above (L2-L3), and the interspace below (L4-L5) to find the widest space. Mark the entry site with a thumbnail or a marker. To help open the interlaminar spaces, ask the patient to practice pushing the entry site area out toward the practitioner.

Open the spinal tray, change to sterile gloves, and prepare the equipment. Open the numbered plastic tubes, and place them upright (see the image below). Assemble the stopcock on the manometer, and draw the lidocaine into the 10-mL syringe.

Use the skin swabs and antiseptic solution to clean the skin in a circular fashion, starting at the L3-L4 interspace and moving outward to include at least 1 interspace above and 1 below. Just before applying the skin swabs, warn the patient that the solution is very cold; application of an unexpectedly cold solution can be unnerving for the patient.

Place a sterile drape below the patient and a fenestrated drape on the patient. Most spinal trays contain fenestrated drapes with an adhesive tape that keeps the drape in place.



Use the 10-mL syringe to administer a local anesthetic. Raise a skin wheal using the 25-gauge needle, then switch to the longer 20-gauge needle to anesthetize the deeper tissue. Insert the needle all the way to the hub, aspirate to confirm that the needle is not in a blood vessel, and then inject a small amount as the

needle is withdrawn a few centimeters. Continue this process above, below, and to the sides very slightly (using the same puncture site).

This process anesthetizes the entire immediate area so that if redirection of the spinal needle is necessary, the area will still be anesthetized. For this reason, a 10-mL syringe may be more beneficial than the usual 3-mL syringe supplied with the standard lumbar puncture kit. The 20-gauge needle can also be used as a guide for the general direction of the spinal needle. In other words, the best direction in which to aim the spinal needle can be confirmed if the 20-gauge needle encounters bone in one direction but not in another.

Next, stabilize the 20- or 22-gauge needle with the index fingers, and advance it through the skin wheal using the thumbs. Orient the bevel parallel to the longitudinal Dural fibers to increase the chances that the needle will separate the fibers rather than cut them; in the lateral recumbent position, the bevel should face up, and in the sitting position, it should face to one side or the other.

Insert the needle at a slightly cephalad angle, directing it toward the umbilicus. Advance the needle slowly but smoothly. Occasionally, a characteristic "pop" is felt when the needle penetrates the dura. Otherwise, the stylet should be withdrawn after approximately 4-5 cm and observed for fluid return. If no fluid is returned, replace the stylet, advance or withdraw the needle a few millimeters, and recheck for fluid return. Continue this process until fluid is successfully returned.

For measurement of the opening pressure, the patient must be in the lateral recumbent position. After fluid is returned from the needle, attach the manometer through the stopcock, and note the height of the fluid column. The patient's legs should be straightened during the measurement of the open pressure, or a falsely elevated pressure will be obtained.

Collect at least 10 drops of cerebrospinal fluid (CSF) in each of the 4 plastic tubes, starting with tube 1. If possible, the CSF that is in the manometer should be used for tube 1. If the CSF flow is too slow, ask the patient to cough or bear down (as in the Valsalva maneuver), or ask an assistant to press intermittently on the patient's abdomen to increase the flow. Alternatively, the needle can be rotated 90° so that the bevel faces cephalad.

Replace the stylet, and remove the needle. Clean off the skin preparation solution. Apply a sterile dressing, and place the patient in the supine position.

Precautions

_ *Do not delay in testing the CSF*. Cells and trypanosomes are rapidly lysed in CSF samples. Glucose is also rapidly destroyed, unless preserved with fluoride oxalate _ *Work carefully and economically*. Often only a small quantity of CSF is available for examination. The specimen is difficult to collect so do not waste any of it.

_ *The CSF may contain virulent organisms*. Use pipettes plugged with non-absorbent cotton wool, or use a rubber safety bulb to draw up the fluid in the pipette. *Never* pipette CSF by mouth.

QUANTITATIVE ESTIMATION OF TOTAL WHITE MLOOD CELLS IN HUMAN CEREBROSPINAL FLUID

PURPOSE: Quantitative estimation of total WBC and different white blood cells in human CSF by manual method. This test is useful in the diagnoses of diseases of Central Nervous System due to inflammation of meningitis due to various causes, bacteria, viruses and fungi) and to diagnose obstruction to CSF circulation

PRINCIPLE

• Total WBC count: CSF diluting fluid removes the RBCs and stains only the WRC. CSF specimen is diluted 1:20 with pipette with the diluting fluid and the cells are counted under low power of the microscope using a Neubauer counting chamber.

• Differential count: The blood smear is prepared on a microscopic slide, which is dried, fixed and stained. The fixative does not allow change in the cells and make them adhere to the glass slide. The polychromic staining solutions containing acidic and basic dyes induce multiple colors when applied to cells. Basic components of the call stained by acidic dyes, acidic components by basic dyes and the neutral components by both the dyes. Stained cellular components are the viewed under the microscope.

PRIMARY SAMPLE

• If only cell count is requested, instruct patients to collect at least 2-3 ml of CSF in a clean dry sterile test tube preferably obtained from the laboratory.

• If biochemical analysis and culture and sensitivity tests are requested instruct to collect at least 5 mL of CSF in two separate sterile test tubes

• Process the sample within 30 minutes of collection.

• Never refrigerate the CSF specimen especially when culture and sensitivity tests are to be performed.

• For total WBC count and differential count and culture uncentrifuged CSF specimen.

CONSUMABLES/REAGENTS

• Turk's fluid. Use CSF diluting fluid only if the CSF is cloudy indicating possibility of increased leukocytes

• Leishman stain

• Leishman buffer

EQUIPMENT/INSTRUMENT

• Neubauer Counting chamber

• Light microscope

PROCEDURE

• Appearance: Note whether the uncentrifuged CSF specimen is clear, turbid, cloudy, and bloody or xanthochromic. Observe specimen of CSF for clot formation by leaving the tube for 10 minutes and again after 24 hours to observe for clot formation or appearance of Cob-Web/or leave the supernatant CSF specimen and observe for clot formation for 24 hours (after processing the sediment for cell count).

• Interpretation of results:

- Normal CSF is crystal clear and colorless.

- Purulent/Turbid /cloudy – TB or bacterial pyogenic meningitis.

- Pink/red/ Uniformly Bloody-Subarachnoid bleed /intracerebral bleed.

- Xanthochromic/ yellowish - Old hemorrhage, sever jaundice spinal cord obstruction or obstruction to the flow of CSF

- Normal CSF does not clot or coagulate

- Clot formation is always abnormal and indicates increased protein concentration (mainly due to fibrinogen which is converted to fibrin to form a clot) and is seen in tuberculous or pyrogenic meningitis and spinal cord obstruction

- Delicate and fine clot-TB meningitis

- Large clot-Pyrogenic meningitis

- Complete and spontaneous colt-Spinal cord obstruction.

• Microscopic examination (Cell count-Using Neubauer Chamber)

- Total WBC count

- Use uncentrifuged specimen for total WC count

- If the CSF is clear, use it undiluted: If the CSF is turbid / cloudy make 1:20 dilution by using

a pipette: Draw CSF up to 0.05 mL and dilute with 0.95 mL CSF diluting fluid.

- Mix well and allow to stand for 5 minutes

- Mix the CSF specimen well

-Load the Neubauer chamber with the sample and leave the chamber undisturbed for 5 minutes to allow the cells to settle on the floor of the ruled area of the chamber

- Place the chamber under the stage of the microscope.

- Count the WBCs under low power objective (x10) in 4 squares area counted is sq.mm.

CALCULATION

Total WBC/cu mm = No. of cells counted x dilution x depth/ Area counted

Dilution 1/1, depth 0.1 mm, area 4 sq mm

Undiluted CSF = No. of cells counted x 10/ 4

Diluted CSF (1:20) = No. of cells counted x 10 x 20/4

DIFFERENTIAL COUNT

- **If the CSF contain few cells: < 5cells/ mm^3**
    - **Place the CSF in the cup of cytocentrifuge chamber**
    - **Centrifuge the CSE at 200-1000 rpm for 10 min, the fluid passes through the tube in the chamber towards a glass tube glass slide and is deposited in a concentrated button about 6 mm in diameter.**
- **Fix with methanol and with Leishman stain and Leishman buffer.**
- **Count the cells under 100 x oil immersion objective**
- **If the CSF contains many cells: 5 cells/mm^3**
- **Place 1 drop of mixed, uncentrifuged CSF specimen on a clean glass slide**
- **Make a thin smear and leave to dry**
- **Fix with methanol and stain with Leishman stain and Leishman buffer**
- **Count the cells under 100 x oil immersion objective.**

INTERPRETATION OF RESULTS

- **Normal CSF contains small and lymphocytes 0-5 lymphocytes/cu mm**
- **Normal CSF does not contain neutrophils**

PRECAUTIONS

- **Ensure the counting chamber is clean and dry.**
- **Take care to ensure air bubbles do not enter while drawing blood or the diluting fluid into the pipette**
- **Count after allowing the cells to settle for 2-3 minutes and include all cells lying within the square and also those cells lying on the lines or touching the lines**
- **Use clean slides to get an even smear.**

SAFETY PRECAUTIONS

- **Handle all reagents with care and avoid contact with eye, mouth and skin**
- **Handle all samples as potentially infectious**

• Discard used reagents and sample as per disposal procedure

POTENTIAL SOURCES OF VARIABILITY

• The CSF specimen should be fresh as the blood cells and other organisms sought for diagnosis rapidly lyse on standing bacteria multiply with delay in analysis resulting in changes chemical composition

• The CSF specimen used for culture should not be refrigerated as the pathogens are killed on exposure to cold

• Jerky movement of the spreader side and the loss of contact between the spreader and the smear slide will yield poor smear

• Dirt, dust particles and yeast cells in CSF diluting fluid may cause errors in counting as it may lead to falsely elevated WBC counts. Hence take extra care during preparation and storage of CSF diluting fluid.

DETERMINATION OF GLUCOSE IN THE CEREBROSPINAL FLUID

Principle: Glucose concentrations in the CSF are normally about 60% of those in blood, i.e. 2.5–4.2mmol/l (45–75mg/100ml). In patients with meningitis (especially purulent and tuberculous meningitis), the concentration of glucose in the CSF is greatly reduced.

Method

For determination of glucose concentrations in the CSF, all methods that are used for determination of blood glucose concentrations can be applied. When the orthotoluidine method is used, four times more CSF is needed than in the test on blood.

***Important*: As the glucose in the CSF is rapidly destroyed once the fluid is collected, it is important to carry out the estimation of glucose concentration as soon as possible. If there is likely to be a delay, the CSF should be preserved in fluoride oxalate.**

DETERMINATION OF PROTEIN IN THE CEREBROSPINAL FLUID

Principle: The total protein concentration in the CSF is measured by diluting the CSF in sulfosalicylic acid and comparing the cloudiness produced against a set of

protein standards. A raised globulin level in the CSF is shown by adding the CSF to a phenol solution in the Pandy test (see below).

Materials and reagents

_ CSF: centrifuge the CSF at 2000*g* for 5 minutes and use the supernatant fluid

_ Graduated pipettes

_ Dropping pipettes

_ Test-tubes

_ Test-tube rack

_ Black cardboard

_ Sulfosalicylic acid, 3% solution

_ Pandy reagent

_ Protein standards.

Method for determination of total protein

1. Pipette 3 ml of sulfosalicylic acid into a test-tube that matches the standard tubes.

2. Add 1ml of clear CSF supernatant fluid and mix. Leave the tube for 5 minutes.

3. Compare the cloudiness of the test sample against the protein standards. Record the concentration of protein in the CSF in g/l.

The normal concentration of protein in the CSF is 100–450mg/l. The protein concentration is increased in:

— meningitis, subarachnoid hemorrhage or constriction of the spine;

— African trypanosomiasis.

Method for determination of globulin (Pandy test)

1. Measure 1ml of Pandy reagent into a small test-tube.

2. Place the tube in front of a piece of black cardboard.

3. Using a dropping pipette, slowly add three drops of CSF. Examine the solution after the addition of each drop.

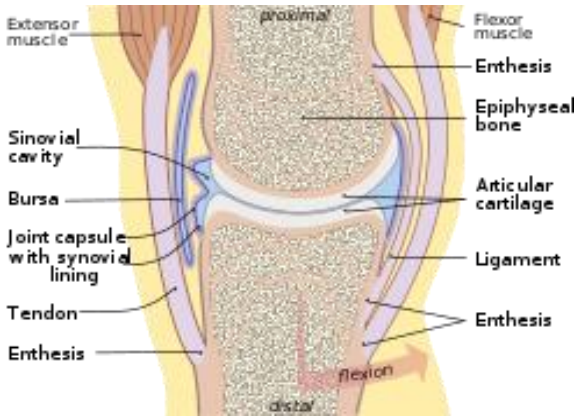
4. Read the results immediately.

If globulin is present, a white cloud forms as the drops of CSF mix with the reagent. If globulin is absent, no white cloud forms as the drops of CSF mix with the reagent, or there is a slight cloudiness that re-dissolves.

Report the test as "Pandy test positive" or "Pandy test negative".

SYNOVIAL FLUID

COLLECTION OF SYNOVIAL FLUID



Joint fluid is called synovial fluid because of its resemblance to egg white. It is a viscous, mucinous substance that lubricates most joints. Analysis of synovial fluid is important in the diagnosis of joint disease. After finding positive results with a "bulge test", the physician will perform an arthrocentesis and aspirate the effected joint. An appropriate gauge needle is attached to a syringe and the entry site is cleansed.

Synovial fluid may be collected by syringe in a procedure termed arthrocentesis, also known as joint aspiration.

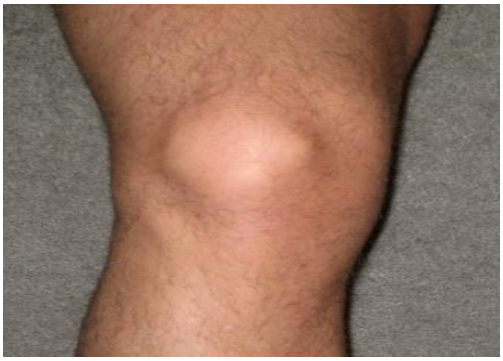
Arthrocentesis

Arthrocentesis is the clinical procedure of using a syringe to collect synovial fluid from a joint capsule. It is also known as joint aspiration. Arthrocentesis is used in the diagnosis of gout, arthritis, and synovial infections such as septic arthritis.

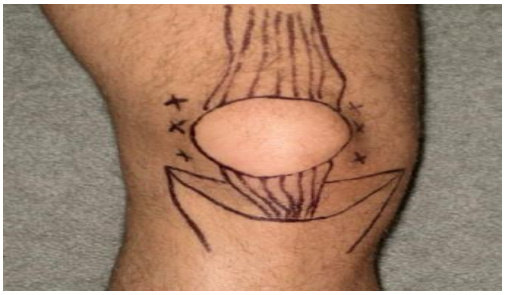
Knee Arthrocentesis Technique

Approach Considerations

The clinician performing the procedure should be familiar with the anatomy of the specific joint and cognizant of the relevant landmarks in order to avoid puncture of tendons, blood vessels, and nerves.



Anterior view of right knee.



Anatomic landmarks for knee arthrocentesis.

Aspiration of Synovial Fluid from Knee

Knee arthrocentesis may be done via a parapatellar approach (which is generally preferred), a suprapatellar approach, or an infrapatellar approach. Once the insertion site is chosen, prepare the skin with sterile solution, allow drying, and then drape.

Using a sterile technique, attach the 18- or 20-gauge needle to the 20-mL syringe, and pull the plunger in order to break resistance. Because the knee may hold up to 70 mL of fluid, using a larger (60-mL) syringe is advisable in certain cases; accordingly, an extra syringe should be available for use if necessary.

Stretch the skin over the insertion site and insert the needle briskly into the joint space while gently aspirating until synovial fluid enters the syringe (in an adult of average size, this usually occurs at 1-2 cm). Relaxation of the quadriceps muscle facilitates insertion of the needle. Placement of a towel under the popliteal region to flex the knee to 15-20° may facilitate entry by opening up the joint space.

For the parapatellar approach, identify the midpoint of either the medial or the lateral border of the patella. Insert an 18-gauge needle 3-4 mm below the

midpoint of either the medial or the lateral border of the patella (see the image below). Direct the needle perpendicular to the long axis of the femur and toward the intercondylar notch of the femur.

Left-knee aspiration via medial parapatellar approach.

For the suprapatellar approach, identify the midpoint of either the superomedial or the superolateral border of the patella. Insert an 18-gauge needle through the midpoint of either set of superior borders. Direct the needle toward the intercondylar notch of the femur. With this approach, the needle enters the suprapatellar bursa. Remember that in 10% of the population, the suprapatellar bursa does not communicate with the knee joint.

For the infrapatellar approach, position the patient sitting upright with the knee bent at 90° over the edge of the bed. Identify either side of the inferior border of the patella and the patellar tendon. Insert an 18-gauge needle 5 mm below the inferior border of the patella and just lateral to the edge of the patellar tendon. Be careful not to go through the patellar tendon while inserting the needle.

If a bone is encountered during needle insertion, pull the needle back, verify the anatomic landmarks, and advance the needle in a corrected direction.

If fluid stops flowing into the syringe, attempt to "milk" the suprapatellar region by applying gentle pressure to the region.

If removal of more fluid is desired, a hemostat can be used to secure the needle in place while the syringe is replaced with a new one.

Once aspiration is complete, the needle is removed and a bandage applied.

LABORATORY TESTING OF SYNOVIAL FLUID

Volume

The amount of fluid contained in joints is usually small. The knee joint normally contains up to 4 mL of fluid. The volume of the aspirate is usually recorded at bedside, but some laboratories may include volume in their reports as well.

Color and clarity

Normal synovial fluid is colorless and clear. Other appearances may indicate various disease states. Yellow/clear synovial fluids are typical in noninflammatory effusions, whereas yellow/cloudy fluids usually involve an inflammatory process. A white/ cloudy synovial fluid may contain crystals; and synovial fluid that is red, brown, or xanthochromic indicates hemorrhage into the joint. In addition, synovial fluid may contain various types of inclusions. Free-floating aggregates of tissue appear as rice bodies. Rice bodies are seen in rheumatoid arthritis (RA) and result from degenerated synovium enriched with fibrin. Ochronotic shards are debris from metal and plastic joint prosthesis. These shards look like ground pepper. Figure compares normal and blood synovial fluids.

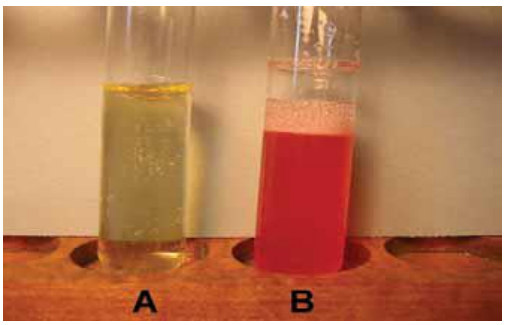


Figure. Synovial fluid. A. Normal. B. Bloody.

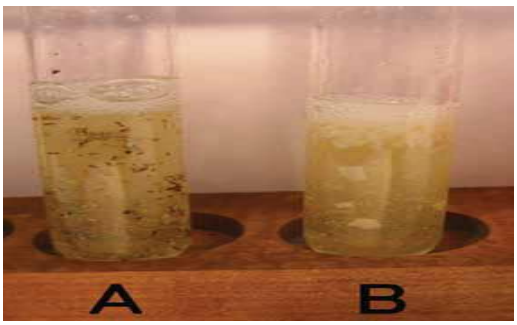


Figure. Synovial fluid inclusions. A. "Ground pepper" ochronotic shards. B. "Rice bodies" fibrin-enriched synovium fragments.

Viscosity

Synovial fluid is very viscous due to its high concentration of polymerized hyaluronate. A string test can be used to evaluate the level of synovial fluid viscosity. After removing the needle or cap from the syringe, synovial fluid is expressed into a test tube one drop at a time. Normal synovial fluid will form a

"string" approximately 5 cm long before breaking. In addition, the fluid may cling to the side of the test tube rather than running down to the bottom. Synovial fluids with poor viscosity will form shorter stings (<3 cm) or run out of the syringe and down the side of the test tube like water. Low viscosity of synovial indicates the presence of an inflammatory process.

Clotting

Clotting of synovial fluid can result when fibrinogen is present. Fibrinogen may have entered into the synovial capsule during damage to the synovial membrane or as a result of a traumatic tap. Clots in specimens interfere with performance of cell counts. Depositing part of the specimen into a tube containing heparin may help avoid clotting of synovial fluid.

Mucin clot

The mucin clot test, also known as Rope's test, is an estimation of the integrity of the hyaluronic acid–protein complex (mucin). Normal synovial fluid forms a tight ropy clot upon the addition of acetic acid. The procedure for mucin clot varies among laboratories as evidenced by differing fluid to acid ratios appearing in various texts. Clinical laboratory professionals should use the procedure adopted by their laboratories. Table below demonstrates this variability. In all cases, the interpretation of clot formation is the same. A good mucin clot indicates good integrity of the hyaluronate. A poor mucin clot, one that breaks up easily, is associated with destruction or dilution of hyaluronate.

Table. Mucin Clot Procedure According to Referenced Texts

AUTHOR	VOLUME OF SYNOVIAL FLUID	VOLUME AND STRENGTH OF ACETIC ACID
Brunzel	One part	Four parts, 2%
Ross and Neely	One part	Four parts, 2%
McBride	Two parts	One part, 3%
Strasinger	Not specified	

CHEMICAL EXAMINATION OF SYNOVIAL FLUID

Protein

Synovial fluid contains all proteins found in plasma, except various high–molecular weight proteins. These high–molecular-weight proteins include fibrinogen, beta 2 macroglobulin, and alpha 2 macroglobulin, and can be absent or present in very low amounts. Most commonly used serum protein procedures can be used to measure synovial fluid protein. The normal range for synovial fluid protein is 1–3 g/dL. Increased synovial fluid protein levels are seen in ankylosing spondylitis, arthritis, arthropathies that accompany Crohn disease, gout, psoriasis, Reiter syndrome, and ulcerative colitis.

Glucose

Synovial fluid glucose levels should be interpreted using serum glucose levels. A fasting specimen should be used or at least one 6–8 hours postprandially. Normally, synovial fluid glucose levels are less than 10 mg/dL lower than serum levels. Joint disorders that are classified as infectious demonstrate large decreases in synovial fluid glucose and can be as much as 20–100 mg/dL less than serum levels. Other groups of joint disorders demonstrate a less of a decrease in synovial fluid glucose, 0–20 mg/dL.

Uric acid

Synovial fluid uric acid normally ranges from 6 to 8 mg/dL. The presence of uric acid in synovial fluid is helpful in diagnosis gout. Usually, crystal identification is used for this determination, but synovial fluid uric acid levels may be performed in laboratories that do not a have light polarizing microscope.

Lactic acid

Lactic acid is rarely measured in synovial fluid but can be helpful in diagnosing septic arthritis. Normally, synovial fluid lactate is less than 25 mg/dL but can be as high as 1000 mg/dL in septic arthritis.

Lactate dehydrogenase

Lactate dehydrogenase (LD) can be elevated in synovial fluid, while serum levels remain normal. Synovial fluid LD levels are usually increased in RA, infectious arthritis, and gout. The neutrophils that are increased during the acute phase of these disorders contribute to this increased LD level.

Rheumatoid factor

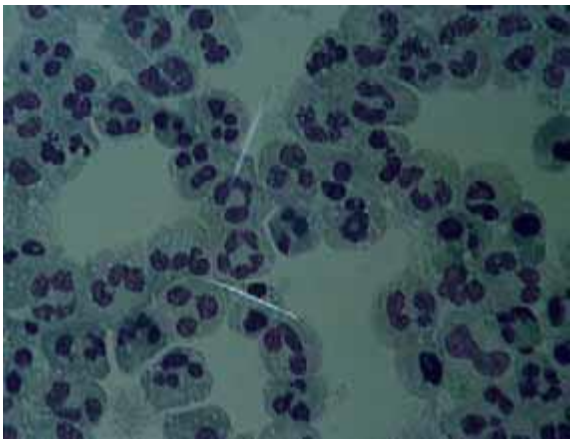
Rheumatoid factor (RF) is an antibody to immunoglobulins. RF is present in the serum of most patients with RA, whereas just more than half of these patients will demonstrate RF in synovial fluid. However, if RF is only being produced by joint tissue, synovial fluid RF may be positive while the serum RF is negative.4 False-positive RF can result from other chronic inflammatory diseases.

MICROSCOPIC EXAMINATION OF SYNOVIAL FLUID

Cell Counts

Synovial fluid cell counts, as all body fluid cell counts, should be performed within 1 hour of collection. Hemocytometer counts and manual differentials are normally performed on synovial fluid.

Crystals



Examination of synovial fluid for crystals is a routine test in most laboratories. Crystal analysis is most commonly used to diagnose gout by the presence of monosodium urate (MSU) crystals.

Figure. Synovial fluid with acute inflammation and monosodium urate crystals. (Wright–Giemsa stain and polarized light).

Other crystals that may be present in synovial fluid include calcium pyrophosphate dehydrate (CPPD) crystals.

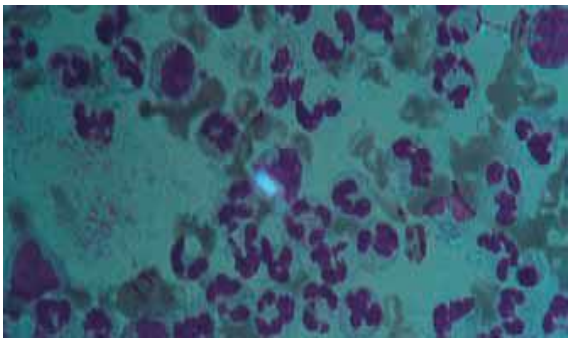


Figure. Synovial fluid with acute inflammation and calcium pyrophosphate dihydrate crystals (Wright–Giemsa stain and polarized light).

Analysis of Synovial Fluid

The aspirated synovial fluid is then analyzed (see Table below).

Table. Characteristics of Synovial Fluid on Analysis

	Appearance	WBCs, cells/µL	PMN cells	Glucose concentration, mg/dL	Protein concentration, g/dL
Normal	Clear	<150	<0.25	Serum glucose	1.3-1.8
Non inflammatory	Clear	<3000	<0.25	Serum glucose	2-3.5
Inflammatory	Cloudy	>3000	<0.75	<25	>4
Purulent	Cloudy	>50,000	>0.9	<25	>4
Hemorrhagic	Bloody	>2000	~0.3	Serum glucose	…

PMN = polymorphonuclear; WBC = white blood cell.

Synovial fluid characteristics associated with septic joint effusion are as follows:

- **Total white blood cell (WBC) count higher than 25,000/µL (sensitivity, 77%; specificity, 73%; positive likelihood ratio [LR $^{+}$], 29; negative likelihood ratio [LR $^{-}$], 0.71)**
- **Total WBC count higher than 50,000/µL (sensitivity, 62%; specificity, 92%; LR $^{+}$, 7.7; LR $^{-}$, 0.42)**
- **Total WBC count higher than 100,000/µL (sensitivity, 22%; specificity, 99%; LR $^{+}$, 2.9; LR $^{-}$, 0.32)**
- **PMN cell proportion 0.9 or higher (sensitivity, 73%; specificity, 79%; LR $^{+}$, 3.4; LR $^{-}$, 0.34)**
- **Synovial glucose or serum glucose concentration lower than 0.5 (sensitivity, 51%; specificity, 85%; LR $^{+}$, 3.4; LR $^{-}$, 0.58)**
- **Protein concentration higher than 3 g/dL (sensitivity, 48%; specificity, 46%; LR $^{+}$, 0.9; LR $^{-}$ -, 1.1)**
- **Lactic dehydrogenase (LDH) concentration higher than 250 U/L (sensitivity, 100%; specificity, 51%; LR $^{+}$, 1.9; LR $^{-}$, 0.1)**

SEMEN ANALYSIS

The most common parameters evaluated during a semen analysis include semen volume, viscosity, pH, and sperm concentration, motility, viability, and morphology. Other indications for performing a semen analysis include determining the effectiveness of a vasectomy, rape-case forensic studies, sperm donor evaluation, and paternity cases.

SPECIMEN COLLECTION AND HANDLING

The preferred method of semen collection is by masturbation. This procedure ensures the opportunity to collect the entire ejaculate. containers should be clean glass or plastic and have a wide opening.

MACROSCOPIC EXAMINATION

■ *Liquefaction*. Once the specimen arrives in the laboratory, it is observed for liquefaction time. Normal liquefaction occurs between 30 and 60 minutes. Liquefaction times beyond 60 minutes are considered abnormal. Specimens that

do not liquefy must be treated with amylase or bromalin to break up mucus in order to obtain accurate sperm counts.

■ *Appearance*. Semen is opaque and can exhibit several normal colors. Typical colors include gray, white, and light yellow. The higher the flavin concentration of semen, the darker the yellow color may be. A highly turbid semen specimen usually contains leukocytes and may indicate a reproductive tract infection or inflammation.

■ *Volume*. Semen volume is measured by using a serological pipette, or small graduated cylinder. Volume is recorded in milliliters to one decimal place (0.1 mL). Normal semen volume ranges from 2 to 5 mL for a complete ejaculate. Volumes both lower and higher than this range have been associated with infertility.

■ *Viscosity*. Viscosity may be assessed while measuring specimen volume or when pipetting the specimen for other tests. Normal semen is slightly viscous and dispenses drop by drop. Increased viscosity is demonstrated by the formation of a string of fluid as the specimen is dispensed from a pipette.

SPERM CONCENTRATION

Automated methods for counting sperm are available, however most laboratories use manual hemocytometer counting techniques. A manual dilution of 1:20 using distilled water to immobilize sperm may be used. A platelet Unopette dilution of 1:100 is used by some laboratories as an alternative to manual dilutions with a volume displacement pipette. Professional judgment should be used when determining the area to count on the hemocytometer. The center square millimeter may be sufficient for accurate counts when the sperm concentration is high.

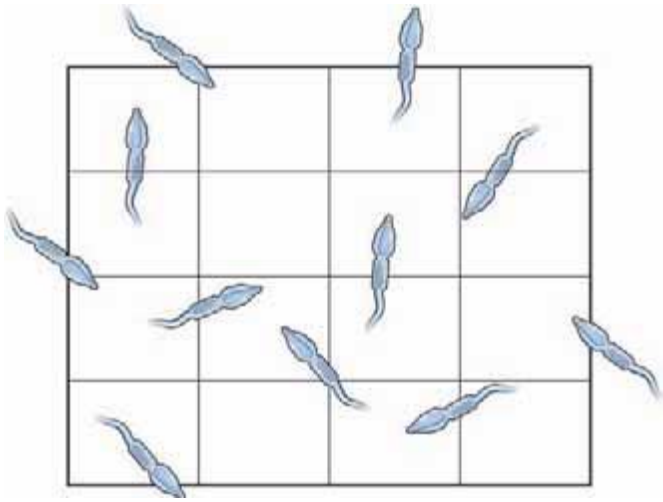


Figure. Inclusion criteria of counting cells. Count cells (sperm heads, not tails) that touch the upper and left boarders of the counting grid. Do not count cells (sperm heads, not tails) that touch the lower and right boarders of the counting grid. Count only complete sperm.

When using a Neubauer hemocytometer, the simplified formula allows for rapid calculation of sperm concentration: *C* = *N* _X *D* X 10/*A*, where *C* is the concentration, *N* is the number of sperms counted, *D* is the dilution factor, and *A* is the area in square millimeters (not number of squares). For example, if the number of sperm counted in nine square millimeters on a 1:100 dilution is 25, the calculation is (25 X 100 X 10)/9 = 2778/mm^3. Sperm concentration is often reported in number per cubic centimeter (cc) or milliliters (mL). Therefore, multiplying by 1000 is necessary to convert the count to the correct unit. In this example, the final count is 2,778,000/cc. Normal sperm concentrations have been reported to range between 20 and 250 million per milliliter.

MOTILITY

Fertilization of an ovum is dependent on the ability of sperm to reach and unite with it. Motility should be evaluated within 1 hour of specimen collection, because motility will decrease over time. One way to evaluate sperm motility is to place a small drop of liquefied semen on a prewarmed slide and cover slipped. Observation of sperm movement is best performed on high dry (45X).

The movement of sperm is evaluated and may be subjectively estimated or counted into three categories. These categories may be called high-motile, low-motile, and nonmotile; or progressive, nonprogressive, and nonmotile. Some laboratories may use as many as five categories: nonmotile, nonprogressive, slow nonlinear progression, moderate linear progression, and strong linear

progression. Some laboratories report the percent of sperm in each category, whereas others report only the percent of motile sperm. At least 80% of the sperm demonstrate some forward progress in a normal semen sample.

More recent use of technology for sperm evaluation includes the use of high-resolution video photography in combination with computer programs that can calculate velocity, linear progression, and motility efficiency and measure patterns of sperm motion.

AGGLUTINATION

Agglutination may be observed while evaluating a wet mount of semen for sperm motility. A few clumps of sperm or sperm sticking to mucus or other cells can normally be seen in a semen sample. However, true agglutination is present if sperm are distinctly clumped head to head or tail to tail, which may indicate the presence of antisperm antibodies. Both IgG and IgA antibodies have been found in the semen of some men with reduced fertility whose sperm demonstrate agglutination. Confirmation with immunologic tests can help determine the specific type of antibody.

VIABILITY

Determining whether nonmotile sperm are viable or nonviable is important in establishing a cause for infertility in males. The membranes of dead sperm are damaged and can easily take up eosin stain. The membranes of viable sperm remain intact and do not allow eosin stain to penetrate, leaving the sperm colorless (they will appear white). Eosin stain can be used alone or in conjunction with nigrosine stain. Nigrosine provides a dark background against which the red-colored dead sperm and the white or colorless sperm can be visualized.

Viable sperm do not take up the eosin stain and remain colorless, thus appearing white while, nonviable sperm take up the eosin stain and appear various shades of red.

At least 100 sperm heads are counted into two categories: red = dead and white = viable. The percent of viable sperm is reported. Normally, > 75% of sperm are viable.

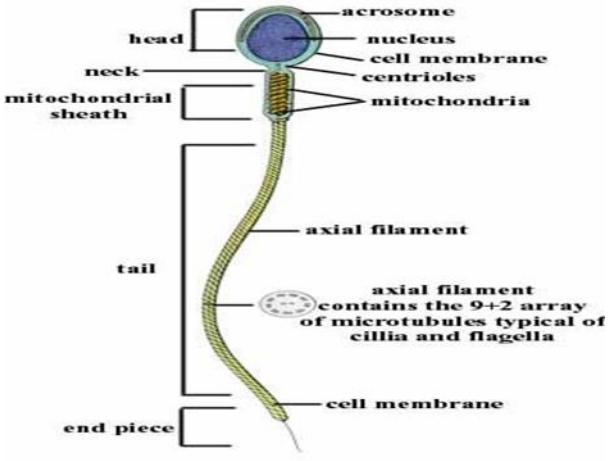
PENETRATION

A procedure for evaluating sperm penetration that is used in the laboratory setting involves the use of bovine cervical mucus. Bovine mucus is commercially available and is contained frozen in flat glass capillary tubes, which are scored at

one end. These tubes are thawed upright with the scored end up to assist with the elimination of air bubbles from the test area. Once thawed and opened at the score marks, the opened ends of the tubes are placed in a sample cup that contains 0.2 mL of fresh semen. This set-up is allowed to incubate at room temperature for 90 minutes. Placing the penetration test set-up inside a closed cabinet helps keep it free from drafts that may alter its temperature. After incubation, the capillary tubes are removed from the specimen, placed on a ruled slide, and observed microscopically. The distance obtained by the vanguard sperm (the sperm that traveled the greatest distance) is recorded for both tubes and the average calculated. Normal sperm should be able to penetrate bovine cervical mucus to at least a distance of 30 mm.

SPERM MORPHOLOGY

Sperm morphology is evaluated by preparing a stained smear of semen and counting and categorizing all forms of sperm seen. The smear may be made by placing a drop of semen on a slide, placing another slide on top, and pulling them apart in opposite directions. The smear may be fixed with a cytology fixative and then stained with Papanicolaou stain. Giemsa or Wright stain may also be used. Sperm morphologies are classified by counting 100–200 sperm using oil immersion. Values for the minimum number of normal sperms vary according to individual laboratories' evaluation criteria. Minimum normal forms for sperm morphology may be >30 to >70%.



Abnormal sperm morphology occurs as an anomaly of either the head or the tailpiece, or both. Head anomalies include acrosomal abnormalities, constricted heads, double headed or double-nucleated heads, enlarged or pinheads, nuclear abnormalities, and vacuolation. Tailpiece anomalies include coiled tailpiece,

cytoplasmic extrusion mass, lengthened or bent neckpiece, midpiece abnormalities, multiple tails, and variation in tail length. In addition, immature forms of sperm may be present.

CHEMICAL ANALYSIS

■ *pH*. The pH of semen should be measured within an hour of collection because semen can become either more acidic (lactic acid production with high sperm counts) or more alkaline (loss of CO2 over time) as the specimen ages. Nitrozine paper is the simplest way to measure semen pH. The pH of fresh semen normally ranges from 7.2 to 7.8.

■ *Acid Phosphatase*. Semen acid phosphatase is used to evaluate the secretory function of the prostate. Normal levels of acid phosphatase are equal or greater than 200 units per ejaculate.

■ *Fructose*. Fructose provides energy for spermatozoa. Semen fructose is produced by the seminal vesicles, with normal levels being equal or greater than 13 µmol per ejaculate and comprises 99% of reducing sugar found in semen.

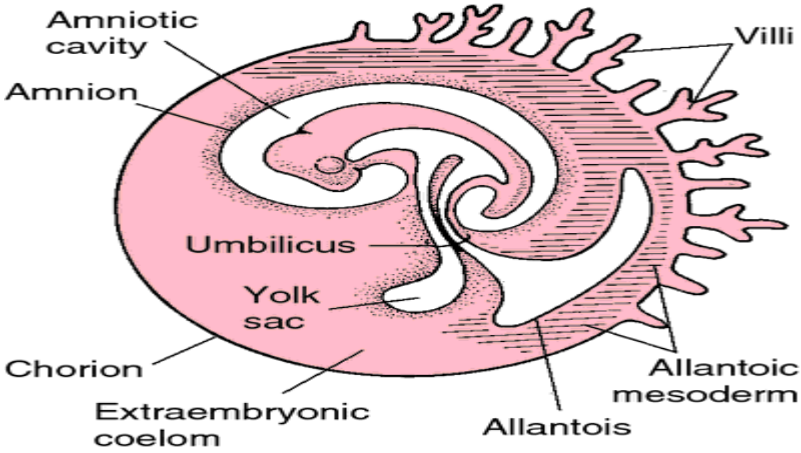
■ *Hormones*. Measuring the level of various hormones is helpful in differentiating among causes of azoospermia. These hormones include testosterone, LH, and FSH. Hyalinization of the seminiferous tubules is accompanied by a decreased to normal testosterone level with an increase in both LH and FSH. Gonadotropin deficiency demonstrates decreased levels of all three of these hormones. In Sertoli-cell-only syndrome, the testosterone and LH levels are normal while FSH is increased. These hormone levels are normal if the cause of azoospermia is ductal obstruction or maturation arrest.

IMMUNOLOGY

Autoimmune antibodies to sperm can form if trauma or infection causes a breakdown of the barrier between sperm and blood. These antibodies are present in both serum and semen. Women can develop isoantibodies to their husbands' sperm. These antibodies may be individual specific or may be reactive to all human spermatozoa. Immunologic testing for antisperm antibodies can be performed as a confirmation when agglutination of sperm is present. Several methods currently exist to test for antisperm antibodies. The Kibrick method involves incubating fresh, liquefied semen with serum from the male or serum from his female partner. Agglutination is observed macroscopically. The Isojima method tests for sperm-immobilizing antibody. Comparison is made between sperm motility of fresh, liquefied semen and that of seamen incubated with either rabbit or guinea pig complement. A sperm immobilization value is

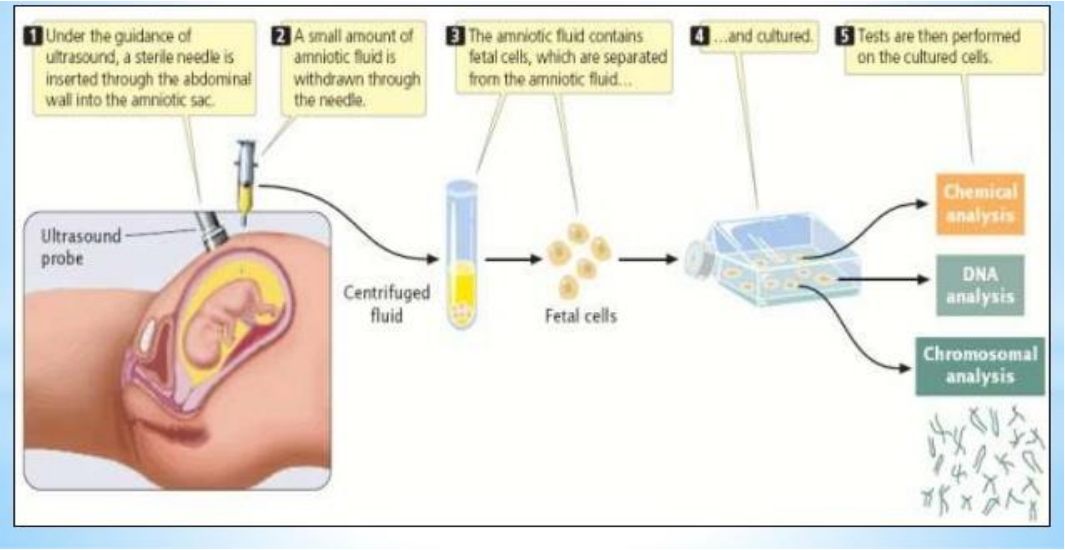
calculated by dividing the percent of motile sperm in the fresh specimen by the percent of motile sperm in the incubated sample. A value of 2 indicates the presence of antibodies. Immunobead assays are used to detect the presence of sperm antibodies on the surface of sperm. These assays can determine whether antisperm antibodies are directed against head, midpiece, or tail and whether the antibodies are IgA, IgG, or IgM. In addition, the immunobead assay method allows for calculating the proportion of sperm in an ejaculate that is antibody bound. Enzyme-linked immunosorbent assay (ELISA) techniques can be used to detect antibodies to prostasomes (prostate-secreted organelles that adhere to spermatozoa).

AMNIOTIC FLUID



Amniotic fluid is found around the developing fetus, inside a membranous sac, called the amnion. The laboratory performs several crucial tests on amniotic fluid to assess the status of the fetus. These tests can be divided into these groups: (a) tests to diagnose genetic and congenital disorders before birth, (b) tests to detect fetal distress from hemolytic disease of the newborn (HDN) or from infection, (c) tests to assess fetal lung maturity, and (d) assessment of the ability of the fetus to survive early delivery.

AMNIOCENTESIS, SPECIMEN COLLECTION AND HANDLING



Amniotic fluid is obtained by needle aspiration into the amniotic sac, usually transabdominally with simultaneous use of ultrasound. The addition of ultrasound has helped make this procedure safer especially if performed after 14 weeks of gestation. Amniocentesis is generally performed between 15 and 18 weeks of gestation for genetic studies although it may be used later in the pregnancy in cases of fetal distress.

The amount collected is usually 10–20 mL (maximum 30 mL), with collection into several different syringes to prevent the contamination of all specimens with the blood from initial puncture. Immediately after collection, the fluid is dispensed into sterile plastic specimen containers. Glass containers are less desirable as cells have more of a tendency to adhere to the glass surface. Normal amniotic fluid is colorless to pale yellow and slightly turbid due to fetal cells, vernix, and hair. Specimens for cell culture and chromosomal studies must be stored at body or room temperature to keep fetal cells alive. Specimens for phospholipid analysis should be transported on ice and centrifuged at 500g and the supernatant saved for testing. If blood is present, the specimen should be centrifuged to prevent hemolysis from altering the test results. All amniotic fluid samples for chemical analysis that must be stored for any length of time must be centrifuged. If samples for chemical analysis need to be stored more than 24 hours, they must be stored frozen.

DIFFERENTIATION OF AMNIOTIC FLUID FROM MATERNAL URINE

A microscopic test, the fern test, is also used to differentiate amniotic fluid from maternal urine. With this test, vaginal fluid is spread out on a glass slide and allowed to dry at room temperature. This slide is observed for fernlike crystals that are a positive screen test for amniotic fluid.

PHYSICAL AND MICROSCOPIC EXAMINATION OF AMNIOTIC FLUID

Normal amniotic fluid is colorless to pale yellow and slightly cloudy. A dark yellow or amber color is associated with bilirubin, whereas a green color indicates meconium, the newborn's first fecal bowel movements. Blood can appear as pink or red and the source of the blood, whether fetal or maternal, can be distinguished by the Kleihauer-Betke test for fetal hemoglobin. A very dark red-brown amniotic fluid is associated with fetal death.

TESTING AMNIOTIC FLUID FOR GENETIC AND CONGENITAL DISORDERS

Valuable cytogenetic information related to the sex of the fetus and to genetic abnormalities can be obtained via amniocentesis. Congenital neural tube disorders can also be detected by amniotic fluid analysis. Amniocentesis is often performed to detect Down syndrome and anencephaly prior to birth.

FETAL DISTRESS TESTING

HDN, also known as erythroblastosis fetalis, is caused when mother develops antibodies to an antigen on the fetal erythrocytes and these maternal antibodies cross the placenta to destroy many fetal red blood cells (RBCs). The destruction of these fetal RBCs results in the appearance of elevated unconjugated bilirubin in the amniotic fluid. Measurement of amniotic fluid bilirubin is performed through spectrophotometric analysis.

Infection

Evidence is mounting of the importance of microorganisms in the amniotic fluid contributing to the incidence of preterm delivery and spontaneous abortion.4 Even bacterial vaginosis and trichomoniasis have been linked to preterm birth. Gram stain, wet mount, culture, and molecular tests may be used on amniotic fluid to look for potential infectious agents.

Respiratory Distress Syndrome

Several fetal lung tests are available to assess fetal lung maturity before birth in order to prevent respiratory distress syndrome by determining the best time for preterm delivery. These tests are: Lecithin: Sphingomyelin Ratio and Phosphatidylglycerol, Amniostat-FLM, Foam Stability, Microviscosity Fluorescence Polarization Assay, Lamellar Bodies and Assessment of Fetal Risk and Survivability with Premature Delivery

SECTION 4: CANCER DETECTION

ENZYME IMMUNOASSAY FOR ALPHA-FETOPROTEIN, CARCINOEMBRIONIC ANTIGEN, TOTAL PROSTATIC SPECIFIC ANTIGEN CANCER: PANEL TEST SYSTEM

Intended Use: The Quantitative Determination of AFP, CEA and PSA Concentration in Human Serum and Plasma by a Microplate Enzyme Immunoassay, Colorimetric. Measurements of these tumor markers are used as an aid in the diagnosis and monitoring of various oncological disorders.

SUMMARY AND EXPLANATION OF THE TEST

Alpha-Fetoprotein (AFP) is a glycoprotein with a molecular weight of 70 kDA. AFP is normally produced during fetal development by the hepatocytes, yolk sac and, to a lesser extent, by the gastrointestinal tract. Serum concentrations reach a peak level of up to 10 mg/ml at twelve weeks of gestation.[1] This peak level gradually decreases to less than 25 ng/ml after one year of postpartum. Thereafter, the levels reduce further to less than 10 ng/ml.

Elevated levels of AFP are found in patients with primary heptatoma and yolk sac-derived germ tumors. AFP is the most useful marker for the diagnosis and management of hepatocellular carcinoma.

AFP is also elevated in pregnant women. Presence of abnormally high AFP concentrations in pregnant women provides a risk marker for Down syndrome.

Carcinoembryonic antigen (CEA) is a glycoprotein with a molecular weight of 180 kDA. CEA is the first of the so-called carcinoembryonic proteins is the most widely used marker for gastrointestinal cancer.

Although CEA is primarily associated with colorectal cancers, other malignancies that can cause elevated levels of CEA include breast, lung, stomach, pancreas, ovary and other organs. Benign conditions that cause significantly higher than normal levels include inflammation of lung and gastrointestinal (GI) tract and benign liver cancer. Heavy smokers, as a group, have higher than normal baseline concentration of CEA.

Prostate Specific Antigen (PSA) is a serine protease with chymotrypsin-like activity. The protein is a single chain glycoprotein with a molecular weight of 28.4 kDA. PSA derives its name from the observation that it is a normal antigen of the prostrate, but is not found in other normal or malignant tissue.

PSA is found in benign, malignant and metastatic prostate cancer. Since prostate cancer is the second most prevalent form of male malignancy, the detection of elevated PSA levels plays an important role in the early diagnosis.

PRINCIPLE

Immunoenzymometric assay:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-marker specific antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

MATERIALS

Reagents for 2 X 96 well Microplate

A. Combi-Cal™ CEA/AFP/PSA Calibrators

Six (6) vials of references for markers at levels indicated below. A preservative has been added. The calibrators, human serum based, were calibrated using a reference preparation indicated in the chart.

Analyte	AFP (ng/ml)	CEA (ng/ml)	tPSA (ng/ml)
A	0	0	0
B	5	5	2
C	25	10	5
D	100	25	10
E	250	100	25
F	500	250	50
Ref #	1st IRP AFP	IRP 73/601	1st IS 96/670

B. AFP Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for AFP in buffer, yellow-orange dye, and preservative. Store at 2-8°C.

C. CEA Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for CEA in buffer, yellow dye, and preservative. Store at 2-8°C.

D. VAST tPSA Enzyme Reagent

One (1) vial containing enzyme labeled antibody and biotinylated monoclonal mouse IgG specific for PSA in buffer, orange dye, and preservative. Store at 2-8°C.

E. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

F. Substrate Solution 'A'

Two (2) vials containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C. (See Reagent Preparation)

G. Substrate Solution 'B'

Two (2) vials containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C. (See Reagent Preparation)

H. Streptavidin Coated Microwells

Two 96-well microplates coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

I. **Stop Solution**

Two (2) vials containing a strong acid (1N HCl). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.

Materials Required But Not Provided:

1.Pipette(s) capable of delivering 0.025, 0.050 & 0.100ml (25, 50 & 100µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive delivery of 0.300ml (300µl) volume with a precision of better than 1.5% (optional).

3.Microplate washer or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability (620nm filter is optional).

5.Container(s) for mixing of reagents (see below).

6.Absorbent Paper for blotting the microplate wells.

7.Plastic wrap or microplate cover for incubation steps.

8.Vacuum aspirator (optional) for wash steps.

9.Timer.

10.Storage container for storage of wash buffer.

11.Distilled or deionized water.

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type, and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required for each tumor marker assayed.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of Wash Concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.

2.Working Substrate Solution – Stable for one (1) year.

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled solution 'B.' Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (*20 - 27° C*).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Select the number of coated wells needed by formatting the microplate for each calibrator, control and patient sample to be tested. Return unused wells and strips to the foil bag, seal and store it at 2-8°C.

2.Pipette 0.025ml (25µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3.Add 0.100ml (100µl) of the appropriate enzyme reagent to each well. It is very important to use the correct 'Enzyme Reagent' for each assay for accurate results.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 60 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100ml (100µl) of working substrate solution to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix by rotation so that a uniform yellow color is obtained.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding stop solution.

Note: It is very important to dispense all reagents in the center of the coated well. Always add reagents in the same order to minimize reaction time differences between wells.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of each corresponding marker in unknown specimens.

1.Record the absorbance obtained from the printout of the microplate reader

2.Plot the absorbance for each duplicate serum reference versus the corresponding marker concentration in appropriate units on linear graph paper (do not average the duplicates of the seru m references before plotting).

3.Draw the best-fit curve through the plotted points.

4.To determine the concentration of corresponding cancer marker for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph.

Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXPECTED VALUES (AFP, CEA & tPSA)

A study of an apparent normal adult population was undertaken to determine expected values for the Cancer Panel VAST® AccuBind® ELISA test system. A total number of 486 apparently normal samples were taken for the study to establish values for these analytes. The expected values are presented in Table 1.

TABLE 1

Expected Values for the Cancer Panel VAST®

Adult Population	AFP (ng/ml)	CEA (ng/ml)	tPSA (ng/ml)
Smokers	< 8.5	< 10.0	< 4.0
Non-Smokers	< 8.5	< 5.0	< 4.0

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range

can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR CANCER ANTIGEN 125 TEST SYSTEM

Intended Use: The Quantitative Determination of Cancer Antigen 125 (CA-125) Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Cancer Antigen 125 (CA-125) is a glycoprotein that occurs in blood as high molecular weight entity (*M*Br$_B$ > *200,*000). High concentrations of this antigen are associated with ovarian cancer and a range of benign and malignant diseases. Although the specificity and sensitivity of CA-125 assays are somewhat limited, especially in early diagnosis of ovarian cancer, the assay has found widespread use in the differential diagnosis of adnexal masses, in monitoring disease progression and response to therapy in ovarian cancer, and in the early detection of recurrence after surgery or chemotherapy for ovarian cancer. Published literature has shown that elevated serum CA-125 levels can be observed in patients with serious endometroid, clear cell and undifferentiated ovarian carcinoma. The serum CA-125 is elevated in 1% of normal healthy women, 3% of normal healthy women with benign ovarian diseases, and 6% of patients with non-neoplastic conditions (including, but not limited to, first trimester pregnancy, menstruation, endometriosis uterine fibrosis, acute salpingitis, hepatic diseases and inflammation of peritoneum or pericardium).

In this method, CA-125 calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of CA-125) are added and the reactants mixed. Reaction between the various CA-125 antibodies and native CA- 125 forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-CA-125 antibody bound conjugate is separated from the unbound enzyme-CA-125 conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of CA-125 levels permits the construction of a dose response curve of activity and concentration. From

comparison to the dose response curve, an unknown specimen's activity can be correlated with CA-125 concentration.

PRINCIPLE

Immunoenzymometric assay:

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-CA-125 antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, a reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A. CA-125 Calibrators

Six (6) vials of references CA-125 Antigen at levels of 0(A), 15(B), 50(C), 100(D), 200(E) and 400(F) U/ml. A preservative has been added. Store at 2-8°C.

Note: The human serum-based standards were made using a >99% pure affinity purified preparation of CA-125. The preparation was calibrated against Centocor CA-125 IRMA test.

B. CA-125 Enzyme-Reagent

One (1) vial containing enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Streptavidin Coated Plate –

One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D. Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E. Substrate A

One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C. See "Reagent Preparation."

F. Substrate B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C. See "Reagent Preparation."

G. Stop Solution

One (1) vial containing a strong acid (1N HCl). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.
Note 3: Above reagents are for a single 96-well microplate
Materials Required but Not Provided:

1.Pipette capable of delivering 0.025 & 0.050ml (25 & 50µl) volumes with a precision of better than 1.5%.

Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.

2.Microplate washers or a squeeze bottle (optional).

3.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

4.Absorbent Paper for blotting the microplate wells.

5.Plastic wrap or microplate cover for incubation steps.

6.Vacuum aspirator (optional) for wash steps.

7.Timer.

8.Quality control materials

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e.>5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store diluted buffer at 2-30°C for up to 60 days.

2.Working Substrate Solution – Stable for one (1) year

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025ml (25µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3.Add 0.100ml (100µl) of the CA-125 Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 60 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of CA-125 in unknown specimens.

1.Record the absorbance obtained from the printout of the microplate reader

2.Plot the absorbance for each duplicate serum reference versus the corresponding CA-125 concentration in U/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3.Draw the best-fit curve through the plotted points.

4.To determine the concentration of CA-125 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in U/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.331l) intersects the dose response curve at 29.3U/ml CA-125 concentration.

Note: Computer data reduction software designed ELISA assays may also be used for the data reduction. ***If such software is utilized, the validation of the software should be ascertained.***

EXPECTED RANGE OF VALUES

The serum CA-125 is elevated in 1% of normal healthy women, 3% of normal healthy women with benign ovarian diseases and 6% of patients with non-neoplastic conditions (including but not limited to first trimester pregnancy, menstruation, endometriosis uterine fibrosis, acute salpingitis, hepatic diseases and inflammation of peritoneum or pericardium).

TABLE I

Expected Values for CA-125 ELISA Test System

Healthy and non-pregnant subjects	**U≤U 35 U/ml**

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

ENZYME IMMUNOASSAY FOR CARCINOEMBRYONIC ANTIGEN

Intended Use: The Quantitative Determination of Carcinoembryonic Antigen (CEA) Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric.

SUMMARY AND EXPLANATION OF THE TEST

Carcinoembryonic antigen (CEA) is a glycoprotein with a molecular weight of 180 kDA. CEA is the first of the so-called carcinoembryonic proteins that was discovered in 1965 by Gold and Freeman. CEA is the most widely used marker for gastrointestinal cancer.

Although CEA is primarily associated with colorectal cancers (CRC), other malignancies that can cause elevated levels of CEA include breast, lung, stomach, pancreas, ovary and other organs. Benign conditions that cause significantly higher than normal levels include inflammation of lung and gastrointestinal (GI) tract and benign liver cancer. Heavy Smokers, as a group, have higher than normal baseline concentration of CEA. Serum values in healthy adults are normally ≤ 5.0 ng/ml however, serum values exceeding 5 times the normal reference range are taken as indicative of malignancy. Also, values seen in malignant and non- malignant conditions can overlap thus making CEA a not very dependable marker for malignancy. However, the real importance of CEA testing lies in patient prognosis, status assessment and monitoring. Monitoring CEA levels during chemotherapy and before surgery can be informative; the

failure of CEA levels to fall during pre-operative radiotherapy usually indicates the presence of a tumor outside the field of radiation and a poor prognosis. Levels have been seen to drop to normal in 4-6 weeks after a successful resection of CRC.

In this method, CEA calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies, directed against distinct and different epitopes of CEA, are added then the reactants mixed. Reaction between the various CEA antibodies and native CEA forms a sandwich complex that binds with the streptavidin coated to the well.

After the completion of the required incubation period, the enzyme-CEA antibody bound conjugate is separated from the unbound enzyme-CEA conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce color.

The employment of several serum references of known carcinoembryonic antigen (CEA) levels permits the construction of a dose response curve of activity versus concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with CEA concentration.

PRINCIPLE
Immunoenzymometric assay:
The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-CEA antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme- labeled antibody and a serum containing the native antigen, a reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen

concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

REAGENTS

Materials Provided:

A.Carcinoembryonic antigen (CEA)

Six (6) vials of references CEA Antigen at levels of 0(A), 5(B), 10(C), 25(D), 50(E) and 250(F) ng/ml. Store at 2-8°C. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the 1st International Reference Preparation (IRP# 73/601).

B.CEA Enzyme Reagent

One (1) vial containing enzyme labeled antibody, biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C.Streptavidin Coated Plate

One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

D.Wash Solution Concentrate

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

E.Substrate A

One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

F.Substrate B

One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

G.Stop Solution

One (1) vial containing a strong acid (1N HCl). Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

Material Required but Not Provided:

1.Pipette(s) capable of delivering 0.025ml (25µl) and 0.050ml (50µl) volumes with a precision of better than 1.5%.

2.Dispenser(s) for repetitive deliveries of 0.100ml (100µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.

3.Microplate washers or a squeeze bottle (optional).

4.Microplate Reader with 450nm and 620nm wavelength absorbance capability.

5.Absorbent Paper for blotting the microplate wells.

6.Plastic wrap or microplate cover for incubation steps.

7.Vacuum aspirator (optional) for wash steps.

8.Timer.

9.Quality control materials

PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

REAGENT PREPARATION

1.Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at 2-30°C for up to 60 days.

2.Working Substrate Solution – Stable for one year

Pour the contents of the amber vial labeled Solution 'A' into the clear vial labeled Solution 'B'. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2 - 8°C.

Note 1: Do not use the working substrate if it looks blue.

Note2: Do not use reagents that are contaminated or have bacteria growth.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C).

*****Test Procedure should be performed by a skilled individual or trained professional*****

1.Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.

2.Pipette 0.025 ml (25µl) of the appropriate serum reference calibrator, control or specimen into the assigned well.

3.Add 0.100 ml (100µl) of the CEA Enzyme Reagent to each well. It is very important to dispense all reagents close to the bottom of the coated well.

4.Swirl the microplate gently for 20-30 seconds to mix and cover.

5.Incubate 60 minutes at room temperature.

6.Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with

7.Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8.Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

9.Incubate at room temperature for fifteen (15) minutes.

10.Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.

11.Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Carcinoembryonic antigen in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.

2. Plot the absorbance for each duplicate serum reference versus the corresponding CEA concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).

3. Draw the best-fit curve through the plotted points.

4. To determine the concentration of CEA for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration

(in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.391 Abs) intersects the dose response curve at (22.5 ng/ml) CEA concentration

EXPECTED RANGES OF VALUES

Nearly 99% of non-smokers have CEA concentrations less than 5ng/ml. Similarly, 99% of smokers have concentrations less than 10ng/ml.

TABLE I

Expected Values for the CEA Elisa Test System

Non-smokers	**<5ng/ml**	**Smokers**	**<10ng/ml**

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

QUANTITATIVE ESTIMATION OF ACID PHOSPHATASE

Purpose: Quantitative estimation of acid phosphatase in serum testing for the released phenol from the substrate disodium phenyl phosphate in acid pH. The levels are helpful in diagnosis and therapy of certain types of cancers especially metastasizing prostatic cancer.

Principle: Disodium phenyl phosphate in hydrolyzed by acid phosphatase at pH 5.0 with liberation of phenol and formation of sodium phosphate. The phenol thus produced reacts with amine antipyrine and gives red or purple color, which could be measured spectrophotometrically.

Performance specifications:

1. Linearity: This method is linear for acid phosphatase up to 10 KA/dL of serum

2. Measurement range: This method has a measurement range of 1-15 KA/L of acid phosphatase in serum.

Primary sample:

1. Use only serum as specimen

2. Collect 4 mL of venous blood in a plain red color vacutainer tube

3. Allow the tube to stand for 30 minutes and separate the by centrifugation at 2500 rpm for 10 minutes

4. Do not use lysed serum for testing as it may give very high results

5. Do not use contaminated/turbid samples for testing

6. Process the sample on the same day within 3 hours of collection

7. If analysis is not done on the same day/within 3 hours collection, separate the serum and store it at 2-8°C for up to 1 day.

Type of container and additive: For serum use a plain vacutainer tube for collecting venous sample.

Reagents:

1. Buffer: Citric acid and sodium citrate buffer.

2. Dissolve 4.2 g of citric acid in water add 7.6 mL of 1 N NAOH and made up to 100 mL with distilled water pH 4.9 (use sodium hydroxide for adjusting the pH)

3. Substrate: Dissolve 0.218 g of disodium phenyl phosphate in 100 mL - the distilled water. Bring the solution quickly to boiling to sterilize, cool and add a little chloroform.

4. 0.5 N sodium hydroxide: 2 g of sodium hydroxide in 100 mL of distilled water.

5. 0.5 M sodium bicarbonate: 42 g of sodium bicarbonate in 1 liter. Store in amber colored bottle.

6. 4-aminoantipyrine (AAP): 0.6% in water

7. Potassium ferricyanide: 2.4 g in 100 mL water

Instrument: Spectrophotometric

Procedure:

Reagent	Reagent Blank	Test Blank	Test
Buffer (mL)	3.0	3.0	3.0
Substrate (mL)	--	3.0	3.0
Distilled water (mL)	3.0	--	--
	Incubate for 5 minutes at 37°C		
Serum (mL)	--	--	0.1
	Incubate for 60 minutes at 37°C		
0.5 N NaOH (mL)	0.8	0.8	0.8
0.8 0.5 M NaHCO3 (mL)	1.2	1.2	1.2
4-aminoantipyrine (mL)	1.0	1.0	1.0
Serum (mL)	--	0.1	0.1
Potassium ferricyanide (mL)	1.0	1.0	1.0

Read at 510 nm.

Standardization: Stock standard: 100 mg phenol in 100 mL of 0.1 NHC

Working standard: 2 mL of stock is diluted to 100 mL. 1 mL contains 20 mg.

Reagents	B	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Working standard (mL)	--	0.125	0.25	0.375	0.5	0.625	0.75	0.875	1.0
Distilled water (mL)	1.0	0.875	0.75	0.625	0.5	0.375	0.25	0.125	--
Buffer (mL)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
NaOH (mL)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
NaHCO3 (mL)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2

4AAP (mL)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Potassium ferricyanide (mL)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**

Read at 510 nm against blank.

Reference range: 1 to 5 KA units/dL.

Critical/Alert level values: Above 5 KA units/dL.

Potential sources of variability: Lysed serum specimens may give falsely elevated values.

ACID PHOSPHATASE (TARTRATE-LABILE) FOR PROSTATIC CANCER

Purpose: Qualitative estimation of acid phosphatase in serum by testing for the released phenol from the substrate disodium phenyl phosphate in acid pH. The levels are helpful in diagnosis and therapy of certain types of cancers especially metastasizing prostatic cancer in serum. In the presence of tartrate, testing at acid pH and by difference, the levels give the tartrate-labile acid phosphatase which is specific for prostate and is helpful in the diagnosis of metastasizing prostatic cancer.

Principle: Prostatic acid phosphatase is strongly inhibited by L (+) tartrate, which has no action on red cell acid phosphatase.

Primary sample:

1. Use only serum as specimen

2. Collect 4 mL of venous blood in a plain vacutainer tube.

3. Allow the tube to stand for 30 minutes and separate the serum by centrifugation at 2500 rpm for 10 minutes

4. Do not use lysed serum for testing as it may give very high results.

5. Do not use contaminated/turbid samples for testing.

Type of container and additive: Use plain vacutainers collection blood samples

Reagents:

1.Reagents same as for the acid phosphatase except the buff which is as follows.

2. Tartrate buffer: Dissolve 15 g of L (+) tartaric acid in about 70 mL of water add 125 mL of normal sodium hydroxide and adjust pH to 4.9 makes up to 100 mL.

Techniques: In addition to the two tubes for test and blank used in process no. 25 (Acid Phosphatase), a third tube containing 2.ml of buffer substrate, 1 drop of the tartrate solution and 0.1 mL of serum is also incubated and put though in exactly the same way as for acid phosphatase.

Calculation: (Reading of the test - Reading of test containing tartrate/Reading of standard - Reading of standard blank) X10

Critical/Alert level values: Below and above the reference range.

Reference range: 0-0.8 KA/dL

Potential sources of variability: Lysed serum specimens may give falsely elevated values.

BIBLIOGRAPHY

Bishop, Michail l., Edward P. Fody ,L. Schoeff. CLINICAL CHEMISTRY, 8th edn. Walter Kluwer,2017:1066pp.

Cheesbrough, Monica. District Laboratory Practice in Tropical Countries, Part 2,2nd edn. Cambridge University Press, 2006:442pp

Cheesbrough, Monica. District Laboratory Practice in Tropical Countries Part 1, 2nd edn. Updated, Cambridge University Press, 2009:462pp.

Fischbach Frances T. A Manual of Laboratory and Diagnostic Tests, 7th edn. Lippincott Williams & Wilkins Publishers, 2003, 755pp.

Fischbach, Frances T, Marshall Barnett Dunning III. A manual of Laboratory and Diagnostic Tests, 9th Ed. Wolters Kluwer Health | Lippincott Williams & Wilkins. 2015:1282pp.

Gupte, Satish. The Short Textbook of Medical Laboratory for Technicians, 2nd edn., Jaypee Brothers Medical Publishers Pvt. Ltd., 2014:258 pp.

Kemal, Jelalu. Laboratory Manual and Review on Clinical Pathology. OMICS Group eBooks USA.2014:32pp.

Varley, H. Practical Clinical Biochemistry, 4th edn. Interscience Publishers, Inc., New York-London 2005:946pp.

Mundt, Lillian A. Kristy Shanahan. *Graff's* Textbook of Routine Urinalysis and Body Fluids 2nd edn. by Lippincott Williams & Wilkins, Philadelphia, 2011: 354pp.

Varley, H. Practical Clinical Biochemistry, 4th edn. Interscience Publishers, Inc., New York-London 2005:946pp.

Ramakrishnan, S., K.N. Sulochana. Manual of Medical Laboratory Techniques, Jaypee Brothers Medical Publishers Pvt. Ltd., 2012: 453pp.

Sood, Ramnik. Concise Book of Medical Laboratory Technology, Methods and Interpretations, 2nd edn., Jaypee Brothers Medical Publishers Pvt. Ltd., 2015:1074pp.

Varley, H. Practical Clinical Biochemistry, 4th edn. Interscience Publishers, Inc., New York-London 2005:946pp.

Varley, H. Practical Clinical Biochemistry, 4th edn. Interscience Publishers, Inc., New York-London 2005:946pp.

Walters, Norma J. , Anna P. Reynolds, Barbara H. Estridge. Basic Medical Laboratory Techniques 4th edn., Thomson Learning USA, 2000:620PP.

WHO, Manual of Basic Techniques for a Health Laboratory 2nd edn, 2003:343pp.

Wu, A. Tietz Clinical Guide to Laboratory Tests, 4th edn., WB Saunders, Co, Philadelphia, 2006:1856pp.