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MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS

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غير مسؤول عن الافكار والرؤى التي يتضمنها الكتاب
والمسؤول عن ذلك الكاتب او الباحث فقط.

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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

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MANUAL OF MEDICAL LABORATORY DIGNOSTIC TESTS/ PART I

CONTENTS

- **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

QUNTITATIVE ESTIMATION OF SERUM BILIRUBIN USING SEMI-AUTOANALYZER

MANUAL DETERMINATION OF TOTAL AND DIRECT BILIRUBIN

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: HEMATOLOGY

EXAMINATION OF BLOOD SPECIMENS

COLLECTION OF BLOOD

Blood must be collected with care and adequate safety precautions to ensure test results are reliable, contamination of the sample is avoided and infection from blood transmissible pathogens is prevented. Protective gloves should be worn when collecting and handling blood samples. Lancets, needles, and syringes must be sterile, and dry, and blood collecting materials must be discarded safely to avoid injury from needles and lancets.

Capillary blood

Capillary blood is mainly used when the patient is an infant or young child and the volume of blood required is small, e.g. to measure hemoglobin, perform a WBC count, and to make thick and thin blood films. In medical laboratories, capillary blood is also used to monitor anemia during pregnancy and post-operatively. Hemoglobin and PCV values are slightly higher in capillary blood than in venous blood. Thick blood films for malaria parasites are best made from capillary blood (anticoagulated blood is more easily washed from slides during staining).

Technique for collecting capillary blood

Make sure the puncture area is warm to allow the blood to flow freely. On cold days soak the hand or foot of an infant in warm water prior to collecting a sample.

1- Cleanse the puncture area with 70% ethanol. Allow the area to dry.

2- Using a sterile picker or lancet, make a rapid puncture, sufficiently deep to allow the free flow of blood.

3- Wipe away the first drop of blood with a dry piece of cotton wool and use the next few drops for the test. Do not squeeze too hard because this will result in an unreliable test result.

4- When sufficient blood has been collected, press a piece of dry cotton wool over the puncture area until bleeding stops.

Venous blood

Anticoagulated venous blood is used when more than 100 µl of whole blood is required or when serum from a clotted blood sample is needed, e.g. for compatibility tests or antibody tests. Venous blood is preferable to capillary blood for the reasons previously described, particularly when the patient is an adult and several tests are required.

Anticoagulants

For hematological tests, the anticoagulants used are EDTA (ethylenediamine tetra-acetic acid), also called sequestrene, and *tri*-sodium citrate. These chemicals prevent blood from clotting by removing calcium.

_ EDTA anticoagulated blood can be used for most tests, e.g. hemoglobin, PCV, WBC count, platelet count, reticulocyte count, and reporting blood cell morphology. It is not suitable for coagulation tests.

The ICSH (International Committee for Standardization in Hematology) recommends the use of dipotassium EDTA* at a concentration of 1.5± 0.25 mg/ml of blood

-Trisodium citrate, 32 g/l, is used to anticoagulated blood for:

-Measuring the ESR, with 1.6 ml of venous blood (or previously collected EDTA blood) being mixed with 0.4 ml of sodium citrate anticoagulant.

Note: ESR measurements are being discontinued in many tropical countries.

-Coagulation tests, with 9 ml of venous blood being mixed with 1 ml of sodium citrate anticoagulant.

Heparin anticoagulant: Heparinized blood is mainly used for clinical chemistry tests and immunophenotyping. It is not recommended for routine hematological tests because it causes cells to clump and heparin gives a blue background to blood films.

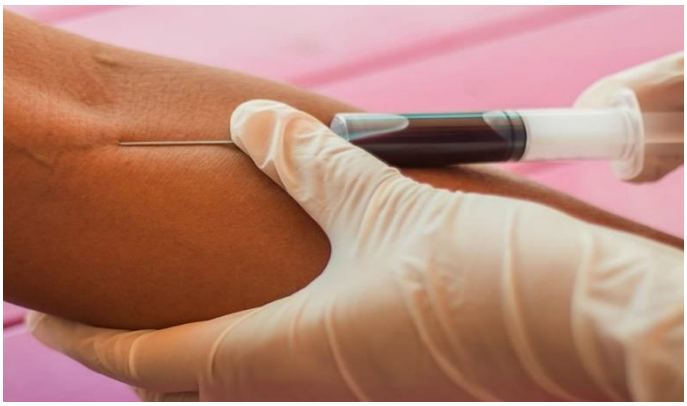
Technique for collecting venous blood

Laboratory staff must not collect venous blood unless they have been adequately trained in the procedure Newly qualified staff must be supervised until they have gained sufficient experience. When venous blood is required from infants, this should be collected by a medical officer. Do not collect blood for hematological tests from intravenous lines.

1- Select a sterile, dry, preferably plastic syringe of the capacity required, e.g. 2.5 ml, 5 ml, or 10 ml. Attach to it a 19 or 20 SWG needle (preferably a disposable one). If the patient is a child or adult with small veins, use a 23 SWG needle.

Note*: When not using a disposable syringe or needle, check the syringe for good suction and the needle for any blockage, directing the syringe and needle *safely away from the patient*. Ensure all air is expelled from the syringe. *Whenever possible use a disposable needle and syringe.

Evacuated tube collection systems: These disposable blood collecting containers are available from several manufacturers but they are more expensive to use for collecting venous blood than a syringe and needle. The container has a vacuum which is used to draw the blood into the container. One end of the needle is situated in the patient's vein and the other end through the cap of the container. Evacuated collection systems minimize contact with blood, help to ensure the correct amount of blood is added to anticoagulant, and simplify multiple sample collection.



2- Apply a soft tubing tourniquet or Velcro fastening arm band to the upper arm of the patient (see Fig.) to enable the veins to be seen *and felt*. Do not apply the tourniquet too tightly or for longer than 2 minutes. Ask the patient to make a tight fist which will make the veins more prominent.

3- Using the index finger, feel for a suitable vein, selecting a sufficiently large straight vein that does not roll and with a direction that can be felt*.

***If a vein cannot be felt, apply a pressure cuff above the elbow and raise the pressure to 80 mm (deflate the cuff once the needle is in the vein).**

4- Cleanse the puncture site with 70% ethanol and allow to dry. Do not re-touch the cleansed area.

5- With the thumb of the left hand holding down the skin below the puncture site, make the venipuncture with the bevel of the needle directed upwards in the line of the vein. Steadily withdraw the plunger of the syringe at the speed it is taking the vein to fill*. Avoid moving the needle in the vein.

***If the plunger is withdrawn too quickly this can cause hemolysis of the blood and the collapse of a small vein.**

6- When sufficient blood has been collected, release the tourniquet and instruct the patient to open his or her fist. Remove the needle and immediately press on the puncture site with a piece of dry cotton wool. Remove the tourniquet completely. Instruct the patient to continue pressing on the puncture site until the bleeding has stopped.

7- Remove the needle from the syringe and carefully fill the container(s) with the required volume of blood. Discard the needle safely. *Do not* attempt to re-sheath it because this can result in needle-stick injury.

***Important*: Do not fill a container with the needle attached to the syringe. Forcing the blood through the needle can cause hemolysis.**

8- Mix immediately the blood in an EDTA or citrate anticoagulated container. When required, make a thick blood film from the blood remaining in the syringe. Immediately label carefully all the blood samples.

9- Check that bleeding from the venipuncture site has stopped. Cover the area with a small dressing.

Avoiding hematoma when collecting venous blood

Bleeding from a vein into the surrounding tissue (hematoma) can cause unnecessary distress to a patient and result in subsequent bruising. Hematoma can be avoided by ensuring an appropriate vein is selected and the needle is well positioned in it and not accidentally pulled out of the vein when withdrawing the plunger of the syringe. When removing the needle, always release the tourniquet *first* and apply pressure immediately to the puncture site, maintaining it until the bleeding has stopped completely (*always* check).

Avoiding hemolysis of blood samples

Hemolysis can be avoided by:

● Checking that the syringe and needle are dry and that the barrel and plunger of the syringe fit well.

● Not using a needle with too fine a bore.

● Not withdrawing the blood too rapidly or moving the needle once it is in the vein. Frothing of the blood must be avoided.

● Removing the needle from the syringe before dispensing the blood into the specimen container and allowing the blood to run gently down the inside wall of the container.

● Adding the correct amount of blood to anticoagulant. Do not shake the blood but gently mix it with the anticoagulant.

● Using clean dry glass tubes or bottles for blood from which serum is required. Allow sufficient time for the blood to clot *and* clot retraction to take place. Red cells are very easily hemolyzed by the rough use of an applicator stick to dislodge a clot.

● Centrifuging blood samples for a minimum period of time. Centrifuging for 5 minutes at about 1000 g is adequate to obtain serum or plasma.

● Not storing whole blood samples in, or next to, the freezing compartment of a refrigerator.

COMPLETE BLOOD COUNT

Purpose: Quantitative enumeration of different formed elements of blood and hemoglobin in whole blood by automated cell counter by the principle of Impedance Variation. This test includes total White Blood Cell WBC count, total Red Blood Cell RBC count, hemoglobin, Packed Cell Volume PCV/ hematocrit Hct, mean corpuscular volume MCV, mean corpuscular hemoglobin MCH, mean corpuscular hemoglobin concentration MCHC, platelet count. It is the enumeration of cellular elements of blood, evaluation of red cell and determination of cell morphology by means of stained smears

Principle

Coulter Method

The Coulter method accurately counts and sizes cells by detecting and measuring changes in electrical resistance when a particle (such as a cell) in a conductive liquid pass through a small aperture. As each cell goes through the aperture, it impedes the current and causes a measurable pulse. The number of pulses signals the number of particles. The height of each pulse is proportional to the volume of that particle.

While number of pulses indicates particle count, the amplitude to the electrical pulse produced depends on the cell's volume. Theoretical analysis of the behavior of particles within an aperture shows that the height of the electrical pulse produced by the cell is the characteristic that most nearly shows proportionality to the cell volume.

WBCs are counted after lysing the RBCs by a detergent (ionic or not ionic) which does not lyse the WBCs themselves. After dilution (1:250 for WBC count 1:6250 for RBC count Indices and platelet count) the diluted sample is directed to two sides of the instrument and particles on the cells side that are larger than 36 fl are counted as RBCs. Cells from are counted as platelets. For WBC counting lysing reagent is added sample in the system to lyse RBCs and causes differential shrinking WBCs, thereby allowing them to be separated into a partial di part differential) and white cells are shown in 3 groups lymphocytes (35- 90 fl) mononuclear cells (90-160 fl) and granulocytes (160-450 fl).

Hct is calculated based on RBC pulse height value. It is height of impulse generated by a passage of cells through a micro aperture, which is directly proportional to the volume of analyzed cells. RBC are lysed and the freed hemoglobin combines with potassium cyanide to form cyanmethemoglobin, which is measured by Spectrophotometry at 525 nm.

MCH and MCHC are calculated from RBC count, Hb and Hct

Hct: calculated from RBC and MCV

HCV: Derived from RBC histogram

MCH: calculated from HGB and RBC

MCHC: calculated from HGB and Hct

MPV and PLT: Derived from PLT histogram

Primary Sample

- **Use whole blood as sample collected in K_2 EDTA.**
- **Mix well by rotation using hemomixer before process for 5 minutes**
- **Process the samples within 2 hours of collection**
- **Do not use decomposed of contaminated sample**
- **Reject clotted samples**

Consumables/ Reagents

- **Cell diluent**
- **Lytic reagent**
- **Shutdown diluent**

Procedure

- **Check the background values and QC values to see whether within the range specified.**

• Run any one of the three levels (low normal and for analyzing the samples to verify whether the system is acceptable limits

• Press the ID key to enter the lab reference number

• Press the enter key to save the lab reference number

• Mix the blood samples thoroughly using hemomixer before processing for 5 minutes

• Keep the sample tube under the sample probe and press the key located behind the sample probe.

• Result will be displayed on the LCD screen

Potential Sources of Variability

The following factors will affect counting:

• Fibrin micro clots or cryoprecipitate in the specimen

• Inadequate lysis of RBC

• Inadequate dilution of specimen and inadequate calibration of the analyzer

• Deterioration of diluting solution

• Presence of dust particles in the diluent-Ensure the diluent and buffer solutions used are free of dust particles especially for platelet counting

• Partial or total obstruction of the aperture

• Wrong threshold setting of the instrument

• Carry over from one to next measurement

• Fluctuation of electric current

Reference Range

Parameter	Range
WBC count	Adults 4000-11000 cells/ cu.mm At birth 10,000-25,000 cells/cu.mm Intents (one year) 6000-18000 cells/cu.mm 4-7 years 6,000-15.000 cells/cu.mm 8-12 years 4.500-13.300 cells/cu.mm
RBC count	Male: 4.5 to 6.5 x 10^6 cells/cu.mm Female: 3.9 to 56 x 10^6 cells /cu.mm
Hemoglobin	At Birth: 13.6 – 19.6 g% At 1 year: 11.3 – 13.0 g% 10-12 years: 11.5 – 14.8 g% Women: 11.5 – 16.5 g% Men: 13.5 – 18.0 g%
Platelet count	150,000 - 450,000/cumm
Hematocrit	At birth:44 63% At 1 year:35% At 10 years:37.5% Women:30-40% Men:40-54%
MCV	76-96 um^3
MCH	27-32 pg
MCHC	30-35%

NEUBAUER CHAMBER (Hemocytometer) FOR RED BLOOD CELL COUNT

The main function of the red blood cell (RBC or erythrocyte) is to carry oxygen from the lungs to the body tissues and to transfer carbon dioxide from the tissues

to the lungs. This process is achieved by means of the Hb in the RBCs, which combines easily with oxygen and carbon dioxide and gives arterial blood a bright red appearance. To enable use of the maximal amount of Hb, the RBC is shaped like a biconcave disk—this affords more surface area for the Hb to combine with oxygen. The cell is also able to change its shape when necessary to allow for passage through the smaller capillaries.

The RBC test, an important measurement in the evaluation of anemia or polycythemia, determines the total number of erythrocytes in a microliter (cubic millimeter) of blood.

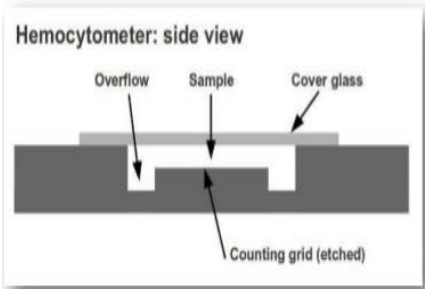
Procedure

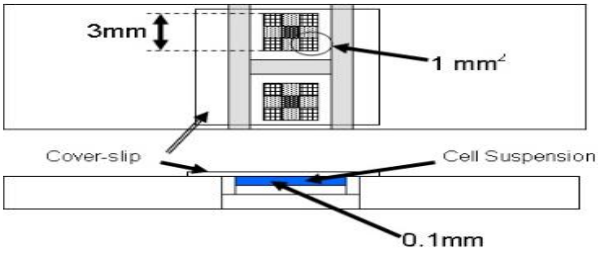
Reagents and instruments:

1. **Neubauer Chamber (Hemocytometer) & coverslips.**

- **Improved Neubauer Chamber**

A thick glass slide with H shaped moats in it. The area between two lines of H (center) is 0.1 mm in depth. Moat prevents mixing of 2 samples on either side of chamber.



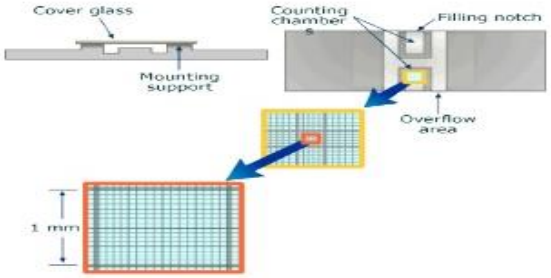


2. **RBC diluting fluid/solution. Consists of 3.2 g of Na-citrate and 1.0 ml of formaldehyde solution made up to 100 ml with D.W.**

Sample: Obtain 5 mL of whole blood in a lavender-topped tube (with EDTA).

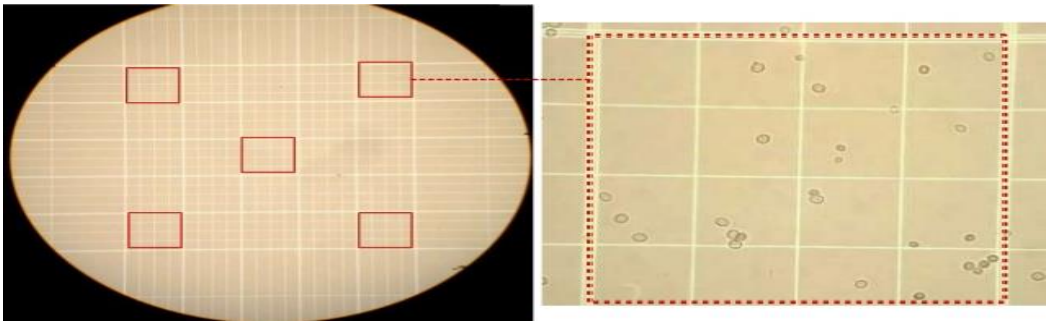
Method

- **Prepare a plastic tube (labeled).**
- **Prepare 1:200 blood dilution (4 ml of diluent + 20 µl Blood).**
- **So, add diluent to the tube. Mix the sample (5 times); then aspirate 20µl and transfer to the tube and mix.**
- **Clean the Hemocytometer and coverslip with 70% ethanol followed by D.W & leave to dry. --Place a coverslip on the Neubauer chamber.**
- **Then, fill the chamber with the diluent (10 microliter) in each side. Leave chamber in humidity (petri-dish with wet filter paper) for 1-2 min.**
- **Condenser slightly lowered. Iris diaphragm should be almost closed.**
- **Place chamber on microscope stage. Start with 10X to focus; then with 40X count RBCs.**



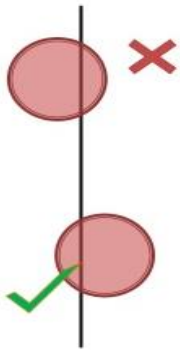
-RBCs should be counted in the central square of the chamber.

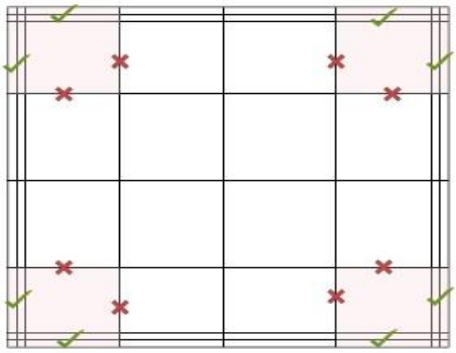
-Select 5 small squares (One at each corner and one in the center).

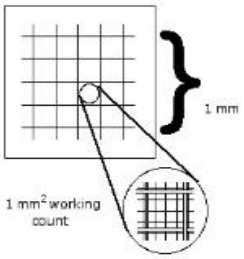


-Count all cells in specified squares, and multiply by the proper conversion factor; the number of cells per cubic millimeter can be determined.

-Count all cells within 16 squares and those lying on middle lines, EXCEPT …







- **Calculation:**

RBCs (10^{12}/L) = No. of RBCs counted X Dilution X 10^6 / Volume (µl)

Dilution = 200

Depth of the chamber = 0.1 mm.

Volume of 5 small squares = 0.02 µl

Age group RBC count:

So, Red cell count/ liter = N x 0.01 x 10^{12} i.e. RBCs= N x 10^{12}/L

Adult Male 4.7 – 6.1 X 10^{12} /L

Adult Female 4.2 – 5.4 X 10^{12} /L

Newborn 4.4 -5.8 X 10^{12} /L

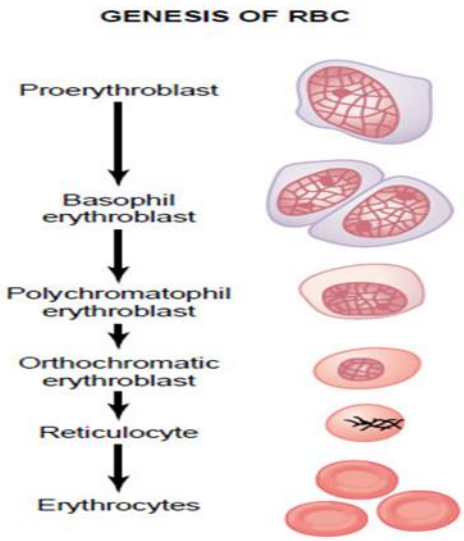
Infant/ children 3.8 – 5.5 X 10^{12} /L

MICROSCOPIC DETERMINATION OF THE RETICULOCYTE NUMBER COCENTRATION/ FRACTION

Reticulocytes are immature erythrocytes that pass into the bloodstream from the bone marrow. The number of reticulocytes in the blood indicates the degree of

activity of the bone marrow in the production of erythrocytes, and when the marrow is very active (as in anemia) their number increases. Reticulocytes contain fine, deep-violet granules arranged in a network (reticulum). They do not contain a nucleus.

Principle



The fine granules in reticulocytes can be stained with brilliant cresyl blue. A blood film is stained with this dye and a certain number of erythrocytes observed under the microscope. From this observation, either:

- **the number of reticulocytes per liter of blood, or**
- **the proportion of erythrocytes that are reticulocytes is calculated.**

Materials and reagents

- **Microscope**
- **Microscope slides (grease-free)**
- **Glass spreader**
- **Test-tubes**
- **Test-tube rack**
- **Funnel**
- **Filter-paper**
- **Two Pasteur pipettes with teats**
- **Hand tally counter, if available**
- **Saturated solution of brilliant cresyl blue.**

Method

1- **Filter a little of the cresyl blue solution into a test-tube. In the bottom of another tube place two drops of filtered cresyl blue solution.**
2- **Collect a few drops of blood from the patient's finger with a Pasteur pipette, or use venous blood collected in EDTA dipotassium salt solution and mix well.**
3- **Add two drops of blood to the tube containing cresyl blue solution.**

4- **Mix by gently shaking the tube. Plug the tube with non-absorbent cotton wool. Leave for 15 minutes.**
5- **Take the tube and shake it gently. Remove one drop of the mixture. Place it on a slide ready for spreading.**
6- **Make a thin smear of the mixture with the spreader. Leave the smear to air-dry.**

Microscopic examination

Examine the smear using the X100 oil-immersion objective. Look at the end of the smear, where the erythrocytes should be well separated from each other. Erythrocytes stain pale blue. Examine at least 100 erythrocytes. Keep a careful count of the total number of erythrocytes examined and the number of these that are reticulocytes. (Counting is easier if the size of the microscope field is reduced. This can be done by placing in the eyepiece a small circular piece of stiff black paper in which a hole of about 5mm in diameter has been punched.)

Some hematologists prefer reticulocytes to be reported in terms of the number concentration (number of reticulocytes per liter of blood), while others prefer them to be reported in terms of the number fraction (the proportion of erythrocytes that are reticulocytes). Depending on the practice in your laboratory or the specification of the requesting physician, make the appropriate calculation.

Traditionally, reticulocytes have been reported in the form of percentages (i.e. the proportion, expressed as a percentage, of the erythrocytes that are reticulocytes). If 500 erythrocytes are observed on the blood film and *n* of them are reticulocytes, the percentage of erythrocytes is calculated by multiplying *n* by 0.2.

Example:

Of 500 erythrocytes examined, 25 are reticulocytes. The percentage of reticulocytes is then 25 X 0.2 = 5%. The normal range for newborn infants is 2.0–6.0%, and that for adults and children is 0.2–2.0%.

Calculation

To calculate the reticulocyte number concentration, you must know the total erythrocyte number concentration. If *C* is the total erythrocyte number

concentration (omitting the "X10^{12}X l") and *n* is the number of reticulocytes seen on observing 500 erythrocytes, the reticulocyte number concentration is *C* X 2*n* X 10^9X l.

Example:

total erythrocyte number concentration = 4.5 X 10^{12}/l

number of reticulocytes seen in counting 500 erythrocytes = 6

reticulocyte number concentration = 4.5 X (2 X6) X10^9 /l

= 4.5X12 X 10^9/l

= 54 X 10^9/l (report this result).

To calculate the reticulocyte number fraction, you do not need to know the erythrocyte number concentration. If *n* is the number of reticulocytes seen in examining 500 erythrocytes, the reticulocyte number fraction is 2*n* X 10^{-3}.

***Example*:**

number of reticulocytes seen in counting 500 erythrocytes = 6

reticulocyte number fraction = (2 X 6) X 10^{-3} = 12 X 10^{-3}

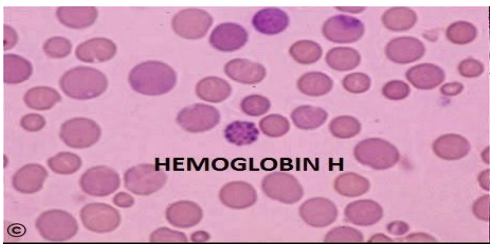
Table. Reticulocyte number concentrations and reticulocyte number fractions, by age group

Age group	Reticulocyte number concentration[a]	Reticulocyte number Fraction
Infants (newborn)	100–300 \ 10^9/l	20–60 \ 10^{-3}
Children	8–110 \ 10^9/l	2–20 \ 10^{-3}
Adults	8–110 \ 10^9/l	2–20 \ 10^{-3}

[a] Approximate values. The concentration depends on the erythrocyte number concentration.

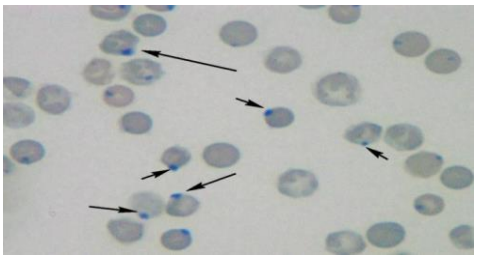
Note: If more than 500 erythrocytes are examined on the blood film, the calculation will have to be adjusted accordingly.

Other structures that can be seen in blood films stained with brilliant cresyl blue. The blood film stained with brilliant cresyl blue that is used for determining the reticulocyte number concentration and reticulocyte number fraction may also show the following structures:



Hemoglobin H bodies

Hemoglobin H bodies appear as pale blue dots, variable in size. Unlike the reticulum of the reticulocytes, they occur in most of the erythrocytes. They are found in a-thalassemia or hemoglobin H disease.



Heinz bodies

Heinz bodies appear as blue granules, variable in size, lying to one side of the erythrocyte near the cell membrane. They occur in glucose-6-phosphatase dehydrogenase deficiency following treatment with certain drugs.

RED BLOOD CELL INDICES

Red cell indices most frequently used in the investigation of anemia are:

- **Mean cell hemoglobin concentration (MCHC)**
- **Mean cell volume (MCV)**
- **Mean cell hemoglobin (MCH)**
- **Red Cell Size Distribution Width (RDW)**

- Mean cell hemoglobin concentration (MCHC)

The MCHC measures the average concentration of Hb in the RBCs. The MCHC is most valuable in monitoring therapy for anemia because the two most accurate hematologic determinations (Hb and Hct) are used in its calculation.

Procedure

1- **The MCHC is a calculated value. It is an expression of the average concentration of Hb in the red blood cells and, as such, represents the ratio of the weight of Hb to the volume of the erythrocyte.**
2- **Use the following formula:**

MCHC (g/dL) = Hb (g/dL) X 100 / Hct (%)

The MCHC gives the concentration of hemoglobin in g/l in 1 liter of packed red cells. It is calculated from the hemoglobin (Hb) and PCV as follows: MCHC g/l

Example

If the Hb of an anemic patient is 81 g/l and PCV is 0.34, the MCHC is: 238 g/l*

***If using g/dl divide the g/l figure by 10.**

Interpretation of MCHC values

A guideline reference range for MCHC in health is 315–360 g/l (31.5–36.0 g/dl). These figures should be checked locally.

- Mean cell volume (MCV)

The mean red cell volume (MCV) provides information on red cell size. It is measured in femtolitres (fl) and is determined from the PCV and electronically obtained RBC count. It can be calculated as follows:

MCV fl*= PCV (l/l)/ RBC X10^{12}/l

***A femtolitre (fl) is 10^{15} of a liter.**

There is some variation in reference ranges for MCV depending on the method used by manufacturers of blood cell analyzers to obtain the MCV value and how an instrument has been calibrated. A guideline reference range is 80–98 fl.

- ## Mean cell hemoglobin (MCH)

The MCH gives the amount of hemoglobin in picograms (pg) in an average red cell. This index is of value in diagnosing severely anemic patients. It is calculated from the hemoglobin and electronically obtained RBC count:

Procedure

The MCH is a calculated value. The average weight of Hb in the RBC is expressed as picograms of Hb per RBC. The formula is:

MCH (pg/cell)=Hb (g/dL) X 10 / RBC (10^{12}/L)

***A picogram (pg) is 10^{12} of a gram.**

***Note*: Most electronic blood cell analyzers calculate the MCH as one of their parameters, providing a print-out of the value in pg.**

A guideline reference range for MCH in health is 26–34 pg/cell or 0.40–0.53 fmol/cell (normally higher in newborns and infants)

Red Cell Size Distribution Width (RDW)

This automated method of measurement is helpful in the investigation of some hematologic disorders and in monitoring response to therapy. The RDW is essentially an indication of the degree of anisocytosis (abnormal variation in size of RBCs). Normal RBCs have a slight degree of variation.

Procedure

1- **Remember that the CV of RDW is determined and calculated by the analyzer.**
2- **Use the CV of RDW with caution and not as a replacement for other diagnostic tests.**

3- **Use the following calculation:**

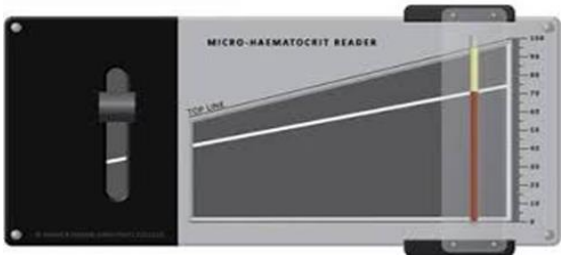
RDW (CV%) = Standard deviation of RBC size X 100 / MCV

PACKED CELL VOLUME (PCV) /Hematocrit

Principle: The packed cell volume is that proportion of whole blood occupied by red cells, expressed as a ratio (liter/liter). Anticoagulated blood in a glass capillary of specified length, bore size, and wall-thickness is centrifuged in a microhematocrit centrifuge at RCF 12 000–15 000 xg for 3–5 minutes to obtain constant packing of the red cells. A small amount of plasma remains trapped between the packed red cells. The PCV value is read from the scale of a microhematocrit reader or calculated by dividing the height of the red cell column by the height of the total column of blood.

Equipment

- **Microhematocrit centrifuge**
- **Capillary tubes for measuring PCV**



- ***Sealant***
- ***Microhematocrit reader***

Test method

Whenever possible perform the test in duplicate.

1- **About three quarters fill either:**
   a plain capillary with *well mixed* EDTA anticoagulated blood (tested within 6 hours of collection), or a heparinized capillary with capillary blood.

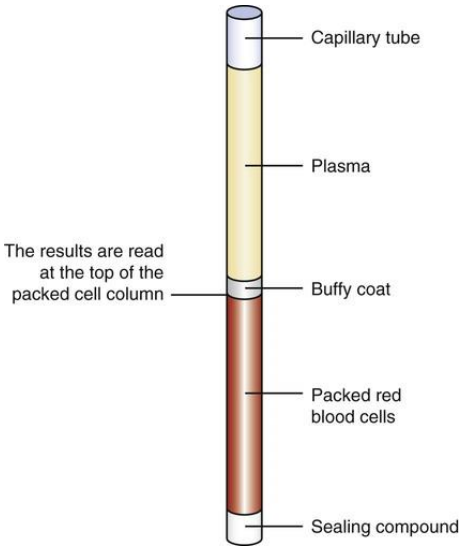
***Leave 10–15 mm of the capillary unfilled.**

2- **Seal the *unfilled* end of the capillary using a sealant material.**
3- **Carefully locate the filled capillary in one of the numbered slots of the microhematocrit rotor with the sealed end against the rim gasket (to prevent breakage). Write the number of the slot on the patient's form. Position the inner lid carefully to avoid dislodging the tubes.**
4- **Centrifuge for 5 minutes (RCF 12 000– 15 000 xg).**

***Note*: If the PCV is more than 0.50, centrifuge for a further 3 minutes to ensure complete packing of the red cells.**

5- ***Immediately after centrifuging*, read the PCV. First check that there has been no leakage of blood from the capillary or breakage.**

To read the PCV in a hand-held microhematocrit reader, align the base of the red cell column (above the sealant) on the 0 line and the top of the plasma column on the 100 line. Read off the PCV from scale. The reading point is the top of the red cell column, just below the buffy coat layer (consisting of WBCs and platelets).



***When no reader is available*: Use a ruler to measure the length of the total column of blood (top of plasma to bottom of red cell column) in mm and the length of the red cell column (base to below buffy coat layer). Calculate the PCV as follows:**

PCV= Length of red cell column (mm)/ Length of total column (mm)

PCV reference range (guideline figures), l/l*

Children at birth 0.44–0.54

Children 2–5 y 0.34–0.40

Children 6–12 y 0.35–0.45

Adult men. 0.40–0.54

Adult women 0.36–0.46

ESTIMATION OF THE HEMOGLOBIN CONCENTRATION

Hemoglobin is the red pigment contained in erythrocytes. It consists of protein chains and iron-containing molecules.

Units of measurement

The SI unit for expressing hemoglobin concentrations is millimole per liter (mmol/ l). When this unit is used, it is necessary to specify the chemical structure to which it applies. In practice, this means that the term "hemoglobin (Fe)" should be used instead of the simple term "hemoglobin". However, as an interim measure, before making the change to millimole per liter, some laboratories are using the unit "gram per liter" (g/l). When this unit is used, the simple term "hemoglobin" suffices, and it is not necessary to say "hemoglobin (Fe)". Values in grams per liter may be converted into values in millimoles per liter by multiplying by 0.062.

Example:

hemoglobin 150g/l / 0.062 = hemoglobin (Fe) 9.3mmol/l

In this manual calculations and values are usually expressed in both forms. It should be noted that if the unit "gram per liter" is used, the values are 10 times greater than values in the traditional unit "gram per 100ml". For example, 150g/l = 15.0 g/100ml.

Photometric techniques

In photometric techniques the absorbance of hemoglobin in a blood sample is measured electronically using a filter colorimeter or a direct read-out hemoglobin meter. Techniques include dilution techniques in which blood is measured into a

measured volume of diluting fluid, and no dilution techniques which do not require prior dilution of the blood.

- **Dilution Techniques**

These include:

- **Hemiglobincyanide photometric method**

Principle: The blood is diluted in Drabkin diluting fluid, which hemolysis the red cells and converts the hemoglobin into hemiglobincyanide (cyanmethemoglobin). The solution obtained is examined in a spectrophotometer (or colorimeter). Its absorbance is proportionate to the amount of hemoglobin in the blood. The hemoglobincyanide photometric method gives the most accurate hemoglobin estimations. It should be used wherever possible.

Materials and reagents

- Spectrophotometer (or colorimeter)

_ Spectrophotometer (or colorimeter) cuvettes

_ Test-tubes

_ Test-tube rack

_ Blood (Sahli) pipettes, 0.2ml

_ Drabkin diluting fluid

_ Reference solution, which may be:

— the fresh hemiglobincyanide reference solution used to calibrate the instrument,

— a reference solution previously calibrated against the hemiglobincyanide reference solution, or a blood sample of known hemoglobin concentration.

FIG: Blood (Sahli) pipettes, 0.2ml

A calibration curve must be prepared before the spectrophotometer (or colorimeter) can be used for hemoglobin estimation. From such a curve a graph can be prepared and a table made for the hemoglobin values.

Important:

At the beginning of each day:

_ Clean the matched spectrophotometer (or colorimeter) cuvettes.

_ Fill one of the cleaned tubes with fresh Drabkin diluting fluid, which is used to zero the spectrophotometer (or colorimeter).

_ Read a reference solution (see above).

Table. Preparing serial dilutions of reference solution

Tube number	Volume of reference solution (ml)	Volume of Drabkin diluting fluid (ml)	Dilution
1	4.0	0.0	1:2
2	2.0	2.0	1:3
3	1.3	2.7	1:4
4	1.0	3.0	

Calibration of the spectrophotometer (or colorimeter) using hemiglobincyanide reference solution (or a reference solution previously calibrated against hemiglobincyanide reference solution)

1. Calculate the hemoglobin value of the reference solution in grams per liter by using the following formula:

concentration in mg/100 ml X 10^a /1000^c) 251^b

where:

a = the factor for converting 100 ml to 1 liter;

b = the dilution factor when 0.02 ml of blood is diluted with 5ml of Drabkin diluting fluid;

c = the factor for converting milligrams to grams.

Since 10 X 251/1000 is very nearly 2.5, the above formula can be simplified as follows (If a dilution of 1 in 200 is used (i.e. 0.02 ml of blood and 4 ml of Drabkin diluting fluid), multiply by 2.0 instead of 2.5.):

hemoglobin value of reference solution in grams per liter = concentration in mg/100 ml \ 2.5

Example:

concentration of reference solution = 60mg/100ml hemoglobin value = 60 \ 2.5 = 150g/l

2. Prepare a series of dilutions of the reference solution in four test-tubes (labelled 1–4). Pipette into each tube the amounts shown in Table above.

3. Mix the contents of the tubes and allow to stand for 5 minutes.

4. Read the dilutions in the spectrophotometer (or colorimeter):

(a) Set the wavelength to 540 nanometers (nm) or place a green filter in the spectrophotometer (or colorimeter).

(b) Fill a matched cuvette with Drabkin diluting fluid and place in the spectrophotometer (or colorimeter).

(c) Zero the spectrophotometer.

(d) Read the contents of tubes 1 to 4, using a cuvette.

Make sure the needle returns to zero between each reading with Drabkin diluting fluid.

5. Prepare a graph, plotting the readings of the diluted reference solutions against their respective hemoglobin concentrations.

6. From the graph make a table of hemoglobin values from 20 to 180 g/l.

Calibration of the spectrophotometer (or colorimeter) using a blood sample of known hemoglobin concentration

1. Obtain a sample of blood of known hemoglobin concentration (e.g. 168 g/l).

2. Switch on the spectrophotometer (or colorimeter) and set to wavelength 540nm.

3. Pipette 8 ml of Drabkin diluting fluid into a test-tube. Add 0.04ml of well-mixed blood. Be sure to wipe the outside of the pipette beforehand to avoid adding excess blood. Mix the haemiglobincyanide solution by inverting several times. Leave to stand for 10 minutes.

4. Zero the spectrophotometer using Drabkin diluting fluid.

5. Read and record the absorbance of the haemiglobincyanide solution prepared above.

6. Prepare a series of dilutions of the haemiglobincyanide solution in four test tubes (labelled 1–4) as shown in Table below.

7. Read and record the absorbances of the diluted solutions.

Table. Sample spectrophotometer readings for different dilutions of reference solution

Dilution undiluted	Haemoglobin concentration (g/l)	Absorbance at 540nm
1 : 2	150	35.0
1 : 3	150/2 = 75	17.5
1 : 4	150/3 = 50	11.5
	150/4 = 37.5	8.5

8. Plot a graph of absorbance against hemoglobin concentration, using ordinary graph paper. Draw a straight line starting at the origin passing as close to each point as possible. Extend the line so that you can read absorbances for hemoglobin values greater than 168 g/l.

Table. Preparing serial dilutions of haemiglobincyanide solution

Tube no.	Volume of haemiglobincyanide solution (ml)	Volume of Drabkin dilution fluid (ml)	Concentration of hemoglobin (g/l)
1	4.0	1.0	13.4
2	3.0	2.0	10.1
3	2.0	3.0	6.7
4	1.0	4.0	3.4

a) In this example, it is assumed that the hemoglobin concentration of the haemiglobincyanide solution is 168 g/l.

A reference table of values is prepared using the graphs obtained from either of the above methods:

_ Draw up a table of absorbance readings starting from 0.00, 0.01, 0.02 and ending at 1.50.

_ Determine the hemoglobin concentrations for each of the absorbances from the graph.

Precautions

_ Potassium cyanide is very poisonous. It must be kept in a locked cupboard at all times when not in use. Wash your hands immediately after handling it.

_ Store Drabkin diluting fluid in a brown reagent bottle because it decomposes on exposure to light. If a brown reagent bottle is not available, use a clear glass bottle carefully wrapped in silver foil.

_ Drabkin diluting fluid should be clear and pale yellow. If it becomes turbid, or loses its color, it should be discarded. The clarity of the diluting fluid can be checked by measuring its absorbance in a spectrophotometer at 540 nm against water as a blank. The absorbance must read zero.

_ Once the haemiglobincyanide solution has been prepared, the hemoglobin estimation must be carried out within 6 hours.

_ Drabkin diluting fluid remains stable for several months when stored at cool temperatures. If the room temperature exceeds 30 °C, store it in a refrigerator at 4–6°C. Do not freeze, as this may cause decomposition of the compound. Always allow the diluting fluid to warm to room temperature before use.

Method

1. Pipette 5 ml of Drabkin diluting fluid into a tube. Draw venous or capillary blood to the 0.02-ml mark of a blood (Sahli) pipette. Do not allow air bubbles to enter. With venous blood ensure that it is well mixed by inverting the bottle containing it and the anticoagulant repeatedly for about 1 minute immediately before pipetting it.

2. Wipe the outside of the pipette. Check that the blood is still on the 0.02-ml mark. Squeeze the bulb of the pipette to expel the blood into the Drabkin diluting fluid and rinse the pipette by drawing up and expelling the fluid in the tube three times.

3. Mix the contents of the tube and leave for 5 minutes.

4. Zero the colorimeter using Drabkin diluting fluid. Read the absorbance of the patient's diluted blood in the spectrophotometer test-tube or cuvette.

If cloudiness appears in the diluted blood, this may be attributable to abnormal plasma proteins or to a high concentration of white cells. Centrifuge the diluted blood at 2000*g* for 5 minutes before taking a reading.

Using the table prepared from the calibration curve, record the concentration of hemoglobin in g/l.

Reference range

Table. Normal hemoglobin concentrations (reference ranges), by age group

Age group concentration (mmol/l)	Haemoglobin (Fe)	Haemoglobin concentration (g/l)
Newborn infants	8.4–12.1	136–196
Infants (1 year)	7.0–8.1	13–130
Children (10–12 years)	7.4–9.2	115–148
Women	7.4–9.9	120–160
Men	8.1–11.2	130–180

-Alkaline hematin D method

Principle: When a blood sample is added to an alkaline solution containing a non-ionic detergent, the hemoglobin is converted to alkaline hematin D-575, which is a stable colored compound. The absorbance of the alkaline hematin D-575 is measured using a haemoglobinometer or colorimeter. The spectrophotometer and haemoglobinometer directly determine the hemoglobin (Hb) concentration of the blood sample, whereas with a colorimeter, the hemoglobin concentration of the blood sample is obtained from the absorbance using a prepared calibration curve or table of values.

The alkaline hematin D (AHD) method offers several advantages over the hemoglobin cyanide method:

_ It is as accurate, but less expensive.

_ The calibration procedure uses chlorhemin, a stable crystalline compound that is commercially available.

_ The AHD reagent does not include potassium cyanide, which is highly toxic, in contrast to Drabkin diluting fluid for the hemoglobin cyanide method.

_ The AHD reagent can be prepared using chemicals that are generally available locally.

Materials and reagents

_Spectrophotometer, haemoglobinometer or colorimeter

_ Test-tubes

_ Test-tube racks

_ Corks or rubber stoppers

_ Cuvettes

_ Grease pencil

_ Cotton wool or gauze

_ AHD standard (supplied by the central laboratory)

_ AHD reagent.

Calibration of the spectrophotometer or haemoglobinometer

1. Note the concentration of the AHD standard indicated on the label, e.g. 160g/l at a 1:150 dilution.

2. Pipette 20ml of AHD standard into a clean test-tube containing 3 ml of AHD reagent.

3. Stopper the test-tube using a clean cork or rubber stopper and mix by inversion. Leave the tube to stand for 2–3 minutes.

4. Fill a clean cuvette with the undiluted AHD reagent. Dry the outside of the cuvette with cotton wool or gauze and place it in the cuvette chamber. Adjust the spectrophotometer or haemoglobinometer to read zero (blank).

5. Replace the undiluted AHD reagent in the cuvette with the diluted AHD standard solution; repeat the measurement procedure and adjust the spectrophotometer or haemoglobinometer to read the correct haemoglobin concentration indicated on the label, e.g. 160 g/l.

Calibration of the colorimeter

1. Switch on the colorimeter and set the wavelength to 540nm. Allow the colorimeter to warm up for the time recommended by the manufacturer.

2. Arrange six test-tubes in a test-tube rack. Label the test-tubes 1, 2, 3, 4, B and N.

3. Pipette 5 ml of AHD reagent into the test-tube marked B.

4. Pipette 3 ml of AHD reagent and 20ml of AHD standard into the test-tube marked N.

5. Dilute the reference solution in test-tube N as described in Table below.

6. Pipette the indicated volumes of AHD reagent and reference solution into test tubes 1–4. Stopper each tube and mix by inversion.

7. Calculate the hemoglobin concentrations in the test-tubes as follows:

hemoglobin concentration = concentration of reference solution X dilution factor

For example:

tube N: 160 g Hb/l

tube 1: 160 g Hb/l X 4/5 = 128 gHb/l

tube 2: 160 g Hb/l X 3/5 = 96 g Hb/l

tube 3: 160 g Hb/l X 2/5 = 64 g Hb/l

tube 4: 160 g Hb/Lx 1/5 = 32 g Hb/l

tube B: 0 g Hb/l

Table. Preparation of serial dilutions of AHD reference solution for calibration of a colorimeter

Test-tube	1	2	3	4
AHD reagent (ml)	1	2	3	4
AHD reference solution (ml)	4	3	2	1
Total volume (ml)	5	5	5	5

8. Pour the AHD reagent from test-tube B into a clean cuvette. Dry the outside of the cuvette with cotton wool or gauze. Place the cuvette into the cuvette chamber, close the cuvette chamber and adjust the colorimeter to read zero absorbance (blank).

9. Replace the AHD reagent in the cuvette with the reference solution from test tube 4. Record the absorbance. Pour the solution back into test-tube 4.

10. Repeat the procedure using test-tubes 3, 2, 1 and N, respectively in sequence.

11. Plot a graph of the absorbance values against the hemoglobin concentration (g/l) for the standard and test samples (N and tubes 1–4, respectively). Starting from the origin, draw a straight line joining through as many of the points as possible.

***Note*: Always prepare a new calibration curve whenever you use a different colorimeter, type of cuvette, or method for hemoglobin measurement.**

Method

Method using a spectrophotometer or haemoglobinometer

1. Switch on the spectrophotometer or haemoglobinometer. Allow it to warm up for the time recommended by the manufacturer (usually 10 minutes).

2. Arrange the test-tubes in a test-tube rack: one for each sample to be tested, one for the blank and two for the control samples.

3. Using a grease pencil, label the test-tubes with the appropriate laboratory numbers of the samples to be measured, B for the blank, and C1 and C2 for the control samples.

4. Pipette 3 ml of AHD reagent into each test-tube.

5. Pipette 20ml of blood collected in EDTA from a patient into the AHD reagent of the appropriate tube. Flush the pipette carefully five times with the AHD reagent.

6. Pipette 20ml of AHD standard into test-tubes C1 and C2.

7. Plug all the test-tubes with a clean cork or rubber stopper and mix by inversion. Leave the tubes to stand for 2–3 minutes.

8. Pour the AHD solution from test-tube B into a clean cuvette. Dry the outside of the cuvette with cotton wool or gauze. Make sure that there are no air bubbles in the solution. Place the cuvette in the cuvette chamber and adjust the spectrophotometer or haemoglobinometer to read zero.

9. Repeat the procedure with the solution in test-tubes C1 and C2, respectively. If the readings of the two controls differ by less than 2.5%, measure the hemoglobin concentration of all the test samples. Record all the results.

Method using a colorimeter

The AHD method is also applicable using a colorimeter. The measurement procedure is the same as that described for a spectrophotometer or haemoglobinometer. However, the absorbance in a colorimeter does not increase linearly with hemoglobin at elevated concentrations. Therefore, a calibration curve must be used to relate the absorbance readings to the hemoglobin concentration, as described above.

Results

Report the results in g/l. Example: “haemoglobin = 89 g/l”.

Errors in hemoglobin estimation

Errors in sampling:

— inadequate flow of blood from the finger prick;

— excessive squeezing of the finger after pricking;

— prolonged use of a tourniquet when collecting venous blood, which leads to concentration of blood cells;

— insufficient mixing of venous blood, which has sedimented after collection;

— small clots in venous blood due to inadequate mixing with EDTA after collection;

— adding too little or excess blood to Drabkin diluting fluid;

— air bubbles trapped in pipettes.

Faulty or dirty equipment, such as:

— broken or chipped pipettes;

— dirty pipettes;

— dirty cuvettes;

— dirty filters;

— a defective spectrophotometer, haemoglobinometer or colorimeter.

Faulty technique:

— using a dilution factor different from the one for which the spectrophotometer, haemoglobinometer or colorimeter was calibrated;

— inadequate mixing of reagent;

— placing the cuvette in the chamber with the frosted sides facing the light path;

— air bubbles in the cuvette;

— using a standard filter from another spectrophotometer or haemoglobinometer for adjustment;

— using the wrong filter for the colorimeter.

***Note*:**

If the spectrophotometer, haemoglobinometer or colorimeter requires frequent recalibration, e.g. every 2–3 days, change the bulb and repeat the procedure for internal quality control. If the problem of frequent recalibration persists, send the machine to a servicing agent.

- **DHT 523 Haemoglobinometer (Direct read-out Hb meter)**

This modern electronic direct read-out hemoglobin meter is precalibrated by the manufacturer and therefore requires no calibration standard solutions. A glass control standard is provided for checking the performance of the meter.

Diluting fluid: Weak 0.4 ml/l (0.04%) ammonia Reagent No. 12 water. The reagent is stable when kept in a tightly stoppered bottle. Renew every 6 weeks.

***Note*: Weak ammonia water lyzes red cells rapidly, is stable, and the ammonia solution used in its preparation is easily available and inexpensive. It does not require refrigeration. The test requires only 2 ml of ammonia diluting fluid.**

Principle of operation

Fig: DHT 523 Haemoglobin Meter.

Light emitted from the LED passes through the blood sample and then through the interference filter, restricting the wavelength to peak at 523 nm within a narrow band. Light passing through this narrow band filter falls on the photodiode (see Fig.). This converts it to an electrical signal for the control and measurement system to calculate and display directly as a hemoglobin concentration in g/l. The meter has a stated photometry precision of __5% (CV) and 2% accuracy of

method. The meter must not be placed in direct sunlight and the operating environment should be within a temperature range of 10–35 C with an upper humidity limit of 80% noncondensing.

Test method

1- Measure carefully 20 µl (0.02 ml, 20 cmm) of capillary blood or *well-mixed* venous blood and dispense it into 2 ml of the ammonia diluting fluid.

***Important*: The volume of blood used must be exactly 20 µl.**

2- Stopper the tube and mix. The solution can be read immediately. The color is stable for 6–8 hours.

3-Check the performance of the meter by inserting the *Control Standard* glass provided in the cuvette aperture. The reading must correspond to the stated value, ±5.

***Note*: Inserting a cuvette starts the measuring process. There is an audible signal as the meter reads the *Control Standard* or patient's sample. Immediately the value is shown on the digital display and held in memory for 30 seconds after the cuvette is removed. The last reading can be recalled by pressing a membrane key on the instrument. The *DHT Haemoglobinometer* has automatic zeroing. In between readings the meter remains in a standby mode.**

4-Transfer the patient's sample or control blood sample to a clean 10 mm light-path cuvette. Hold the cuvette only by its non-optical sides and ensure there are no air-bubbles in the sample.

5-Place the cuvette in the cuvette holder, wait for the audible signal, and read the hemoglobin value from the display.

6-Return the sample to its tube and allow the cuvette to drain, e.g. invert it on a paper towel.

- **No-Dilution Techniques**

  - **HemoCue Haemoglobin Technique**

The most recently developed *HemoCue* hemoglobin meter is the model 201$^+$. It differs from previous models in being smaller, having an internal performance check system (control cuvette is no longer needed), and a new cuvette holder which prevents contamination of the optronic unit and is easier to clean. The 201$^+$ meter can also store up to 600 test results. The microcuvettes are specially designed for use with the model 201$^+$ (cannot be used in previous *HemoCue* models). They are individually packaged and available in a smaller pack size (25 per pack). While the new model 201$^+$ *HemoCue* is less expensive, the price of the microcuvettes remains high. The 201$^+$ *HemoCue* meter measures 85 X 160 X43 mm and weighs 350 g (batteries included). It can be operated from mains electricity using the mains adapter supplied or from four AA batteries (not provided).

Principle of HemoCue

Undiluted whole blood is drawn into a chemically coated single-use microcuvette. The red cells are lyzed by sodium deoxycholate and the hemoglobin reacts with sodium nitrite (forming methemoglobin) and sodium azide to give azidemethaemoglobin which is measured by the meter at wavelengths 570 nm and also at 880 nm to compensate for any turbidity in the sample, e.g. caused by high WBC count or raised lipids.

The concentration of hemoglobin is digitally displayed in grams per liter or if preferred, g/dl. The measuring range of the *HemoCue* meter is 0–256 g/l (25.6 g/dl) with an accuracy of _1.5% compared with the ICSH method. There is no interference from bilirubin. The meter is calibrated by the manufacturer. A performance check (*self-test*) is built into the meter. Every time the meter is turned on (and every 2 hours during operation), a performance check is carried out. The operating environment should be within the temperature range 18–30 ºC with an upper humidity limit of 90% non-condensing.

Test method

1- Pull out the microcuvette holder to its loading position.

2- Press and hold down the left button of the meter until the display is activated. The meter automatically carries out a performance check. After 10 seconds the meter will show three flashing dashes, indicating it is ready for use.

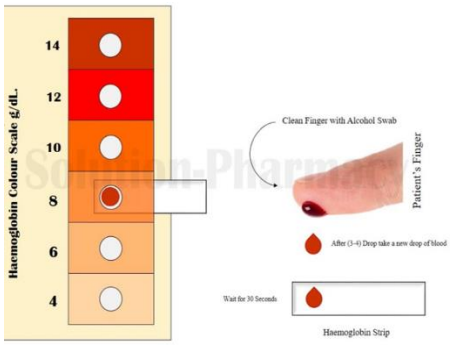
3- Fill a microcuvette in one continuous process with capillary blood or *well mixed* venous blood.

4- Wipe off excess blood from the outside of the microcuvette tip, making sure no blood is withdrawn.

6- **Within 10 minutes of filling the microcuvette, measure the hemoglobin. Place the microcuvette in the microcuvette holder and push in the holder to its measuring position. After 15–60 seconds, the hemoglobin value will be displayed (it will remain displayed for as long as the holder is left in its measuring position).**

- **Haemoglobin Colour Scale**

This technique for estimating hemoglobin is based on comparing the color of a drop of blood absorbed on a particular type of chromatography paper, against a printed scale of colors corresponding to six different levels of hemoglobin: 4, 6, 8, 10, 12, and 14 g/dl (intermediate readings can be made between any two adjacent shades of color). Although similar in principle to the obsolete Tallqvist blotting paper method (discredited due to inaccuracy and imprecision) the new *Haemoglobin Color Scale* uses modern materials and techniques to provide a simple, inexpensive, method for estimating hemoglobin in community health care where photometric measurement is not possible.



Exact details of how to estimate hemoglobin using the *Haemoglobin Color Scale* are supplied with the Scale. From the above information it is clear that to obtain reliable test results it is *essential* to use only the special test papers supplied and *follow exactly* the instructions given for collecting a blood sample, amount of blood to use, and how to compare the color of the blood with the color standards of the *Color Scale*.

HAMOGLUBIN ELECTROPHORESIS

Haemoglobin electrophoresis is used to separate and identify the different hemoglobin by their migration within an electric field. Haemoglobin variants separate at different rates due to differences in their surface electrical charge as determined by their amino acid structure.

Alkaline cellulose acetate electrophoresis

Several techniques are available to separate hemoglobin variants by electrophoresis. For routine work, electrophoresis in an alkaline buffer at Ph 8.4–8.6 using a cellulose acetate membrane is adequate. This gives good separation of HbA, HbF, HbS, and HbC. On alkaline electrophoresis HbD and HbS have the same mobility and HbC, HbE and HbO also co-migrate. In specialist laboratories agarose gel electrophoresis at an acid pH (6.0) can be used to separate this hemoglobin and also to provide a clear separation of HbF from HbS and HbC.

Equipment

Most recently developed equipment for cellulose acetate alkaline electrophoresis is designed for use with plastic supported (*Mylar*-backed) cellulose acetate membranes. Such equipment is not suitable for use with unsupported (non-*Mylar*-backed) cellulose acetate membranes. Unsupported cellulose acetate membranes are becoming increasingly difficult to obtain* as more laboratories change to using *Mylar*-backed membranes because they provide rapid separation of the different abnormal hemoglobin and HbA2 with greater resolution.

***A source of unsupported cellulose acetate membranes is Sartorius AG. The membranes measure 78 _ 150 mm, code No. SM 12200–78 _ 150K and are packaged 50 per box. These membranes can be used in older electrophoresis tanks.**

Equipment for performing alkaline cellulose acetate electrophoresis using *Mylar*-backed supported cellulose acetate membranes is available from Helena BioSciences. The essential components are:

● An electrophoresis chamber (tank), model *Zip Zone Chamber* (Cat. No. 1283) as shown

Fig. Helena BioSciences *Zip Zone* electrophoresis chamber suitable for alkaline cellulose acetate hemoglobin electrophoresis.

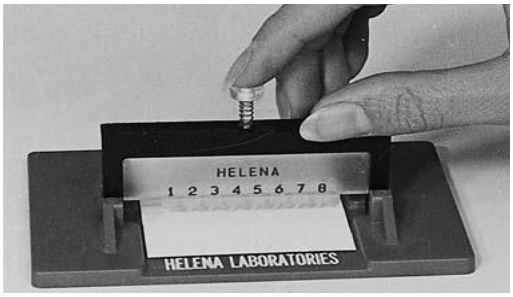


Fig. Applicator system used with the *Zip Zone* electrophoresis chamber. *Courtesy of Helena BioSciences*.

● A regulated Power Supply capable of delivering 350 V 50 mA is required for cellulose acetate hemoglobin electrophoresis. An example of a suitable Power Supply is the model EV 243 variable voltage Power Supply available from Helena BioSciences.

Fig. EV 243 Power Supply suitable for cellulose acetate hemoglobin electrophoresis with integral digital timer.

● Accessories for applying the samples, including:

– *Zip-Zone* 8 unit applicator (Cat. No. 4080)

– *Zip-Zone* well plate (Cat. No. 4081)

– *Zip-Zone* aligning base (Cat. No. 4082)

Cellulose acetate (Mylar-backed) membranes*

***Referred to by Helena BioSciences as *Titan 111-H Acetate plates (Cat. No. 3022),* they are designed for use in the *Zip- Zone* electrophoresis chamber.**

***Titan 111-H acetate plates* (membranes) measure 60 _ 76 mm. Each membrane is sufficiently wide for 8 samples to be applied. They are supplied 25 membranes (plates) per box.**

Reagents

Tris-EDTA-borate buffer, pH 8.5 Reagent No. 86 Keep refrigerated at 4–8˚C. It has good stability.

Controls

Controls should include the following hemolysates:

HbAA: Normal adult blood

HbAS: Blood from a person with sickle cell trait

HbAF: Blood from an infant below 3 m

HbAC: Blood from a person with HbC trait (when HbC is found locally)

Control hemolysates should be prepared from HIV and hepatitis HBsAg negative persons (see end of subunit). When stored at 4 _–8 _C they are stable for several weeks and when stored frozen they can be kept for several months.

***Note*: Various controls for hemoglobin electrophoresis are also available from Helena BioSciences (*Hemo Controls*), e.g. *Hemo* Hb A, F, S, A2, *Hemo* Hb A, A2, *Hemo* A, F, S, C, and *Hemo* A, S, A2.**

Method

The procedure for performing alkaline cellulose acetate hemoglobin electrophoresis using Helena BioSciences equipment and *Mylar*-backed supported cellulose acetate membranes (*Titan 111 cellulose acetate plates*) is supplied with the membranes. The following is a summary of the method used to separate the different haemoglobins (e.g. Hb A, F, S, and C), excluding staining and densitometry to quantitate the relative percentage of each hemoglobin band*.

***Staining is required when a permanent record of the results of hemoglobin electrophoresis is required. When using *Mylar*-backed cellulose acetate membranes, separation of the different haemoglobins is rapid and clear, enabling results to be read visually without the need for staining.**

Furthermore, some laboratories have found that they can reuse membranes by allowing the original samples to 'run off'. Quantifying the different hemoglobin bands may be indicated in certain hemoglobin disorders. It requires use of a clearing agent, drier, and densitometer (available from Helena BioSciences).

1- Prepare the cellulose acetate membrane (*Titan 111 cellulose acetate plate*) exactly as described in the Helena BioSciences procedure.

2- Pour 100 ml of the Tris-EDTA-borate buffer into each of the outer sections of the *Zip-Zone* electrophoresis chamber.

3- Wet two wicks (as supplied) in the buffer and drape one over each support bridge, ensuring each makes contact with the buffer and that there are no air bubbles under the wicks. Cover the chamber to prevent evaporation.

4- Transfer 5 µl of each hemolysate sample (tests and controls) into the *Zip-Zone* well plate.

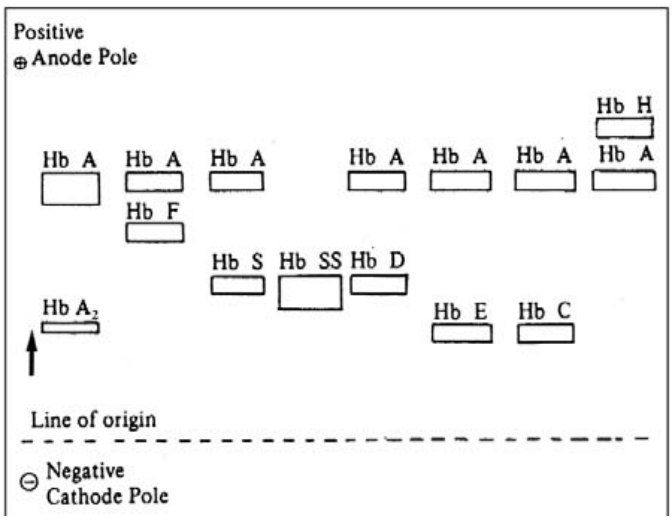
5- Place a cellulose acetate membrane (plate) in the *Zip-Zone* aligning plate and apply the samples using the 8-unit applicator *exactly* as described in the Helena BioSciences procedure.

6- Immediately place the cellulose acetate membrane (plate) in the electrophoresis chamber, *cellulose acetate side down*.

7- Connect the chamber to the Power Supply and electrophorese the plate for 25 minutes (or shorter) at 350 volts and 50 mA.

***Note*: To economize, some laboratories apply a second row of samples to the membrane (about half-way down). When this is done, more careful attention must be paid to timing the 'run'. As soon as the controls and samples show adequate clear to read separations, electrophoresis can be stopped.**

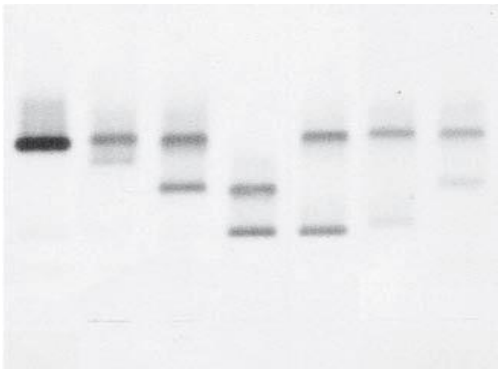
Results



The relative mobilities of haemoglobins A, F, S, D, E, C and H after alkaline electrophoresis are shown in Plate a

In alkaline buffer, HbS and HbD have similar mobility and HbC, HbA2, HbE, and HbO have similar mobility. Separation of some haemoglobins on a *Titan 111 cellulose acetate plate* are shown in Plate b. Acid citrate agar electrophoresis can help to differentiate some of the haemoglobins which co-migrate.

Plate(a). Separation of haemoglobins by electrophoresis at alkaline pH. *Note*: HbS and HbD have the same mobility but can be differentiated by a sickle cell test. HbE and C also co-migrate but the global distribution of these two haemoglobins is different.



AA AF AS SC AC AE AD

A

SD

CE

Plate (b). Separation of haemoglobins on *Mylar*-backed cellulose acetate membrane (plate) by alkaline electrophoresis. *Courtesy of Helena BioSciences*.

$HbG^{Philadelphia}$: This alpha globin abnormal hemoglobin is found in Africans and may cause confusion when interpreting electrophoresis test results. HbAA + HbGPhil looks like HbAS on electrophoresis. With HbAS + HbGPhil, the hybrid molecule S/G has a mobility similar to HbC. HbSS + GPhil looks like HbSC from which it may be distinguished by the solubility test which gives an HbSS result.

ERYTHROCYTE SEDIMENTATION RATE (ESR)

The erythrocyte sedimentation rate (ESR) is a common hematological test for nonspecific detection of inflammation that may be caused by infection, some cancers and certain autoimmune diseases. It can be defined as the rate at which Red Blood Cells (RBCs)sediment in a period of one hour.

Principle: When anticoagulated blood is allowed to stand in a narrow vertical glass tube, undisturbed for a period of time, the RBCs – under the influence of gravity- settle out from the plasma. The rate at which they settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour(mm/hr). This mechanism involves three stages:

- **Stage of aggregation: It is the initial stage in which piling up of RBCs takes place. The phenomenon is known as Rouleaux formation. It occurs in the first 10-15 minutes.**
- **Stage of sedimentation: It is the stage of actual falling of RBCs in which sedimentation occurs at constant rate. This occurs in 30-40 minutes out of 1 hour, depending upon the length of the tube used.**

- **Stage of packing: This is the final stage and is also known as stationary phase. In this, there is a slower rate of falling during which packing of sedimented RBCs in column occurs due to overcrowding. It occurs in final 10 minutes in 1 hour.**

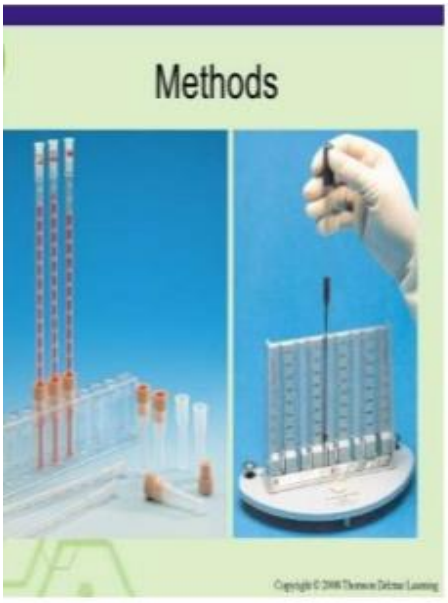
Methods of ESR Determination

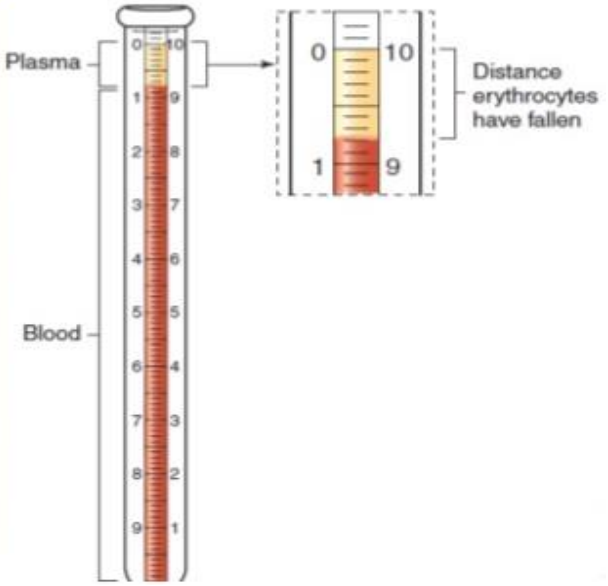
There are two main methods to determine ESR:

- **Wintrobe's method**
- **Westergren's method**

Each method produces slightly different results. Mosely and Bull (1991) concluded that Wintrobe's method is more sensitive when the ESR is low, whereas, when the ESR is high, the Westergren's method is preferably an indication of patient's clinical state.

Wintrobe Method





This method uses Wintrobe's tube, a narrow glass tube closed at the lower end only. The Wintrobe's tube has a length of 11 cm and internal diameter of 2.5 mm. It contains 0.7-1 ml of blood. The lower 10 cm are in cm and mm. The marking is

0 at the top and 10 at the bottom for ESR. This tube can also be used for PCV. The marking is 10 at the top and 0 at the bottom for PCV.

Requirements:

- **Anticoagulated blood (EDTA, double oxalate)**
- **Pasteur pipette**
- **Timer**
- **Wintrobe's tube**
- **Wintrobe's stand**

Procedure:

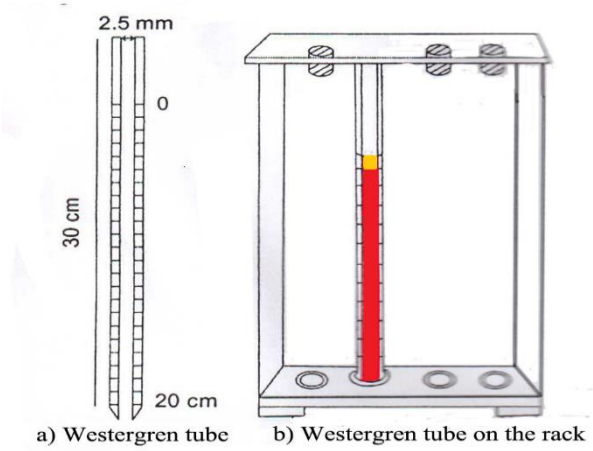
1. **Mix the anticoagulated blood thoroughly.**
2. **By using Pasteur pipette, fill the Wintrobe's tube upto '0' mark. There should be no bubbles in the blood.**
3. **Place the tube vertically in ESR stand and leave undisturbed for 1 hour.**
4. **At the end of 1 hour, read the result.**

Normal Value:

For males: 0-9 mm/hr
For females: 0-20 mm/hr

WESTERGREN'S METHOD

It is better method than Wintrobe's method. The reading obtain is magnified as the column is lengthier. The Westergren tube is open at both ends. It is 30 cm in length and 2.5 mm in diameter. The lower 20 cm are marked with 0 at the top and 200 at the bottom. It contains about 2 ml of blood.



a) Westergren tube b) Westergren tube on the rack

Requirements:

- **Anticoagulated blood (0.4 ml of 3.13% trisodium citrate solution + 1.6 ml blood)**
- **Westergren tube**
- **Westergren stand**
- **Rubber bulb (sucker)**

Procedure:

1. **Mix the anticoagulated blood thoroughly.**
2. **Draw the blood into the tube up to 0 mark with the help of rubber bulb.**
3. **Wipe out blood from bottom of the tube with cotton.**
4. **Set the tube upright in stand. Make sure the pipette fits snugly to eliminate possible leakage and that the pipette is in vertical position.**
5. **Leave the tube undisturbed for 1 hour.**
6. **At the end of 1 hour, read the result.**

Normal Value:

For males: 0-10 mm/hr
For females: 0-15 mm/hr

Clinical Significance of ESR

The erythrocyte sedimentation rate (ESR) is a non-specific test. It is raised in a wide range of infectious, inflammatory, degenerative, and malignant conditions associated with changes in plasma proteins, particularly increases in fibrinogen, immunoglobulins, and C-reactive protein. The ESR is also affected by many other

factors including anemia, pregnancy, haemoglobinopathies, hemoconcentration and treatment with anti-inflammatory drugs.

Causes of a significantly raised ESR:

- **All types of anemias except sickle cell anemia**
- **Acute and chronic inflammatory conditions and infections including:**
  – HIV disease
  – Tuberculosis
  – Acute viral hepatitis
  – Arthritis
  – Bacterial endocarditis
  – Pelvic inflammatory disease
  – Ruptured ectopic pregnancy
  – Systemic lupus erythematosus
- **African trypanosomiasis (rises rapidly)**
- **Visceral leishmaniasis**
- **Myelomatosis, lymphoma, Hodgkin's disease, some tumors**
- **Drugs, including oral contraceptives**

Causes of Reduced ESR:

- **Polycythemia**
- **Poikilocytosis**
- **Newborn infants**
- **Dehydration**
- **Dengue hemorrhagic fever**
- **and other conditions associated with hemoconcentration**

Automated Analysis of ESR

Today, there are automated ESR systems that provide faster results and address laboratory safety by minimizing contact with blood samples. Automated Systems Based on the Westergren Method

- **Streck, Inc. (Omaha, NE) offers the CE-marked Streck ESR-Auto Plus (as shown in the Fig.)**

COUNTIG WHITE CELLS (WBC; Leukocyte Count)

Leukocytes fight infection and defend the body by a process called *phagocytosis,* in which the leukocytes actually encapsulate foreign organisms and destroy them. Leukocytes also produce, transport, and distribute antibodies as part of the immune response to a foreign substance (antigen).

The WBC serves as a useful guide to the severity of the disease process. Specific patterns of leukocyte response can be expected in various types of diseases as determined by the differential count (percentages of the different types of leukocytes).

Principle of test

Whole blood is diluted 1 in 20 in an acid reagent which hemolyzes the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted. White cells are counted microscopically using an Improved Neubauer ruled

counting chamber (hemocytometer) and the number of WBCs per liter of blood calculated.

***Note*: When after examining a stained blood film, many nucleated red cells are present (more than 10%), the WBC count should be corrected.**

Blood sample: EDTA anticoagulated blood or capillary blood can be used for counting white cells. Heparin or sodium citrate anticoagulated blood must not be used. The count should be performed within 6 hours (blood should not be refrigerated).

Equipment

- ***Counting chamber (hemocytometer)*.**
- ***Counting chamber cover glasses***
- ***Pipettes/calibrated capillaries and safe filling device***
- ***Hand counter***

Reagent

WBC diluting fluid* Reagent prepared by:

- **Adding glacial acetic acid 2.5 ml**
- **2 drops of 1% gentian violet**
- **Made up to 100 ml with distilled water**
- **Store at room temp 25-35˚C**

***This is a weak acid solution to which gentian violet is added which stains the nucleus of white cells.**

Test method

1- Measure 0.38 ml of diluting fluid and dispense it into a small container or tube.

2- Add 20 µl (0.02 ml, 20 cm) of *well-mixed* EDTA anticoagulated venous blood or free flowing capillary blood and mix.

***Important*: The volume of blood used in the test must be correct.**

3- Assemble the counting chamber:

– Make sure the central grid areas of the chamber and the special hemocytometer cover glass are completely clean and dry.

– Slide the cover glass into position over the grid areas and press down on each side until rainbow colors (Newton's rings) are seen. Prior moistening of the chamber surface on each side of the grid areas will help the cover glass to adhere to the chamber.

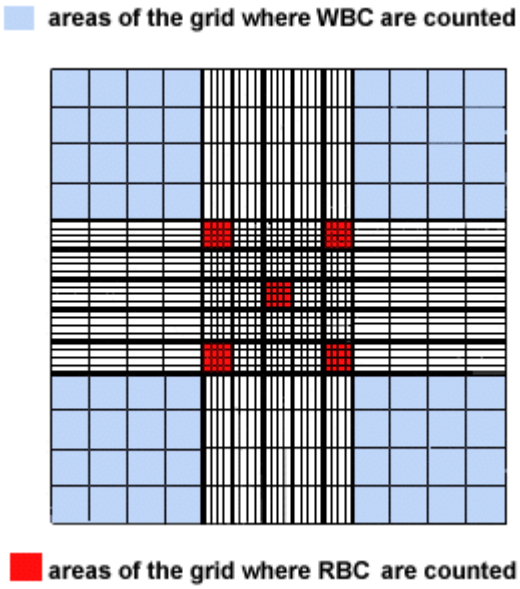
4- Re-mix the diluted blood sample. Using a capillary, Pasteur pipette, or plastic bulb pastette held at an angle of about 45˚, fill one of the grids of the chamber with the sample, taking care not to overfill the area.

Important: The chamber must be refilled if the sample overfills into the channel beyond the grid or an air bubble forms in the grid area.

5- Leave the chamber undisturbed for 2 minutes to allow time for the white cells to settle.

Note: To prevent drying of the fluid, place the chamber in a petri dish or plastic container on dampened tissue or blotting paper and cover with a lid.

6- Dry the underside of the chamber and place it on the microscope stage. Using the 10X objective with the condenser iris closed sufficiently to give good contrast, focus the rulings of the chamber and white cells. Focus the cells until they appear as small black dots.



7- Count the cells in the four large corner squares of the chamber marked W1, W2, W3, W4 in (total area of 4 mm2). Include in the count the cells lying on the lines of two sides of each large square.

8- Report the number of white cells per litre of blood using the following simple calculation:

– Divide the total number of cells counted by 2.

– Divide the number obtained by 10.

The number obtained X10^9 is the white cell count.

Example

Cells counted in 4 squares = 84

84 / 2 = 42

42 / 10 = 4.2

WBC count = 4.2 cells* X *10^9/l

WBC calculation details

WBC count (per liter) =Cells counted X 20* X10^6 / 4‡ X 0.1¶

where* = 1 in 20 dilution of blood, ‡ = 4 mm^2 area counted, ¶ = 0.1 mm depth of chamber.

9- After performing the count, before the sample dries, dismantle the chamber, wash and dry it. Store it with the cover glass in a safe place.

Counts higher than 50.0 X10^9/l

When a count is higher than 50.0/10^9/l, repeat the count using 0.76 ml of diluting fluid and 20 µl of blood. Multiply the result by 2. Very high WBC counts are found in some forms of leukemia. Always examine a stained thin blood film.

Counts lower than 2.0 X 10^9/l

When a count is lower than 2.0 X 10^9/l, repeat the count using 0.38 ml of diluting fluid and 40 µl of blood. Divide the result by 2.

Correcting a WBC count when there are many nucleated RBCs

When more than 10 nucleated red blood cells (RBCs) per 100 WBC are present in the blood film, correct the WBC count as follows:

Corrected WBC= Uncorrected WBC count X 100 / Nucleated RBCs* + 100

***Number of nucleated RBCs per 100 WBC as seen in stained blood film.**

Reference Values

Normal

Black adults: 3.2–10.0 _ 10 3 cells/mm 3 or _ 10 9 /L or 3200–10,000 cells/mm 3

Adults: 4.5–10.5 _ 10 3 cells/mm 3 or _ 10 9 /L or 4500–10,500 cells/mm 3

Children:

0–2 weeks: 9.0–30.0 _ 10 3 cells/mm 3 or _ 10 9 /L or 9000–30,000 cells/mm 3

2–8 weeks: 5.0–21.0 _ 10 3 cells/mm 3 or _ 10 9 /L or 5000–21,000 cells/mm 3

2 months–6 years: 5.0–19.0 _ 10 3 cells/mm 3 or _ 10 9 /L or 5000–19,000 cells/mm 3

6–18 years: 4.8–10.8 _ 10 3 cells/mm 3 or _ 10 9 /L or 4800–10,800 cells/mm 3

DIFFERENTIAL WHITE CELL COUNT

A differential white cell count provides information on the different white cells present in the circulating blood, i.e. neutrophils, lymphocytes, monocytes, eosinophils, basophils (rarely seen). Providing the total WBC count is known, the absolute number of each white cell type, i.e. number of each cell per liter of blood, can be calculated and an assessment made of whether the number of a particular cell type is increased or decreased (compared with the accepted reference range).

Method

As previously discussed, it is only possible to report blood films reliably providing the thin blood film is well made and correctly stained. Allow the stained film to dry completely before examining it.

1- Place a drop of immersion oil on the lower third of the blood film and cover with a clean cover glass.

2- Examine the film microscopically. Focus the cells using the 10_ objective with the condenser iris closed sufficiently to see the cells clearly. Check the staining and distribution of cells.

3- Move to a part of the film where the red cells are just beginning to overlap and bring the 40X objective into place. Open the iris diaphragm more.

4- Systematically examine the blood film and count the different white cells seen in each field, preferably using an automatic differential cell counter, or if this is not available.

Differential WBC cell counter

Electronic and mechanical differential cell counters are available. A 6-digit mechanical cell counter is shown in the figure. It is

recommended because it is robust, easy to use and does not require batteries or mains electricity to operate it. When a total of 100 cells have been counted, there is an audible signal. The numbers of each cell type are read from the display windows. Turning the knob on the side returns all the displays to zero.

5- Calculate the absolute number of each white cell type by multiplying the number of each cell counted (expressed as a decimal fraction) by the total WBC count.

Example

If: Percentage of neutrophils counted = 80% i.e. 0.80 when expressed as a decimal fraction.

If total WBC count = 8.6 X 10^9/l

Absolute neutrophil count is 0.80 X 8.6 = 6.9 X 10^9/l

Reporting absolute numbers of each cell type

When only the percentage of each cell type is reported in a differential WBC count and not the number per liter of blood (absolute count), this can be misleading, e.g. in the above example, a neutrophil percentage count of 80% is 'abnormal' but when expressed in absolute numbers the neutrophil count is 6.9 X 10^9/l which is within the normal reference range for an adult

Differential WBC reference range*

***These values are guideline figures only. They should be checked locally.**

Adult	Absolute	Number Percentag
Neutrophils	1.5–7.5 X 10^9/l	(40–75%)
Lymphocytes	1.2–4.0 X 10^9/l	(21–40%)
Monocytes	0.2–1.0 X 10^9/l	(2–10%)
Eosinophils	0.02–0.6 X 10^9/l	(1–6%)
Basophils	0.01–0.1 X 10^9/l	(0–1%)

CHILDREN (2–6 y)	Absolute	Number Percentag
Neutrophils	1.5–6.5 X 10^9/l	(20–45%)
Lymphocytes	6.0–8.5 X 10^9/l	(45–70%)
Monocytes	0.1–1.0 X 10^9/l	(2–10%)
Eosinophils	0.3–1.0 X 10^9/l	(1–6%)
Basophils	0.01–0.1 X 10^9/l	(0.1–1%)

- In an adult, lymphocytes are mainly of the small type whereas in a child, large lymphocytes predominate.

ABSOLUTE EOSINOPHIL COUNT

Purpose: Quantitative estimation of Absolute Eosinophil Count in human by manual method using Hemocytometer. Increased Eosinophil count is observed in wide variety of conditions like allergy, drug reaction parasitism vascular disease and in certain leukemias. Eosinophils are decreased in hyper-adrenalism and Cushing's syndrome.

Principle: One diluting the blood with eosinophil diluting fluid, the RBCs and other leukocytes except eosinophils gets lysed. Eosinophils take the eosin stain and appear red. These cells are then counted by using a Neubauer chamber under low power (10 X) of Microscope.

Performance Specification

Definition of terms: EDTA - Ethylene Diamine Tetra Acetic acid, RBC Red Blood Corpuscles; WBCs - White Blood Corpuscles, mm^3 Cubic millimeter.

Primary Sample

- Use whole blood as specimen for the test
- Collect 2 mL of blood in K_2EDTA vacutainer
- Do not use dotted specimens or contaminated serum

- **Process the specimen on the same day within 2 hours of collection.**
- **Mix the blood sample gently by rotation for 2 minutes in a hemomixer and between the palms of hand prior to testing.**

Reagents/Consumables

• Diluting fluid (Hingleman's solution) prepared by adding

- Yellow eosin - 0.5g

- 95% phenol - 0.5g

- Formalin - 0.5 ml

- Distilled water - 99 mm

• Micropipettes and glass test tubes and pipettes

Equipment

• Light microscope

• Neubauer chamber

Procedure

• Pipette 0.36 mL of eosinophil diluting fluid in a test tube and add 0.04 ml of K2 EDTA blood.

• Mix well for not more than 30 seconds and keep for 10 mts and charge Neubauer counting chamber.

• Keep it in a moist chamber for 2-3 min

• Count the cells under low power objective with reduced light in 9 squares of Neubauer chamber

Calculations

Total no. of eosinophils, cu mm (ul) = No. of cells counted x 10 / 0.9

Dilution = 10

Volume of fluid = area counted x depth

=9 sq. mm x 0.1

=0.9

Interpretation of Results

The results are calculated as per the above formula and the number of eosinophils is reported as per cu.mm of whole blood.

Precautions

- **Ensure the counting chamber is clean and dry**
- **Take care to ensure air bubbles do not enter while diluting blood**
- **Load the counting chamber in one application and ensure fluid does not overrun the surrounding moat**
- **Ensure there are no air bubbles under the cover slip and that the diluting fluid does not over run the cover slip**
- **Use a cover slip that is thick and flat**
- **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.**

Safety Precautions

- **Handle all samples as potentially infectious**
- **Handle all reagents with care and avoid contact with eye, mouth and skin**
- **Do not perform mouth pipetting**
- **Discard used reagents and sample as per disposal procedure**

Potential Sources of Variability

- **Eosinophils disintegrate in the diluting fluid hence counting done within 30 minutes of the diluting the sample with the diluting fluid**

Reference Range

40-440 cells/cu mm.

SEROLOGICAL TECHNIQUE FOR ISOLATION OF LYMPHOCYTE POPULATIONS

In studies on humans, peripheral blood lymphocytes are most readily available sources of cells. Lymphocytes and their specific subpopulations can be isolated by: Fluorescent activated cell sorter (FACS), density gradient separation and rosetting.

Activities for which the lymphocytes can be separated could be:

To detect the ability of a given B cell to produce a given antibody,

To detect the ability of a given T cell to produce particular Cytokines,

To test the ability of a given cell to be stimulated by a given mitogen.

The study of human T cells is best performed using purified cells, since the presence of other cell types may have indirect effects on T cell function. However, for any kind of functional assay on T cell specificity antigen - presenting cells are necessary.

A. peripheral blood Mononuclear Cells (PBMC) Isolation

The mononuclear cell fraction containing monocytes and lymphocytes is separated from polymorphonuclear cells and red blood cells by density gradient centrifugation.

Equipment and reagents

- **Suppl. RPMI - 1640 medium: RPMI - 1640 (LifeTechnologies Inc, Gaithersburg, MD) contaning 2mML - glutanine (Biochrom seromed, Berline, Germany), 25mM N-(2 hydroxyethyl) piperazine -N-**

(2ethancesulfonic acid) (HEPES) (Biochrom), 100U ml penicillin, 100gml streptomycin (Pen-strep, Biochrom)

- **Fetal calf serum (FCS) (e.g. Life Technologies Inc.) Which has been inactivated by heat (560c, 30 minutes) before use.**
- **Heat-inactivated human serum, blood group AB (HUS, obtained from a local blood transfusion center).**
- **Ficoll - Hypaque (Histopaque - 1077, sigma).**
- **50 and 15ml conical centrifuge tubes (e.g. Greiner, Nurtingen Germany).**
- **Temperature controlled centeifuge with GH - 3.7 - horizontal rotor (e.g. heraeus or Beckman).**
- **Trypan blue, haemocytometer:**

Procedure

- **Peripheral blood is collected in sterile heparinized tubes. Heparinized whole blood (10ml) is mixed with 15ml suppl. RPMI - 1640.**
- **The mixture is carefully layered over 15ml of Ficoll - Hypaque in a 50-ml conical centrifuge tube.**
- **Spin for 20 min at 2000 rpm (900g at 4OC).**
- **The layer between Ficoll and the upper layer (containing RPMI - 1640 and serum) contains the mononuclear cell (MNC) fraction.**
- **Using a pipette remove 80% of the upper layer and recovers the interface (MNC) layer. Transfer the latter to a new 50-ml conical tube, and fill the tube with suppl. RPMI**
- **1640/5% fetal calf serum (FCS) and centrifuge for 10min at 1300rpm (400g, 180c).**
- **After the supernatant has been removed, the MNC pellet is resuspended in suppl. RPMI-1640/5% FCS, and washed twice. For the last wash 15ml conical tube can be used.**

- **Finally the cells are resuspended in 1ml suppl. RPMI - 1640/10% heat - inactivated human AB seum (HUS).**

Counting and markers of cell death

Cell suspension (20ul) is diluted with 20ul 0.5% aqueous Trypan blue. The stained (dead) and non-stained (viable) cells are counted in a haemocytometer.

B. Separation of T and Non - T Cells from Monounclear Cells

The E - rosetting Technique

The E-rosetting technique describes a procedure for separating T cells and non -T cells from a population of MNCs (e.g. peripheral blood or synovial fluid MNCs). This method is based on the ability of human T cells to bind to sheep erthrocytes via their CD2 molecule. Neuraminidase treatment of sheep red blood cells (SRBCs) enhances the binding of SRBCs to T lymphocytes. First neuraminidase treated SRBCs are prepared. Secondly, SRBCs and MNCs are mixed to form rosettes (E+, which are then isolated from the non – resetting population (E-, i.e., B cells and monocytes) by Ficoll gradient centrifugation. In the last step, bound SRBCs are separated from the rosetted T cells by hypotonic lysis.

Equipment and reagents for E – resetting

- **SRBCs (eg. From Biologische Arbeitsgemeinschaft Hessen. Germany): sterile PBS suppl, RPMI -1640 FCS, heat inactivated (Life Technologies inc.) Test - Neuraminidase (Centeon L.L.C., king of Prussia, PA): Ficoll density 1.09 (Biochrom).**

- **15ml conical centrifuge tubes (e.g. Greiner or Falcon). Temperature controlled centrifuge (eg. Beckman or Heraeus).**

Preparation of Neuraminidase - treated SRBC

A suspension of SRBCs (2ml) and sterile PBS (10ml) are placed in a 15ml conical centrifuge tube and spun at 2000 rpm (900g) for 10min, where after the PBS supernatant is removed and the cells are resuspended in PBS. This washing procedure is repeated twice. Before treatment with neuraminidase, washed SRBCs can be stored at 40C for 3 days. Part of the dry SRBC pellet (300µl) is incubated with 4.6ml RPMI -1640 and 100µl neuraminidase in a water bath

(370C, 30 min), washed twice with RPMI -1640 (2000rpm. 10min), and finally resuspended in RPMI -1640 to a total volume of 5ml. The suspension is stored at 40Cuntil use.

Rosette formation and Ficoll density gradient centrifugation

1. MNCs are prepared by standard Ficoll – Hypaque centrifugation, washed, counted and suspended in suppl. RPMI - 1640/10% (10x106 cells mL-1) The neuramindase - treated SRBCs are mixed with the MNCs (20 - 30 min, room temperature) to allow Erosette formation, whereafter the mixture is layered over a Ficol solution (density 1.09) in a 15ml conical centrifuge tube. The volumes of SRBCs medium and Ficoll used in this protocol depnd on the number of MNCs to be separated. The tubes are centrifuged for 30 min at 2800 rpm.

2. Remove and decant about 80% of the upper layer (RPMI - 1640/ 10% HUS) from the centrifuged suspension. The E-rosette negative (monocytes/ B cell enriched) layer (E) is recovered from the interface layer with a pipette, transferred to a 15-ml conical tube, and washed with a pipette, transferred to a 15ml conical tube, and washed with suppl, RPMI - 1640/5% FCS.

3. The E-rosette-positive (T cell) pellet (E+) is suspended in 1ml RPMI - 1640/10% FCS in the 15-ml tube. Cold distilled water (2ml) is added for hypotonic lysis of SRBCs and mixed gently. After a few seconds, add 8ml RPMI -1640/10% FCS. Transfer this suspension to a 50 - ml tube containing 40 ml RPMI -1640 / 10% FCS and centrifuge for 10min at 1300rpm.

C. Separation of T cell subsets

Purification of T - cell populations by indirect antibody panning

T cells expressing particular cell surface markers, such as the CD4, CD8, αß - TCR or TCR molecules can be selected by their capacity to bind to an antibody coated plastic plates. For example to purify CD8+ T cells, isolated T cells (E+ cells) are treated with a mouse anti - human monoclonal antibody against the CD4 molecule, and then incubated on plastic dishes that have been coated with an anti - mouse IgG antibody. The T-cell populations that are not CD4 positive (i.e. the αßTCR CD8+ and the γδTCR CD8+ subpopulations), and do not therefore bind the mouse anti human CD4 antibody, will not adhere to the coated plate.

These CD4- cells can be selected physically from the adherent CD4+ subpopulation.

Equipment and reagents

- **T-cell population (E+ cells)**
- **Appropriate monoclonal antibody (eg. OKT4 or OKT8 hybridism supernatant containing anti -CD4 or anti - CD8 antibodies, or commercially available anti -CD4, anti -CD8 antibody); suppl. RPMI -1640; FCS, heat inactivated PBS, sterile**
- **Plastic six -well plates (Microplate Standard Greine): 15ml conical centrifuge tubes (e.g. Falcon); sterile rubber scraper; temperature-controlled centrifuge (e.g. Beckman or Heraeus).**

I. Procedure for the separation of T cells into CD4+ and CD8+ T cells.

1. Preparation of the panning plate Goat anti-mouse Ig is diluted to 10µg ml-1 in suppl. RPMI -1640 and added to the wells of a plastic six - well plate (15ml per well). To Separate 2 x 106 to 3 x 106 T cells, one well of the panning plate is needed. Incubate overnight at 40C or for 60min at room temperature. Remove unbound Ig by using a sterile

pipette and gently wash the plate by adding 3ml PBS to each well (wash three times), Add 2ml suppl. RPMI - 1640 /5% FCS and keep the plate at 40C until the T cells are added to the plate (at least 30 min).

2. Pre-treatment of the T cells. Prepare the monoclonal antibody (e.g. Sterile filtrated) OKT4 Hybridoma supernatant containing these antibodies, or commercially available anti - CD4 antibodies diluted in sterile PBS at a concentration appropriate for flow cytometry according to the manufacturer's instructions). Count the T cell population and place the cells in a 15 - ml centrifuge tube in suppl. RPMI - 1640/5% FCS; spin at 1300 rpm (400g) and 40C.Decant the supernatant and resuspend the cell pellet in1

- 2 ml of the monoclonal antibody diluted in PBS. Incubate the tube containing the cells for 30min on ice (iced water) and then fill with suppl. RPMI - 1640/ 10% FCS. Centrifuge for min at 1300 rpm (400g) and 40C. After the supernatant has

been removed, the cell pellet is resuspended in suppl. RPMI- 1640/10%FCS, and the wash repeated once. Finally, the cells are resuspended in suppl. RPMI -1640 (1.5ml per 2x106 to 3 x 106 cells).

3. Incubation the coated plate with the pre-treated T cells: Remove the RPMI 1640 /5% FCS from the coated wells of the six well panning plate with a sterile pipette, and immediately add the pre-treated T cells in RPMI 1640 (1.5ml per well) spin the plate for 10min at 300 rpm and 40C carefully remove the plate from the centrifuge and incubate 30 min at 40c.

4. Collection of the negatively selected cells: Gently swirl the plates for 1 minutes and collect the supernatant containing the non - adherent cells using a sterile pipette. The negative selected, non-adherent (i.e. CD4) T cells are washed twice with suppl. RPMi 1640 / 10% FCS in a 15ml conical tube counted and responded in supp. RPMI 16040/10% human serum or interleukin 2 supplemented media depending on the culture proceeding this is non - adherent populations of cells should be 90 - 95% pure.

5. Collection of positively selected adherent CD4+ T cells: Wash the plates gently with 3 ml suppl. RPMI - 1640/5% FCS per well (2-3 washes) until all non - adherent cells have been removed.

Pitfalls

The purity of the adherent cell population is greater than nonadherent population. However, it must be considered that the function of the adherent T cell population may be altered by the binding of specific antibodies to surface molecules to be positively selected.

II. Immunomagnetic Negative Selection of CD4+ T cells

The protocol below is another cell separation technique mediated by antibody-antigen reactions T cells (E+ cells) are incubated with specific monoclonal antibodies to surface molecules (anti-CD8) to coat unwanted T cells. Magnetic beads coated with goat anti-mouse IgG are then applied to the cell suspension in order to bind the antibody coated cells. After binding; the target cells can be recovered using a strong magnetic field. Negative isolation is a method by which the CD4+ subset is purified from the CD8* subset binding to the coated magnetic

beads. Furthermore, in a positive selection step, the beads can be removed from the CD8+ target cells by a process of detachment.

Equipment and reagents

- **T -Cell population (E+celles)**
- **Appropriate monoclonal antibody (e.g anti CD8 antibody by ptarmigan) goat anti-mouse IgG coated magnetic beads (Dynabeads M-450, Dynal Oslo, Norway). Sterile PBS; FCS, heat inactivated coating medium (Hanks balanced salt solution (HBSS) without Ca2+ , Mg2+ or phenol Red, supplemented with 10% FCS 20mM HEPES). Suppl. RPMI – 1640 HUS heat inactivated.**
- **Magnetic separation device (Dynal MPC-1) mixing device (Dynal MX1. 2 or 3) 15ml centrifugation tubes (e.g. Falcon) vortex mixer, temperature-controlled centrifuge (e.g. Beckman or Heraeus)**

Procedure

All steps in the protocol are done at 40C.

1. Prewash of Dynabeads M-450: transfer the required number of Dynabeads M-450 from the vial to a polypropylene washing tube containing PBS/2% FCS (washing buffer), and place on the Dynal MPC -1 for 2 min, decant the supernatant, resuspend in excess washing buffer, and replace on the Dynal MPC -1. Finally, resuspend in a small volume of coating medium (e.g the volume originally pipettes from the vial).

2. Antibody coating of CD8+ T cells: Resuspend washed T cells in 10ml coating medium at 2 x 10 cells mL-1 in a 15ml conical tube and add 1ml anti-CD8 monoclonal antibody at a 10x saturating concentration. Incubate for 30 min at 40C with gentle tilting and rotation (e.g. in a mixing device).

3. Wash twice in coating buffer (centrifugation at 1000 rpm, 40C) to remove unbound antibody.

4. Add the suspension of washed Dynabeads and incubate for 30min at 40C with gentle tilting and rotation (e.g. in the mixing device) to keep cells and beads in suspension.

5. Place the tube in Dynal MPC and leave it to rest for 2 minutes to magnetically remove the CD8, cells labelled by antibody and coated with beads. Transfer the unbound cells to a fresh tube, perform a second magnetic separation, count, and resuspend in suppl. RPMI 1640/10% HUS. Negatively selected cells obtained by this method are unstipulated, pure and of high yield.

6. For recovery of positively selected CD8+ cells, remove the tube from the Dynal MPC, and wash the resettled cells by resuspending in RPMI-1640/10% HUS.

Repeat step 5 twice. These positively selected cells can be removed from the beads by a process of detachment.

III. Rosette Test

Subsets of T lymphocytes can be identified by their differing membrane structures called markers. Markers are categorized as antigen and receptors and can be detected by rosette technique. The E rosette forming cells were assigned to T cell lineage and the E-rosettes become the principle marker for identification and enumeration of human T cells. The presence of FC receptors for IgG or IgM on T lymphocyte has been correlated with their functional activity. Cells with IgM receptors were shown to provide help for B cell differentiation to plasma cell, whereas cells with IgG receptors were reported to function as suppressors.

1. E-ROSETTE TEST

Spontaneous rosette formation with untreated sheep merythrocytes was performed with some modification.

- **Separate Lymphocytes and adjust the count to 2.5x106 / ml in PBS.**
- **Prepare 1% sheep erythrocyte suspension in PBS after 3 times washing in PBS.**
- **Then 50 micro liters of bovine serum albumin will be taken in tube in which 100µl of lymphocytes suspension and 100ul of 1% sheep RBC suspension will be added.**
- **Then centrifuge for 5 minutes at 1000rpm**

- **After incubation at 40C for 1hour, 0.1% toludine will be added and rosette-forming cells will be counted.**

2. IgM and IgG Rosette test

Step 1: Preparation of anti-ox antibody

- **Collect blood from an ox by vein puncture from external jugular vein.**
- **Separate plasma and cells by centrifuging under sterile condition.**
- **Inject 1ml of sterile packed Ox RBC intraperitoneally into rabbit.**
- **After 10 days, bleed the rabbit and test serum for antiox IgG, Ig A and IgM antibodies.**

Step 2: preparation of Anti-Ox IgG and IgM sensitized cells

- **Suspend 0.5ml of 50% washed red cells separately in 2.5ml of IgG and IgM fraction (IgG and IgM fraction obtained by column chromatography)**
- **Keep the mixture at 370C for 1 hour for IgG sensitization and at 40C for 1 hour for IgM sensitization**
- **Wash cells 3 times with PBS and prepare 1% suspension.**

Step 3: Running IgM/IgG Rosette test

- **Mix 100µl patient T lymphocytes (separated by nylon wool) with 100µl of 1% IgG and IgM sensitized Ox cells in different precipitin tubes.**
- **Centrifuge for 5 minutes at 1000 rpm and incubate for 30 minutes at 370C for the IgG rosette and 40C for IgM rosette.**
- **Add toludine blue dye (0.1%) and count the percentage of rosette forming cells under high power field using ordinary microscope.**

IV. Cell sorting in the fluorescence activated cell sorter (FACS).

A suspension of cells is allowed to react with antibodies that are specific for particular molecules on the surface of one of the cell types in the mixture. The antibody has a fluorochrome attached to it. The suspension is then mixed with a

buffer (sheath fluid) and droplets, each containing a single cell, are generated by ultrasonic vibrations in a nozzle. The droplets pass one by one through a laser beam, a beam of high intensity light of a particular wavelength. As the beam hits the cell, two things happen. The fluorochrome molecules absorb the light, but emit light of another wavelength. The emitted light is focused by collecting lenses on a barrier filter, which only allows light of a certain wavelength to pass through. Lightm detectors (photomultipliers) placed behind the barrier filter can then record whether light of a given wavelength has been emitted from the cell and passed through the filter. At the same time, however, because of the cell curvature and surface unevenness, the light of the laser beam hits the cell at different angles and in turn is reflected from the cell at different angles, i.e. it is scattered. The character of the light scatter depends on the cell's size and density; the larger and denser the cell, the lighter it scatters. The degree of light scatter is estimated by measuring light rays reaching the photomultipliers at two different angles in relation to the laser beam; a low angle (a forward scatter) and a right or obtuse angle (side scatter). The computer then uses these two estimates to determine the size and density of the cell. Based on this information and information regarding the emission of the fluorescent light, the computer checks whether the cell meets certain criteria for a particular cell type and, depending on the outcome, sends a signal to impart a certain electric charge to the droplet. As the droplets pass through an electric field generated by the deflection plates, they are sorted according to their charge and collected in tube.

Methods of Monoclonal Antibody Production

Hybridoma Technique

Large quantities of absolutely pure, specific immunoglobulin directed against an antigen of interest can be produced by fusing a normal plasma cell making the antibody of interest with a myeloma cell with the capacity for prolonged growth in tissue culture. The resulting mixed cell is called hybridoma.

The first stage in making a hybridoma is to generate antibody producing plasma cells. This is done by immunizing a mouse against the antigen of interest and repeating the process several times to ensure that it mounts a good response. Two to four days after administration of antigen, the mouse's spleen is removed

and broken up to form a cell suspension. These spleen cells are suspended in culture medium together with a special mouse myeloma cell line. It is usual to use myeloma cells that do not secrete immunoglobulins since this simplifies purification later on. Spleen cells are fused with a myeloma cell line by the addition of polyethylene glycol (PEG) which promotes membrane fusion. Only a small proportion of the cells fuse successfully. The fusion mixture is then set up in culture with medium containing 'HAT'. HAT is a mixture of hypoxanthine, aminopterin and thymidine. There are two biosynthetic pathways by which cells can produce nucleotides and hence nucleic acids. The myeloma cells are selected so that they lack the enzyme hypoxanthine phosphoribosyltransferase and as a result cannot utilize hypoxanthine in the culture medium to produce inosine, a pryimidine precursor. They are obliged to utilize an alternative biosynthetic pathway involving thymidine. But the aminopterin in the culture is a drug that prevents myeloma cells from making their own thymidine. Since the myeloma cells cannot use hypoxanthine and the aminopetrine stops them from using the alternative synthetic pathway, they cannot make nucleic acids and will soon die. Hybrids made from a myeloma and a normal cell will grow; they possess hypoxanthine phosphoribosyl transferase and can therefore use the hypoxanthine and thymidine in the culture medium and survive. The spleen cells die in culture naturally after 1-2 weeks. Any wells containing growing cells are tested for the production of the desired antibodies (using RIA or ELISAs) and if positive, the cultures are cloned by plating out so that there is only one cell in each well. This produces a clone of cells derived from a single progenitor, which is both immortal and producer of monoclonal antibody.

Recombinant DNA techniques

- **Attempts are also being made to replace altogether the hybridoma method by recombinant DNA techniques.**
- **One such attempt focuses on the gene segments that specify the fragment antigen binding (Fab) of an immunoglobulin molecule, the VH CH1 and VLCL.**
- **These segments can be amplified by PCR from many different mRNA (cDNA) molecules expressed in a population of cells undergoing an immune response. The amplified segments are inserted into a suitable**

vector, cloned and paired randomly (always one, VH CH1 with VLCL, in a suitable vector) and the pairs translated into proteins (Fabs).

- **Screening of this combinatorial library of antibodies with labeled antigen then identifies these combinations that bind this antigen.**
- **The identified VHCH1-VLCL pairs are placed in to an expression vector, either bacterial or mammalian, and used to produce large quantities of antibodies with selected specificity.**

Uses of monoclonal antibodies

- **The greatest impact of Monoclonal antibodies in immunology has been on the analysis of cell membrane antigens.**
- **Because Monoclonal antibodies have a single specificity compared to the range of antibody molecules present in the serum, monoclonal antibodies have multiple clinical applications including:**

o Identifying and quantifying hormones

o Typing tissues and blood

o Identifying infectious agents

o Identifying clusters of differentiation for the classification and follow-up therapy of leukemias and lymphomas

o Identifying tumor antigens and autoantibodies

o Immunotherapy

Effector-Cell Assay

Various methods have been developed for assaying lymphocyte-effector functions, including antibody production, cytotoxicity, and T-cell mediated help and suppression. Individual B cells producing specific antibody or individual T cells secreting particular cytokines may be detected by ELISPOT assay. For detection of antibody –producing cells, the lymphocytes are plated onto an antigen-sensitized plate. Secreted antibody binds antigen in the immediate vicinity of cells producing specific antibody. The spots of bound antibody are

then detected chromatographically using enzyme coupled to anti-immunoglobulin and a chromogen. For detection of cytokine-producing cells, the plates are coated with anticytokine and the captured cytokine is detected with enzyme coupled antibody to a different epitope on the cytokine.

PLATELET COUNT

Platelets, also called thrombocytes are a component of blood whose function (along with the coagulation factors) is to react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot. Platelets have no cell nucleus: they are fragments of cytoplasm that are derived from the megakaryocytes of the bone marrow, and then enter the circulation.

Principle of test: Blood is diluted 1 in 20 in a filtered solution of ammonium oxalate reagent which lyzes the red cells. Platelets are counted microscopically using an Improved Neubauer ruled counting chamber and the number of platelets per liter of blood calculated.

Blood sample: Use EDTA anticoagulated venous blood.

Equipment

An Improved Neubauer ruled *Bright-line* counting chamber and other equipment as described previously for WBC counting are required for counting platelets.

Platelet hemocytometers: Thin glass chambers for counting platelets by phase contrast microscopy are available. Such chambers are expensive and break easily. They are not essential for counting platelets.

Reagent

Ammonium oxalate 10 g/l (1% w/v) diluting fluid.

***Important*: Always filter the fluid before use.**

Test method

Perform a platelet count within 2 hours of collecting the blood.

1- Measure 0.38 ml of filtered ammonium oxalate diluting fluid and dispense it into a small container or tube.

2- Add 20 µl (0.02 ml, 20 cmm) of *well-mixed* anticoagulated venous blood and mix.

3- Assemble the counting chamber and fill it with *well-mixed* sample as described previously in steps 3 and 4 of the method for counting white cells.

4- Leave the chamber undisturbed for 20 minutes. To prevent drying of the fluid, place the chamber in a petri dish or plastic container on dampened tissue or blotting paper and cover with a lid.

5- Dry the underside of the chamber and place it on the microscope stage. Using the 10_X objective, focus the rulings of the grid and bring the central square of the chamber into view. Change to the 40X objective and focus the small platelets. They will be seen as small bright fragments (refractile).

***Note*: If available, use phase contrast microscopy.**

6- Count the platelets in the small squares marked P

7- Report the number of platelets in 1 liter of blood.

This is the actual number of platelets counted X 10^9.

Example

If 150 platelets are counted, the platelet count is 150 X 10^9/l.

Platelet calculation details

Platelet count (per liter) =Cells counted X 20* X 10^6 / 0.2† _ 0.1¶

where * = 1 in 20 dilution of blood, † = 0.2 mm2 area counted, ¶ = 0.1 mm depth of chamber

PERIPHERAL BLOOD SMEAR STUDY

Purpose: Detection of normal and abnormal blood cells:

MAKING, FIXING AND STAINING BLOOD FILMS

Thin blood films can be made from free-flowing capillary blood or *well mixed* EDTA anticoagulated blood. To prevent EDTA associated blood changes it is important to make blood films from EDTA anticoagulated blood with as little delay as possible, i.e. within 1 hour of collecting the blood.

***Caution*: Wear protective gloves when handling blood and follow safe working practices.**

Materials and reagents:

_ Microscope

_ Microscope slides (should be well washed and, if necessary, cleaned with ethanol or ether using a piece of soft cloth)

_ Spirit lamp or Bunsen burner

_ Glass spreader (see below)

_ Blood lancet

_ Two glass rods, either over a sink or over a staining tank

_ Measuring cylinder, 50 ml or 100 ml

_ Beakers or bottles containing clean tap water

_ Wash bottle containing buffered water

_ Interval timer

_ Rack for drying slides

_ Field stain

_ Giemsa stain

_ Leishman stain

_ May–Grünwald stain

_ EDTA dipotassium salt, 10% solution

_ Methanol

_ 70% Ethanol or ether.

To make a spreader, select a slide with a perfectly smooth edge. Make a diagonal mark across the two corners at one end of the slide with a file. Snap off the two filed corners.

Method:

Collection of specimens:

Take the blood from the side of the third or fourth finger. Let the blood flow freely. First take samples for determining the erythrocyte or leukocyte number concentrations, if possible.

***Important*:**

Do not take blood from:

— the index finger or thumb

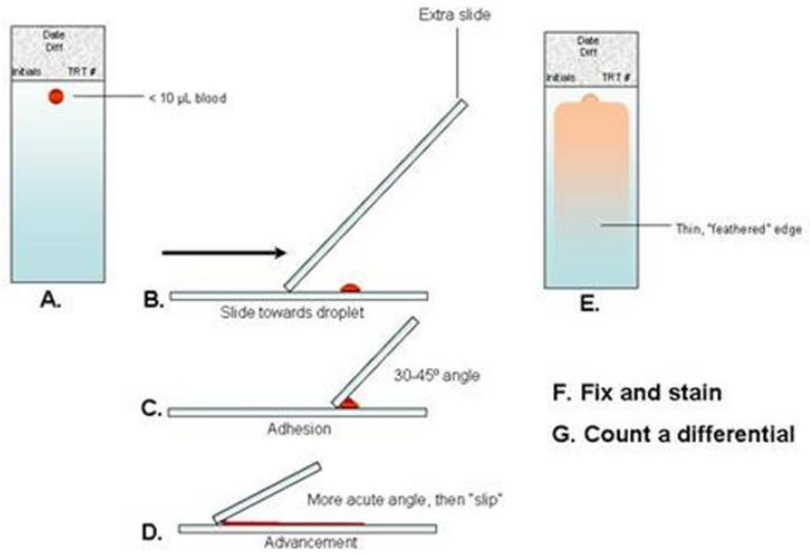
— an infected finger (e.g. paronychia)

— the ear (too many monocytes).

If it is not possible to prepare the film within 1–2 hours of collection of the blood specimen, EDTA dipotassium salt solution should be added. Other anticoagulants such as heparin alter the appearance of leukocytes and thrombocytes and should not be used.

Preparation of the film:

1.Collect a drop of blood of about 4mm diameter by touching it lightly with one end of the slide.



2. Hold the slide with one hand. Using the other hand, place the edge of the spreader just in front of the drop of blood.

3. Draw the spreader back until it touches the drop of blood.

4. Let the blood run along the edge of the spreader.

5. Push the spreader to the end of the slide with a smooth movement (all the blood should be used up before you reach the end). Blood from patients with anemia should be spread more rapidly.

6. Check that the film is satisfactory.

_ There should be no lines extending across or down through the film.

_ The film must be smooth at the end, not ragged and lined as shown in.

_ The film must not be too long.

_ The film must not be too thick.

_ The film must not contain holes because a greasy slide has been used.

A well-spread film is of great importance. A badly spread film will give the wrong leukocyte type number fractions and make it impossible to report erythrocyte morphology.

Drying the film:

Adequate drying is essential to preserve the quality of the film, especially in humid climates. The film can be left to air-dry in dry climates. In the wet season (in the tropics). Dry the film by waving it rapidly about 5cm away from the flame of a spirit lamp or Bunsen burner: hold the slide to the side and slightly above (but never directly over) the flame. If necessary, protect the blood film from flies.

Mark the dry film with the patient's name or number. Write with a lead pencil on the thick part of the film not used for examination. Drying the blood film over a spirit lamp

Fixation of the film:

If the film is intended for determining leukocyte type number fractions, it should be fixed with methanol before staining with May–Grünwald stain (see below).

If the film is intended for detection of parasites, it should be fixed with methanol before staining with Giemsa or Field stain (see below).

Precautions:

Care is required to avoid the formation of deposits of stain, which appear on the film as masses of little black spots. A number of precautions are also required to avoid staining the films too blue, too pink or too dark; these are briefly described below.

_ Use perfectly clean glassware. Wash it every day. *Do not* use acid. Remove stain deposits with methanol.

_ Use neutral water (buffered if possible, except for Field stain).. Acid water produces a film that is too red; alkaline water one that is too blue. Neutral water must be freshly prepared as it becomes acid when exposed to air.

Staining of the film:

Method for Leishman stain

1. Fix the thin blood film with methanol for 2–3 minutes.

2. Prepare a 1 in 3 dilution of Leishman stain using one volume of stain and two volumes of buffered water. Mix.

***Example*: Use 10 ml of stain and 20 ml of buffered water. Prepare sufficient stain for 1 day's use only, as the diluted stain does not keep well.**

3. Cover the slide with the diluted stain for 7–10 minutes.

***Important*: The staining time may need to be adjusted, especially when a new batch of stain is received or the stain has been stored for a long time.**

4. Wash the stain off in a stream of buffered water. Do not tip the stain off as this will leave a deposit of stain on the film.

5. Leave clean water on the slide for 2–3 minutes to differentiate the film. (The time taken for differentiation will depend on the stain and the pH of the water used.)

The pH of the water is of vital importance in differentiating the different types of leukocyte with Leishman stain. It should be between 6.8 and 7.2, and preferably between 7.0 and 7.2.

6. Tip the water off and place the slide in a draining rack to dry.

Method for May–Grünwald and Giemsa stains:

1. Fix the thin film with methanol for 2–3 minutes.

2. Prepare the stains as follows:

_ Dilute May–Grünwald stain 1 in 2 using equal volumes of stain and buffered water. Mix.

***Example*: use 10 ml of stain and 10 ml of buffered water.**

_ Dilute Giemsa stain 1 in 10 using one volume of stain and nine volumes of buffered water. Mix gently.

***Example*: use 2 ml of stain and 18 ml of buffered water.**

***Note*: Prepare only enough stain for 1 day's use, as the diluted stains do not keep well. Prepare the Giemsa mixture slowly and carefully. Shaking causes the stain to precipitate.**

3. Cover the slide with diluted May–Grünwald stain for 5 minutes.

4. Tip the stain off and replace with diluted Giemsa stain for 10 minutes.

***Important*: The staining time may need to be adjusted, especially when a new batch of stain is received or the stain has been stored for a long time.**

5. Wash the stain off in a stream of buffered water. Do not tip the stain off as this will leave a deposit of stain on the film.

6. Leave clean water on the slide for 2–3 minutes to differentiate the film. The time for differentiation will depend on the stain and the pH of the water used. The pH should be between pH 6.8 and 7.0.

7. Tip the water off and place the slide in a draining rack to dry.

Method (rapid) for Field stain:

1. Fix the thin film with methanol for 2–3 minutes.

2. Dip the slide into Field stain B and count up to five. Drain and wash the slide in the first container of tap water.

3. Drain and dip the slide into Field stain A and count up to 10. Drain and wash the slide well in the second container of tap water.

4. Examine the color of the film. It should appear mauve, neither too blue nor too pink.

If the film is not satisfactory, return the slide either to the Field stain A or to the Field stain B for a few more seconds, as needed. If the film is satisfactory, stand the slide in a draining rack to dry.

How to remedy poor results:

Deposits of May–Grünwald stain or neutral water

Deposits caused by May–Grünwald stain or neutral water can be seen with the naked eye in the liquid on the slide. Drain off the stain. Rinse the slide twice in methanol. Dry and re-stain using fresh or filtered May–Grünwald stain.

Deposits of Giemsa stain:

These deposits are seen with the naked eye or under the microscope. Rinse with methanol, but wash off immediately with neutral water. Dry the slide and repeat the staining procedure from the beginning.

Too much blue in the film (basophilic staining):

Prepare a solution of 1% boric acid in 95% ethanol. Rinse the slide twice in this preparation. Wash at once in neutral water. Dry and examine under the microscope without further treatment. Basophilic staining can usually be prevented by using buffered water at a more acid pH and, if necessary, altering the differentiation time.

Poor staining may also be caused by impurities in the dyes — the use of the standardized stain is recommended.

Reporting Blood Films

Reporting Romanowsky stained thin blood films includes:

– Differential white cell count and white cell morphology

– Red cell morphology

– Comments on platelets (venous EDTA anticoagulated blood sample)

– Other abnormalities, e.g. presence of malaria parasites, trypanosomes, microfilariae, *Bartonella*, and *Borrelia*

STAINS FOR DIAGNOSE LEUKEMIAS

Several special WBC staining methods are used to diagnose leukemia, amyloid disease, lymphoma, and erythroleukemia; to differentiate erythema myelosis from sideroblastic anemia; to monitor progress and response to therapy; and to detect early relapse. *Amyloid* refers to starch like substances deposited in certain

diseases (e.g., tuberculosis, osteomyelitis, leprosy, Hodgkin's disease, and carcinoma).

● Sudan Black B (SBB) Stain

The SBB stain aids in differentiation of the immature cells of acute leukemias, especially acute myeloblastic leukemia. The SBB stains a variety of fats and lipids that are present in myeloid leukemias but not present in the lymphoid leukemias.

Procedure

1. Obtain bone marrow aspirate.

2. Prepare slide, stain with SBB, and scan microscopically. Use normal smear control.

Results:

-Positive Reactions

Granulocytic cells (neutrophils and eosinophils)

Myeloblasts

Promyelocytes

Neutrophilic myelocytes

Metamyelocytes, bands, and segmented neutrophils

Eosinophils at all stages

Monocytes and precursors

-Variable Reactions

Basophils

-Negative Reactions (Sudanophobia)

Lymphocytes and lymphocytic precursors

Megakaryocytes and thrombocytes (platelets)

Erythrocytes

Erythroblasts may display a few granules that represent mitochondrial phospholipid components.

● Periodic Acid–Schiff (PAS) Stain

The PAS stain aids in the diagnosis of acute lymphoblastic leukemia (ALL). Early myeloid precursors and erythrocyte precursors are negative. As granulocytes mature, they increase in PAS positivity, whereas mature RBCs stay negative. The PAS stain cannot be used to distinguish between ALL and AML or between benign and malignant lymphocytic disorders.

Procedure

1. Obtain bone marrow aspirate.

2. Prepare slide, stain with PAS, and scan microscopically.

Results:

Lymphoblasts: stain (positive)

Myeloblasts: do not stain (negative)

● Terminal Deoxynucleotidyl Transferase (TDT) Stain

The thymus is the primary site of TDT-positive cells, and TDT is found in the nucleus of the more primitive T cells. A thymus-related population of TDT-positive cells resides in the bone marrow (normally a minor population, 0%–2%). TDT is increased in _ 90% of cases of ALL of childhood. A minor (5%–10%) population of patients with acute nonlymphoblastic leukemia has TDT-positive blasts. TDT-positive blasts are prominent in some cases of chronic myelogenous leukemia (CML), relating to the development of an acute blast phase. TDT has been reported to assist in establishing the diagnosis of ALL. TDT-positive cases of blast-phase CML correlate with a positive response to chemotherapy (vincristine and prednisone).

Procedure

1. Obtain a 5-mL EDTA-anticoagulated peripheral blood sample or a 2-mL EDTA-anticoagulated bone marrow aspirate.

2. Dry slides (store at room temperature for up to 5 days), process, and stain, then examine under the microscope for positive cells.

Results:

Negative in nonlymphoblastic leukemia

Negative in peripheral blood

0% to 2% positive in bone marrow

● Leukocyte Alkaline Phosphatase (LAP) Stain

Neutrophils are the only leukocytes to contain various amounts of alkaline phosphatase.

The LAP stain is used as an aid to distinguish chronic granulocytic leukemia from a leukemoid reaction. A leukemoid reaction is a high WBC that may look like leukemia but is not. In remission of CML, the LAP may return to normal. In the blast phase of CML, the LAP may be elevated.

Procedure

1. Obtain specimen by capillary puncture, venous whole blood (EDTA), or bone marrow aspirate green–topped tube. Prepare smear and air-dry; stain with LAP.

2. Make a count of 100 granulocytes and score (from 0 to 4 _) as to the degree of LAP units.

Results*:* 40–100 LAP units

● Tartrate-Resistant Acid Phosphatase (TRAP) Stain

The malignant mononuclear cells of leukemic reticuloendotheliosis (hairy cell leukemia) are resistant to inhibition by tartaric acid. There is evidence that the reaction is not entirely specific because TRAP reactions have been reported in

prolymphocytic leukemia and malignant lymphoma and in some cases of infectious mononucleosis.

Procedure

1. Obtain venous blood sample (5 mL) or bone marrow smear.

2. Incubate blood smear with TRAP, counterstain, and examine microscopically.

Results:

No TRAP activity

DETECTION OF NORMAL AND ABNORMAL BLOOD CELLS

Microscopic examination:

Using the X40 objective, examine the slides. The cells should appear as described in Table below.

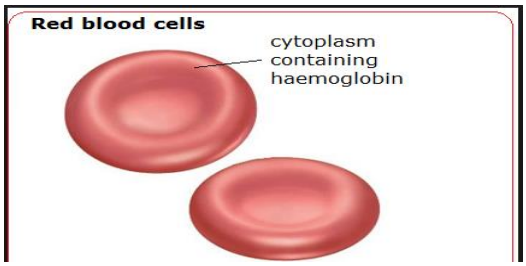
Erythrocytes

In certain diseases, especially anemia, the erythrocytes may have an abnormal shape, size or colour. To check for abnormal erythrocytes, look at the cells just before the thin end of the film; this is where they are spread out, just touching one another, but not overlapping. Do not look at either the thick end, where the cells are too closely packed, or the thin end, where there are not enough cells. The various types of abnormal erythrocytes are described below.

Table. Appearance of blood cells in thin films stained with Leishman stain

Cell type	Appearance
Neutrophils	Cytoplasm stains faint pink and contains small mauve granules

Eosinophils granules	**Cytoplasm stains faint pink and contains large red**
Basophils granules	**Cytoplasm contains numerous dark mauve-blue**
Monocytes	**Cytoplasm stains grey-blue**
Lymphocytes	
large	**Cytoplasm stains clear blue**
small	**Cytoplasm stains dark blue**
Erythrocytes	**Stain pink-red**
Thrombocytes	**Stain mauve-pink**

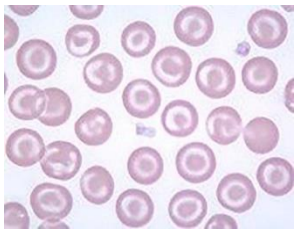


- **Normal erythrocytes**

Size: 6–8mm.

Shape: round, discoid, occasionally slightly irregular.

Cytoplasm: periphery deep pink, center pale pink or colorless.



- **Normal erythrocytes: Target cells**

Size: 6–8mm.

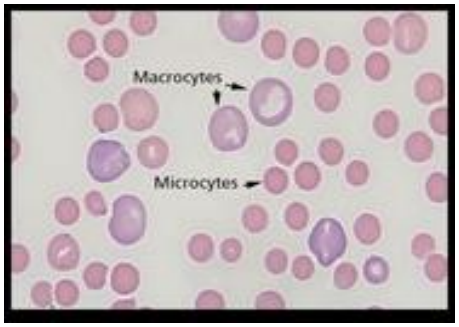
Shape: round or slightly irregular.

Cytoplasm: center and periphery stain well, but between them there is a colorless ring. Seen in thalassemia, vitamin B6 deficiency, haemoglobinopathy, liver diseases, sickle-cell anemia and iron-deficiency anemia.

- **Erythrocytes: Sickle cells**

Shape: elongated and narrow, often with one or both ends curved and pointed.

Seen in sickle-cell anemia and sickle-cell thalassemia, along with nucleated erythrocytes, target cells and often macrocytes.

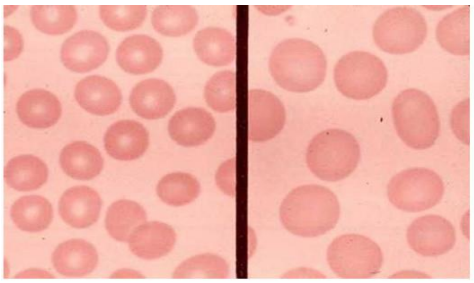


- **Erythrocytes: Microcytes**

Size: small (about 5mm).

Seen often in iron-deficiency anemia, sideroblastic anemia and thalassemia.

Must be distinguished from spherocytes (see below).

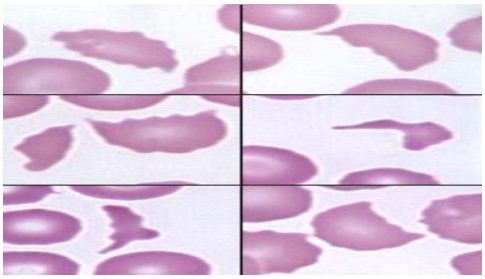


normal red cells macrocytic red cells

- **Erythrocytes: Macrocytes**

Size: large (9–10mm).

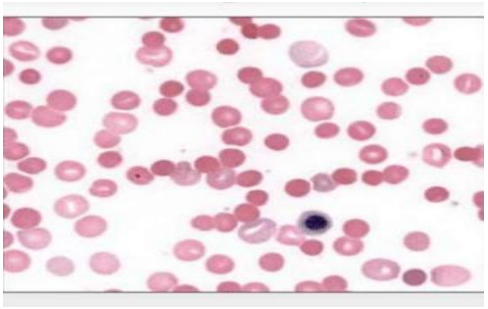
Seen in macrocytic anemias caused by folic acid deficiency, vitamin B12 deficiency and iron-deficiency anemia, and in certain liver diseases. Must be distinguished from reticulocytes (see below).



- **Erythrocytes: Schistocytes**

Size: normal or slightly smaller than normal erythrocytes. Fragmented cells.

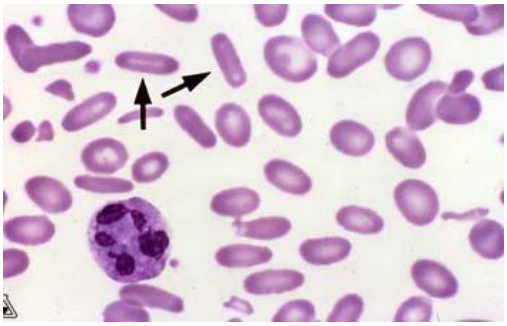
Seen in hemolytic anemias, sickle-cell disease and thalassemia.



- **Erythrocytes: Spherocytes**

Size: small (6mm).

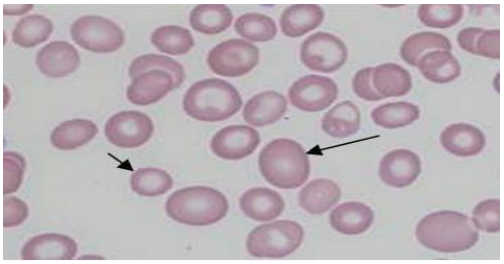
Shape: perfectly round. Cytoplasm: more darkly stained than normal erythrocytes. Seen in hemolytic anemias and hereditary spherocytosis.



- **Erythrocytes: Elliptocytes**

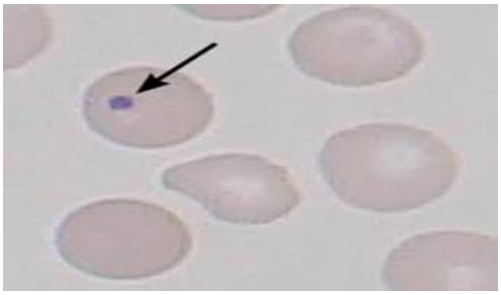
Size: normal (8mm). Shape: oval.

Cytoplasm: stained darker at the periphery (especially at the poles). Seen very occasionally. Found in hereditary elliptocytosis, iron-deficiency anemia, pernicious anemia, sickle-cell disease, thalassemia and myelofibrosis.



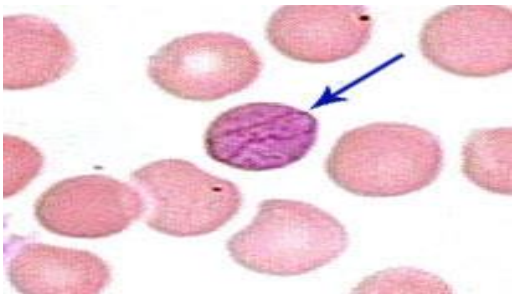
- **Erythrocytes : Anisocytosis**

A condition in which erythrocytes of different sizes are present in the blood, e.g. erythrocytes measuring 9mm are mixed with erythrocytes measuring 6mm. Seen in many types of anemia.



- **Erythrocytes containing Howell–Jolly bodies**

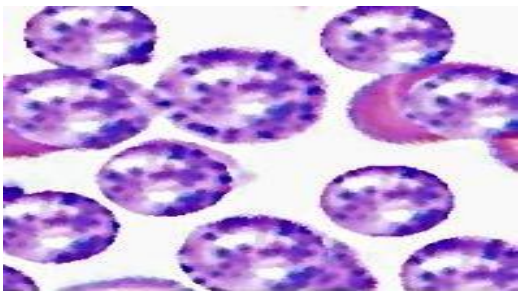
Erythrocytes containing one or more large purple granules (nuclear remnants). Do not confuse with thrombocytes lying on the cells. Seen in haemolytic anemias and megaloblastic anemia, and following splenectomy.



- **Erythrocytes containing Cabot ring bodies**

Erythrocytes containing thin ring-shaped or figure-of-eight structures that stain red with Wright stain.

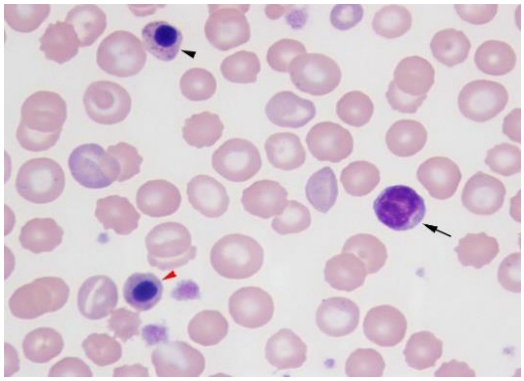
Seen in severe anemias. Do not confuse with malaria parasites.



- **Erythrocytes containing basophilic stippling**

Erythrocytes containing multiple blue–black dots in the cytoplasm. Do not confuse with stain deposits.

Seen in vitamin deficiency, thalassemia and lead poisoning.

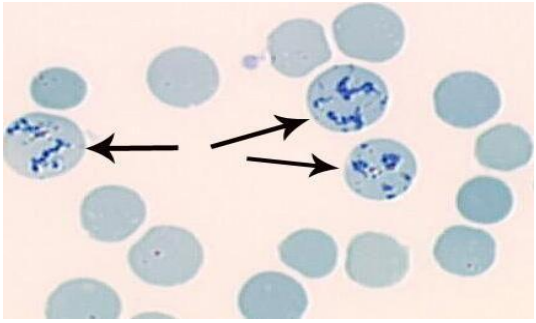


- **Nucleated erythrocytes (normoblasts)**

Size: 8–10mm. Shape: round or irregular.

Nucleus: round, often eccentric, with deep purple, dense chromatin. Cytoplasm: pink or greyish-blue.

Seen in accelerated erythropoiesis in severe anemias, for example sickle-cell anemia, in severe bacterial infections, and in leukemias and cancers.



- **Reticulocytes erythrocytes**

Erythrocytes containing granules (nuclear remnants) that stain dark blue with vital stains such as brilliant cresyl blue and Evan's blue. Reticulocytes usually disappear within 4 hours after the release of the erythrocytes into

The degree of each type of red cell abnormality can be reported in plus signs as follows:

++++ (very marked)

+++ (marked)

++ (moderate)

+ (slight but significant)

Leukocytes:

In contrast to erythrocytes, leukocytes contain a nucleus that may vary in size and shape. As already mentioned, there are five main types of leukocyte — neutrophils, eosinophils, basophils, lymphocytes and monocytes.

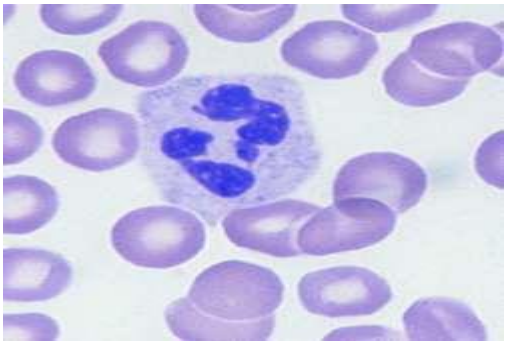
The proportion of each type of leukocyte, known as the leukocyte type number fraction, is of diagnostic importance.

Polymorphonuclear cells (neutrophils, eosinophils and basophils)

Polymorphonuclear cells have:

— a nucleus with several lobes;

— granules in the cytoplasm (hence their usual name, granulocytes).

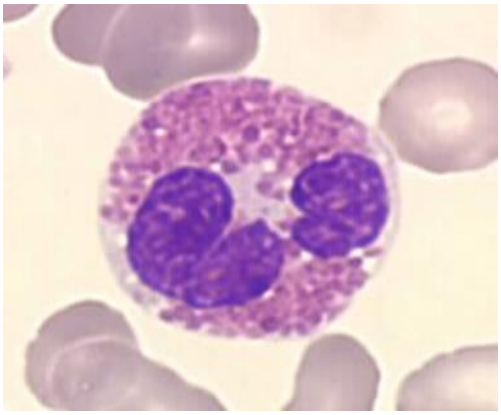


- **Polymorphonuclear neutrophils**

***Size*: 12–15mm.*Shape*: rounded, well defined.**

***Nucleus*: several (2–5) lobes, linked by strands of chromatin. The chromatin appears as a uniform deep purple mass.**

***Cytoplasm*: abundant, pinkish, containing numerous very small, mauve granules. The granules appear brown-violet after staining.**



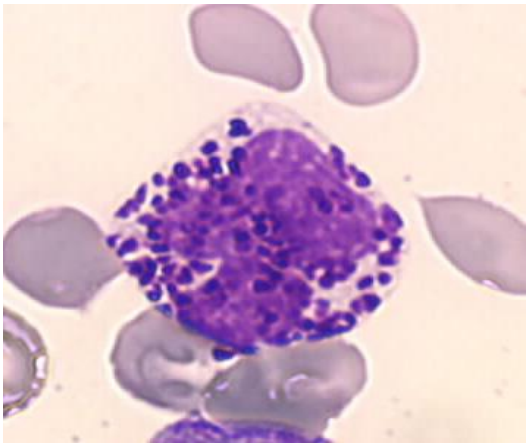
- **Polymorphonuclear eosinophils**

***Size*: 12–15mm.**

***Nucleus*: usually two lobes.**

***Cytoplasm*: very little visible, containing numerous large, round, densely packed, orange-red granules.**

Sometimes the cell appears damaged, with scattered granules.



- **Polymorphonuclear basophils**

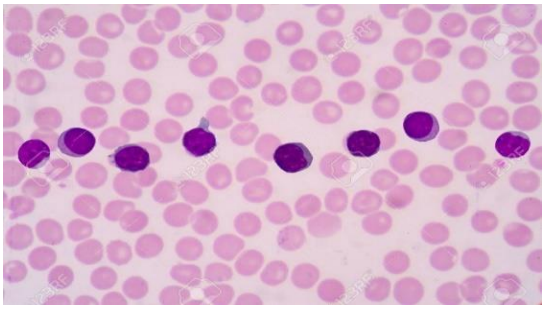
These are the rarest type of granulocyte.

***Size*: 11–13mm. *Shape*: round. *Nucleus*: difficult to see because it is covered by the granules.**

***Cytoplasm*: very little visible, containing numerous very large, round, deep purple granules, less densely packed than those of the**

eosinophils. Small colorless vacuoles are sometimes present.

Lymphocytes and monocytes:



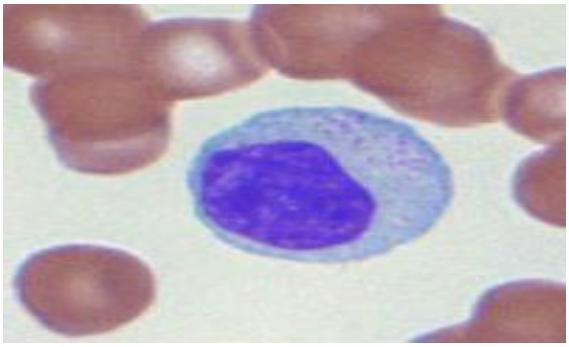
Lymphocytes and monocytes have a compact nucleus and may or may not have granules in the cytoplasm.

- **Small lymphocytes**

***Size*: 7–10mm. *Shape*: round.**

***Nucleus*: large (occupying most of the cell), with densely packed dark purple chromatin.**

***Cytoplasm*: very little visible, blue with no granules.**



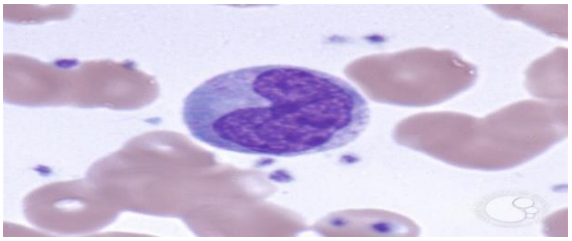
- **Large lymphocytes**

***Size*: 10–15mm.**

***Shape*: round or irregular.**

***Nucleus*: oval or round, may lie to one side of the cell.**

***Cytoplasm*: abundant, pale blue, containing several large, dark red granules.**



- **Monocytes**

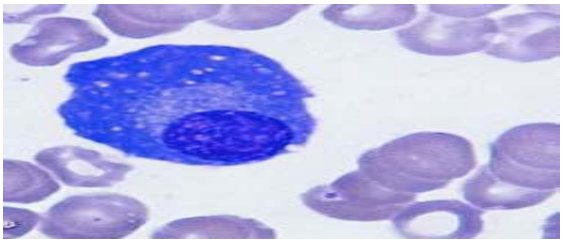
***Size*: 15–25mm (largest of the leukocytes). *Shape*: irregular.**

***Nucleus*: variable, often kidney-shaped with pale mauve chromatin arranged in strands.**

***Cytoplasm*: pale blue, containing fine, dust-like, usually reddish granules.**

Vacuoles are usually present. In patients suffering from malaria the cytoplasm often contains brownish-black masses. These masses are malaria pigment.

Rare or abnormal cells:



- **Plasma cells**

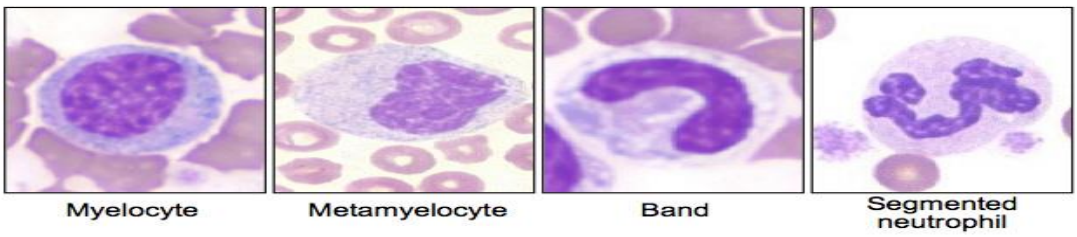
Plasma cells produce antibodies. They may be seen in blood films prepared from patients with measles, tuberculosis, other viral or bacterial infections or multiple myeloma.

***Size*: 12–15mm. *Shape*: round or oval.**

***Nucleus*: round, eccentric, with densely packed chromatin, often in a wheel-like arrangement.**

***Cytoplasm*: dark blue with a pale-staining area round the nucleus. Numerous very small vacuoles are present, which are not easily seen.**

- **Immature granulocytes**



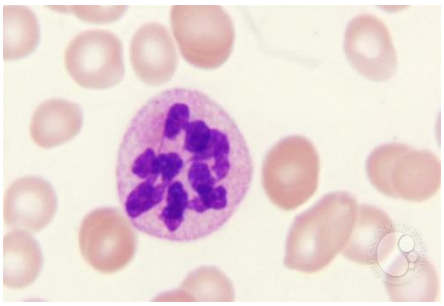
Immature polymorphonuclear granulocytes of the bone marrow pass into the bloodstream in severe bacterial infections. They can be distinguished by the following features: *Size*: 12–18mm.

***Nucleus*: without lobes, with chromatin varying in colour from dark red to purple.**

***Cytoplasm*: pale blue or pink with many large mauve or dark red granules.**

Toxic granulation may be seen, in which the granules are very large and darkly stained.

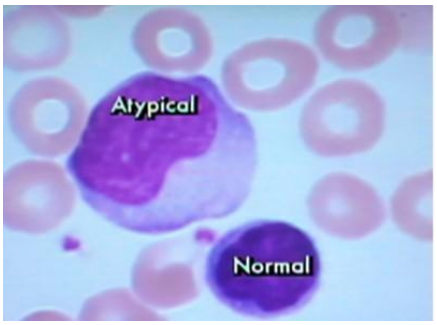
If immature polymorphonuclear neutrophils ("band form" or "stab cells") are seen, report their number fraction as for other types of leukocyte. There are also immature cells without granules and with nucleoli (lymphoblasts).



- **Hyper segmented polymorphonuclear neutrophils**

Hyper segmented polymorphonuclear neutrophils look like normal neutrophils, except that their nuclei have 5–10 lobes and are often larger in size.

Such neutrophils can be seen in patients with macrocytic anemia, caused by folic acid or vitamin B12 deficiency.



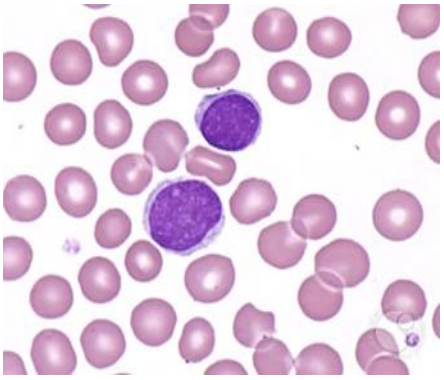
- **Atypical lymphocytes**

Atypical lymphocytes can be seen in viral infections, especially infectious mononucleosis (glandular fever), whooping cough and measles.They are also found in tuberculosis, severe malaria and the acquired immunodeficiency syndrome (AIDS).

***Size*: very variable, 12–18mm. *Shape*: usually irregular.**

***Nucleus*: round or irregular, often lying to one side of the cell; nucleoli may be seen.**

***Cytoplasm*: usually darker blue than that of large lymphocytes; forms a dark edge to the cell. Does not contain granules.**



- **Lymphoblasts**

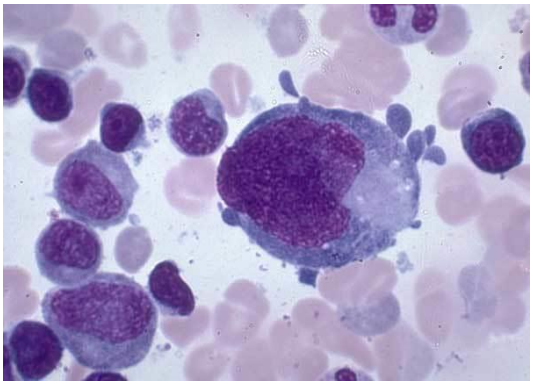
The earliest (most immature) of all the types of leukocyte. Lymphoblasts can be seen in the blood films of patients with leukemia.

***Size*: large, 15–25mm.**

***Nucleus*: large, round, pale mauve, containing 1–5 nucleoli.**

***Cytoplasm*: dark blue, with a clear unstained area round the nucleus. Does not contain granules.**

polymorphonuclear neutrophils:



- **Megakaryocytes**

The parent cells of thrombocytes found in the bone marrow.

***Size*: very large, 60–100mm.**

***Nucleus*: very irregular, greatly lobulated but dense.**

***Cytoplasm*: contains numerous fine granules, mostly dark red, and thrombocytes. The cell wall is not clearly defined. (Very rarely found in the blood.)**

COOMBS TEST/ ANTIGLOBULIN TEST (AGT)

The Coomb's test (also known as Antiglobulin Test or AGT) refers to two clinical blood tests used in immunohematology which are done to find certain antibodies that cause autoimmune hemolysis of red blood cells (erythrocytes). The two types of Coombs tests are:

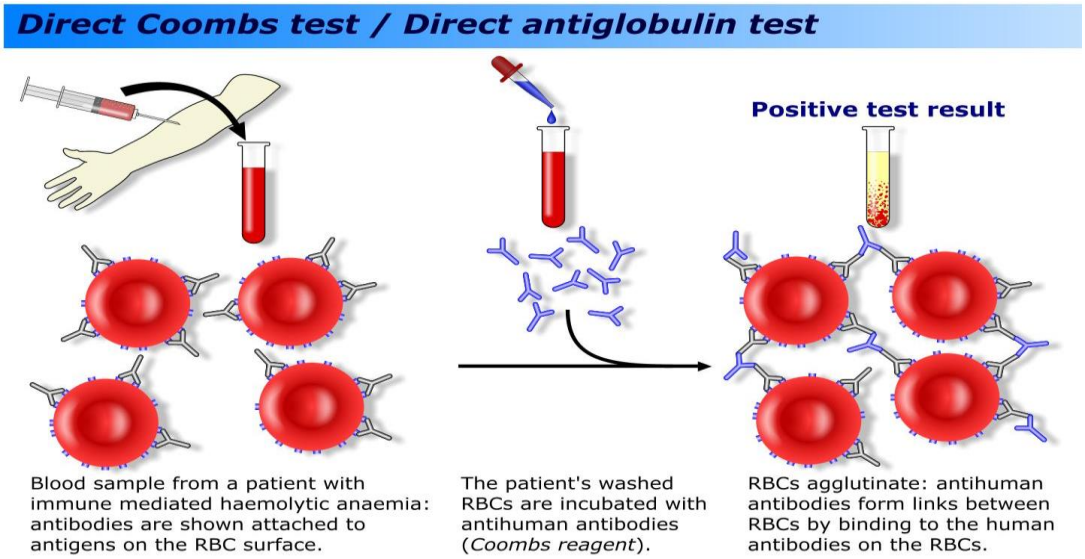
- **Direct Coombs test**
- **Indirect Coombs test**

Principle of Coombs Test: In certain diseases or conditions, an individual's blood may contain IgG antibodies that can specifically bind to antigens on the red blood cell (RBC) surface membrane. Red cells coated with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement. In order for agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the C3b or C3d component of complement, must be added to the system. Because antibodies are gamma globulins, an antibody to gamma globulin can form bridges between red cells sensitized with antibody and cause them to agglutinate.

Direct Coombs Test

The direct Coombs test (also known as the direct antiglobulin test or DAT) is used to detect if antibodies or complement system factors have bound to RBC surface antigens in vivo. A blood sample is taken and the RBCs are washed and then incubated with antihuman globulin. If this produces agglutination of RBCs, the direct Coombs test is positive, a visual indication that antibodies are bound to the surface of red blood cells.

This is the test that is done on the newborn's blood sample, usually in the setting of a newborn with jaundice. The test is looking for "foreign" antibodies that are already adhered to the infant's RBCs, a potential cause of hemolysis.



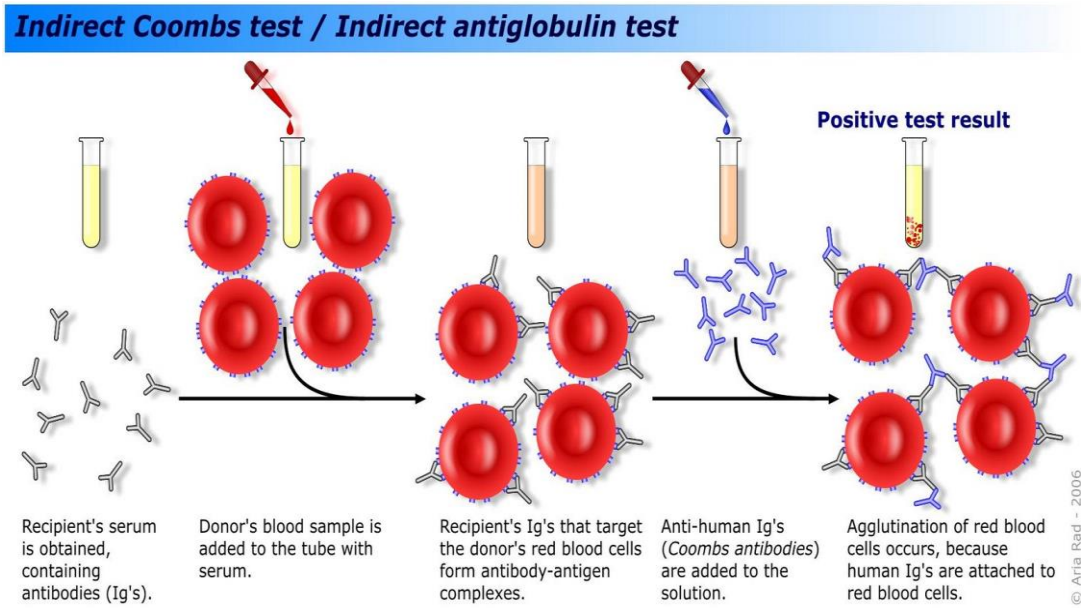
Procedure:

1. **Prepare 5% cell saline suspension of the cells to be tested.**
2. **Label 3 tubes as T, PC and NC.**
3. **In the tube labeled as T (Test), take 2 drops of 5% saline cell suspension to be tested.**
4. **In the test tube labeled as PC (Positive control), take 1 drop of anti D sera and 1 drop of Rh +ve pooled cells.**
5. **In the test tube labeled as NC (Negative control), take 1 drop of normal saline and one drop of Rh +ve pooled cells.**
6. **Add 2 drops of Anti human globulin to each of the tubes.**
7. **Mix well and centrifuge for 1 minute at 1500 rpm.**
8. **Resuspend the cells by gentle agitation and examine macroscopically and microscopically for agglutination.**

Indirect Coombs Test

The indirect Coombs test (also known as the indirect antiglobulin test or IAT) is used to detect in-vitro antibody-antigen reactions. It is used to detect very low

concentrations of antibodies present in a patient's plasma/serum prior to a blood transfusion. In antenatal care, this test is used to screen pregnant women for antibodies that may cause hemolytic disease of the newborn. The IAT can also be used for compatibility testing, antibody identification, RBC phenotyping, and titration studies.



Procedure:

1. **Label 3 tubes as T, PC and NC.**
2. **In the tube labeled as T (Test), take 2 drops of test serum.**
3. **In the test tube labeled as PC (Positive control), take 1 drop of anti D serum.**
4. **In the test tube labeled as NC (Negative control), take 1 drop of normal saline.**
5. **Add one drop of 5% cell saline suspension of pooled O Rh +ve cells in each tube.**
6. **Incubate all the tubes at 37°C for 1 hour.**
7. **Wash the cells 3 times with normal saline.**

8. **Add 2 drops of Anti Human Globulin to each tube.**
9. **Keep for 5 minutes and centrifuge at 1500 rpm for 1 minute.**
10. **Resuspend the cells and examine macroscopically as well as microscopically for agglutination.**

Interpretation

If the blood coagulates, it can be concluded that the patient's red blood cells have been bound by (his/her own) immunoglobulins. Of course, this isn't the normal state of affairs, and implies that the patient is experiencing an autoimmune hemolysis of his/her red cells.

BLEEDING DISORDERS

Tests includes:

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

Abnormal bleeding may be caused by:

– damage to vascular endothelium

– reduction in platelet numbers

– defective platelet function

– disorders of blood coagulation

Laboratory investigations

● **Activated partial thromboplastin time (APTT), other names: Intrinsic pathway coagulation factor profile, aPTT, partial thromboplastin time, PTT, blood coagulation tests. The aPTT is one of several blood coagulations tests. It measures how long it takes your blood to form a clot. Normally, when one of your blood vessels is damaged, proteins in your blood called clotting factors come together in a certain order to form blood clots and quickly stop bleeding. The aPTT test can**

be used to look at how well those clotting factors are working. It's often used with other tests that monitor clotting factors.

● Prothrombin time (PT) and INR (international normalized ratio). Prothrombin time (PT) is a blood test that measures how long it takes blood to clot. A prothrombin time test can be used to check for bleeding problems. PT is also used to check whether medicine to prevent blood clots is working (warfarin oral anticoagulant).

● Thrombin time (TT) test, also known as the thrombin clotting time (TCT) is a blood test that measures the time it takes for a clot to form in the plasma of a blood sample containing anticoagulant, after an excess of thrombin has been added.

- Activated partial thromboplastin time (APTT) test

Principle of test: Kaolin (surface activator) and platelet substitute (phospholipid) are incubated with citrated plasma at 37 ˚C for the time specified in the test method. Calcium chloride ($CaCl_2$) is added and the time taken for the mixture to clot is measured.

Reagents

● Kaolin/platelet substitute mixture*

***Purchase from a reliable manufacturer in lyophilized form and reconstitute as instructed.**

● Calcium chloride, 0.025 mol/l (25 mM)*

***This is best obtained ready-made unless the laboratory has facilities to make the reagent accurately and standardize it.**

Control plasma

A normal control plasma must be run with each batch of tests. Obtain from the nearest Coagulation Reference Laboratory or purchase from a reliable manufacturer. The control plasma should be prescreened for HIV, HBsAg and HCV.

Equipment

● Water bath at 37 ˚C.

● Stop-watch.

Blood specimen: Collect 9 ml of venous blood (well taken with minimum of stasis) into a *plastic* tube containing 1 ml of aqueous *tri*-sodium citrate anticoagulant, 32 g/l. Mix the blood well with the anticoagulant. Without delay, centrifuge the blood at 1 200 g–2 000 g for 15 minutes. This will provide platelet poor plasma. Immediately remove the plasma into a plastic tube (vial) and stopper. If a delay in performing the APTT is unavoidable, refrigerate the sample at 4–8 ˚C. The test should be performed within 1 hour of collecting the blood.

APTT test method

Test the control plasma and patient's plasma in duplicate.

Method using Kaolin/Platelet substitute mixture

1- Pipette 0.2 ml of well-mixed kaolin/platelet substitute in a small glass tube.

2- Add 0.1 ml of plasma, mix, and incubate at 37 ℃ for *exactly* 2 minutes (tilting the tube at intervals).

3- Add 0.1 ml 0.025 mol/l calcium chloride, mix and start the stop-watch. Hold the tube in the water bath and tilt the mixture back and forth, looking for clot formation. When a clot forms, stop the stop-watch and record the time.

4- Report the patient's APTT (average of the duplicate tests) providing the APTT of the normal control plasma is satisfactory.

Reference APTT range

Normal plasma clots in 36–50 seconds (using the method previously described).

-Prothrombin time (PT) test

Principle of the test: Plasma or capillary blood is added to a thromboplastin and calcium chloride reagent at 37 ˚C and the time taken for a clot to form is measured. The clotting time in seconds is converted to the International

Normalized Ratio (INR), usually by reference to a table provided by the manufacturer of the reagent or from the formula.

INR= (PT Patient/ PT Control) ^{ISI*} i.e. prothrombin ratio to the power of the ISI

***International sensitivity Index: This figure is provided by the manufacturer of the thromboplastin reagent. To obtain the INR, calculate the prothrombin ratio, log the ratio, multiply by the ISI, and antilog the result.**

Reagents

Thromboplastin calcium chloride combined reagent.

***Capillary blood PT testing*: This avoids the need to collect venous blood when monitoring patients being treated with warfarin. It should not be used however when a patient is anemic or polycythemia. Plasma should then be used.**

Availability: Lyophilized thromboplastin/calcium chloride reagent that can be used to perform a PT using either plasma or capillary blood is available from Diagnostic Reagents Ltd.

Equipment: As described previously for APTT test.

Blood specimen: Use free-flowing capillary blood or collect and centrifuge venous blood as described previously for the APTT test. Do *not* however refrigerate the venous blood or plasma. Perform the PT test as soon as possible after collecting the blood.

Control plasma

Obtain locally or use the *Diagen Normal Reference Control Plasma* described previously for the APTT test. Alternatively, use the *Rabbit Brain Capillary Reagent Normal Control Plasma* (also supplied by Diagnostics Reagents Ltd).

PT test method

If using patient's plasma, test in duplicate. Test the control plasma in duplicate. The following capillary blood method is based on using *Diagen Rabbit Brain Capillary Reagent*.

1- Pipette 0.25 ml of the thromboplastin/calcium reagent into a small glass tube. Place in a 37 ˚C water bath for 1–2 minutes.

2- Using a calibrated capillary or delivery pipette, add 50 µl (0.05 ml) of capillary blood or plasma, mix, and start the stop-watch. Hold the tube in the water bath and tilt the mixture back and forth looking for clot formation. When a clot forms, stop the stop-watch and record the time in seconds.

3- Convert the clotting time to the INR using the table provided by the manufacturer. Separate INR tables are provided for capillary blood and plasma.

***Note*: The INR conversion table provided by the manufacturer is specific for the batch of thromboplastin supplied with it.**

Reference PT range

Normal plasma samples (patients not on anticoagulant) clot in 11–16 seconds. Each laboratory should establish its own normal reference range.

Coagucheck S meter

This is a hand-held easy to use reflectance meter, available from Roche Diagnostics which provides INR and PT results within 1 minute of applying a 10 µl capillary or venous blood sample. It operates from mains electricity using an adaptor or from 4 AA batteries

Fig.: Coagucheck S meter

-Thrombin time (TT) test

Principle of test: Thrombin is added to citrated plasma at 37 ˚C. The time taken for the mixture to clot is measured and the appearance of the clot noted.

Reagent

The use of a thrombin time test kit is recommended. This provides the correct concentration of thrombin to use in the test, i.e. that which gives a clotting time of 12–15 seconds with pooled normal plasma.

Equipment: As described for the APTT test.

Blood specimen: Collect venous blood into citrate anticoagulant and centrifuge to obtain platelet poor plasma as described for the APTT test. Perform the test with as little delay as possible.

TT test method

The method described is that used in the *Diagen Thrombin Test Time Kit*. Test the patient's plasma and control plasma in duplicate.

1- Pipette 200 µl (0.2 ml) of plasma into a small glass tube. Incubate at 37 ℃ for 1–2 minutes.

2- Add 100 µl (0.1 ml) of thrombin, mix and start the stop-watch. Hold the tube in the water bath and tilt the mixture back and forth, looking for clot formation. When a clot forms, stop the stopwatch and record the time in seconds.

***Note*: In severe fibrinogen deficiency, the TT is greatly prolonged.**

3- Report the patient's TT (average of the duplicate tests) providing the TT of the control plasma is satisfactory.

Reference TT range

Normal plasma samples clot within 12–15 seconds.

Automated coagulation machines or Coagulometers measure the ability of blood to clot by performing any of several types of tests including Partial thromboplastin times, Prothrombin times (and the calculated INRs commonly

used for therapeutic evaluation), Lupus anticoagulant screens, D dimer assays, and factor assays.

MEASURMENT OF THE BLEEDING TIME: Duke method

Principle: A small cut is made with a lancet in the lobe of the ear. Blood flows from the puncture and the time it takes for the bleeding to stop is measured. The test is performed:

— for the diagnosis of certain hemorrhagic disorders;

— before surgical operations;

— before puncture of the liver or spleen.

Materials and reagents:

_ Sterile blood lancets

_ Microscope slides

_ Filter-paper (or blotting paper)

_ Stopwatch, if available, otherwise a watch with a second hand

_ Ether.

Method:

1. Gently clean the lobe of the ear with cotton wool and ether. Do not rub. Allow to dry.

2. Puncture the ear lobe. The blood should flow freely, without any need to squeeze the ear lobe. Start the stopwatch.

3. After 30 seconds collect the first drop of blood on a corner of the filter-paper (or blotting paper). Do not touch the skin with the paper.

4. Wait 30 seconds more. Collect the second drop of blood in the same way, a little further along the strip of paper.

5. Continue to collect one more drop of blood every 30 seconds. The drops become progressively smaller.

6. When no more blood appears, stop the stopwatch (or note the time on the watch).

Another method is to count the number of drops on the filter-paper (or blotting paper) and multiply by 30 seconds.

For example: there are seven drops. The bleeding time is 7 / 30 seconds = 3.5 minutes.

Results

Report the bleeding time to the nearest half minute. Mention also the reference range for the method used. Example: bleeding time 3.5 minutes (normal range, Duke method: 1–5 minutes). If the bleeding time is prolonged, examine a Romanowsky-stained thin blood film to see whether the number of thrombocytes appears to be less than normal (venous blood must be used).

OBSERVATION OF CLOT RETRACTION AND MEASURMENT OF LYSIS TIME

Principle: The tubes with clotted blood are used:

— for observation of the retraction of the clot

— for measurement of the time it takes for the clot to dissolve (lysis).

These tests are carried out for the diagnosis of certain hemorrhagic disorders.

Materials

_ Glass test-tubes, 75mm \ 10 mm, marked to hold 1ml

_ Timer

_ Metal test-tube rack

_ Water-bath

_ Materials to carry out venipuncture.

Method

Collection of specimens

Collect a venous blood specimen from patients. Do not add anticoagulant to the tubes in which you collect the blood.

Observation of clot retraction

1. Place the tube in the water-bath at 23°C (or leave it to stand at room temperature).

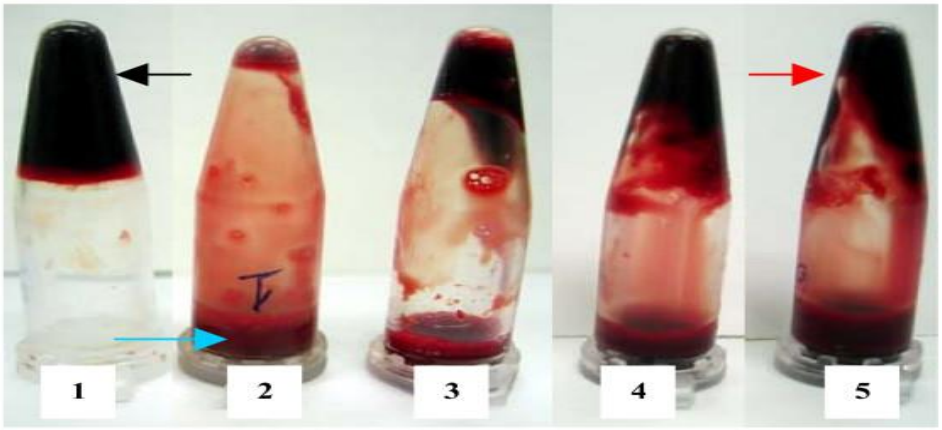
2. Examine the clot after 1, 2, 3 and 4 hours. The clot normally remains solid during the first 4 hours, although it begins to retract, usually in the first hour.

After 4 hours the clot will have completely retracted, the red cell mass separating from the yellow serum.

Measurement of lysis time

1. Place the tube containing the blood in the water-bath at 37°C (or leave it to stand at room temperature).

2. Examine the clot after 12, 24, 48 and 72 hours until lysis occurs; that is, until the clot dissolves completely and all the erythrocytes sink to the bottom of the tube.



Clot-lysis of blood samples of normal subjects (positive and negative control). Tube no. 1 is a control clot (negative control) to which water was added. No clot lysis was observed in tube no.1; a black arrow indicates the intact clot. Tube no. 2–5 (positive control) was lysed by four different concentrations of streptokinase with decreasing order. After dissolution of the clots, tubes were inverted and fluid (blue arrow) along with the remnants of clots (red arrow) could be clearly seen

Results

Normal clot retraction

The red clot is well separated and, at the surface, is attached to the sides of the tube. There may be a small deposit of erythrocytes in the bottom of the tube; it should not be more than 5mm thick.

Abnormal clot retraction

- ### Blood deficient in fibrinogen

If blood is deficient in fibrinogen there is a small red clot at the bottom of the tube, not necessarily attached to the sides of the tube. It is surrounded by sedimented erythrocytes and covered by supernatant.

- ### Blood deficient in thrombocytes

If blood is deficient in thrombocytes there is a red clot that remains almost completely attached to the sides of the tube and that will have retracted very little, if at all. Hardly any serum will have exuded. (Examine a Romanowsky-stained thin blood film, using venous blood)

- **Abnormal plasma proteins**

Abnormal plasma proteins cause coagulation of plasma. This appears as a yellow clot — clotted plasma. Beneath it is a poorly retracted red clot.

- **Hemophilia**

If there is no clot at all or a yellow clot that forms very slowly over the deposit of erythrocytes, the cause is a serious clotting factor deficiency such as that which occurs in hemophilia. Hemophilia is a hereditary hemorrhagic disease affecting males.

Report the clot retraction as:

— "normal"

— "abnormal", with a description of the clot.

Lysis time

A clot normally takes at least 48 hours to dissolve. However, the lysis time may be reduced in certain conditions. For example, in patients with acute fibrinolytic disease the clot may dissolve within 1–4 hours. Report the lysis time of the clot in hours.

EUGLOBULIN LYSIS TIME TEST

Purpose: To determine the euglobulin clot lysis time for the measurement of fibrinolysis.

Principle: The plasma euglobulins are precipitated with 1 % acetic acid and re-suspended in a borate solution. The euglobulins are allowed to clot by the addition of calcium chloride. The clot is incubated, and time of lysis reported

Primary Sample

- **Use citrated plasma as specimen.**
- **Collect 2.7 ml of venous blood in 3.2% trisodium citrate vacutainer**
- **Mix by rotating the tube in between the palms**

• Centrifuge the blood at 4000 rpm for 15 minutes and separate

• Process the sample immediately after separation of plasma

Materials/Reagents

• 3.2% Sodium citrate

• 0.025M Calcium chloride

• 1% Acetic acid

• Borate Solution

• Distilled water

• Test tubes

Equipment

• Water bath

• Centrifuge

Procedure

• Pipette out the patient plasma into test tube (12x100 mm size) market as T

• To another test tube (15x125 mm) marked as T1, pipette out 90 ml of distilled water, 0.5 ml of patient plasma and 1 ml of 0.1 acetic acid and mix well

• Refrigerate the preceding mixture for 30 minutes at 4C to allow for euglobulin precipitation • Centrifuge at 2500 rpm for 5 minutes

• Pour off the supernatant and invert the tube on filter paper to drain

• When drained well revert the tube and add 0.5 mL of the Borate solution and place in a 37C water bath

• Stir the mixture gently with a glass rod

• Add 0.5 ml of 0.025 M calcium chloride to the mixture

• **Incubate the tube in the 37C water bath and periodically check for the clot lysis. When the clot lysis begins check the tube every 5 minutes until the lysis is complete**

• **Repeat the procedure on plasma collected from normal person, marking the tube as C and C1**

Interpretation of Results

Observe the time of completion of clot lysis

Precautions

• **Handle all regents 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**

Reference Range

Normal range: 2-4 hours

FIBRINOGEN ASSAY (CLOT WEIGHT METHOD)

Purpose: Quantitative estimation of fibrinogen in plasma. Low plasma fibrinogen observed in severe liver disease. Reduced levels of plasma fibrinogen observed in typhoid fever. Increased levels are observed in acute infection such as tuberculosis and rheumatoid disease and also during pregnancy.

Principle: The clot formed in the plasma after the addition of calcium Chloride proportional to the concentration of fibrinogen.

Primary Sample

• **Use citrated plasma as specimen**

• **Collect 2.7 ml of whole blood in 32% trisodium citrate vacutainer**

• **Mix by rotating the tube in between the palms**

• Centrifuge the blood at 4000 rpm for 15 minutes and separate the plasma

Reagents

• 3.2% Sodium citrate

• 0.025M Calcium chloride

• Acetone.

• Distilled water

Instruments

• Water bath.

• Hot air oven.

• Balance

Procedure

• Remove the supernatant plasma with a pasture pipette and transfer it to a plain tube marked as T for test and keep it in a beaker containing ice

• Split the ends of an applicator stick and place it in a test tube (12 x 100 mm) labeled as T1.

• Pipette out I mL of plasma and transfer it in T1.

• Add 1 mL of calcium chloride to the above tube and mix well.

• Incubate at 37°C for 15 minutes.

• Harvest the clot gently by winding the stick and remove all the serum by pressing the clot over the wall of the tube.

• Transfer the stick with the attached clot in a tube containing approximately 2 mL of distilled water

• Then remove the stick and slit the sleeve of the clot using a toothpick and peel it off onto a filter paper.

- **Blot dry the clot and transfer it to 5 mL of acetone**
- **Keep it in acetone for 3 minutes till the clot becomes nail hard.**
- **Remove the clot and leave it in hot air oven 160°C for 2 to 3 minutes for acetone to evaporate**

Calculation

Fibrinogen = Clot weight in mg

INTERPRETATION OF RESULTS

The clot formed is weighed using a balance which is proportional to the fibrinogen concentration.

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**

Reference Range

250 to 450 mgs/DL

QUALITATIVE ESTIMATION OF FACTOR XIII ASSAY

Purpose: Qualitative estimation of factor XIII in blood. Moderately decreased factor XIII levels are observed in liver disease, sickle cell anemia and in pregnancy. Activated factor XIII levels are increased in acute myocardial infarction. Factor XIII exists in the plasma in an inactive form and is activated by thrombin during the conversion of fibrinogen to fibrin.

Principle: The plasma is allowed to clot by the addition of calcium chloride. 5 M urea is then added to the clot. In the presence of normal amounts of factor XIII this clot remains un-dissolved for 24 hours in 5 M urea solution.

Primary Sample

Use citrated plasma as specimen. Collect 2.7 mL of venous blood in 3.2% trisodium citrate vacutainer. Mix by rotating the tube in between the palms. Centrifuge the blood at 4000 rpm for 15 minutes and separate the plasma.

Reagents

1. 3.2% Sodium citrate

2. 5 M urea

3. 0.025 M Calcium Chloride

Instrument

Water bath

Procedure

1. Switch on the water bath and adjust the temperature to 37C

2. Pipette 0.5 mL of patient's plasma into a test tube (12x100 mm size) marked T.

3. Then add 0.5 mL of 0.025 M calcium chloride to it.

4. Incubate the resulting fibrin clot at 37°C for 30 min

5. Loosen the clot from the sides of the test tubes by gently tapping the sides tube.

6. Then add 5mL of 5 M urea and shake the clot in the urea solution

7. Allow the tubes to stand in the water bath at 37C.

8. Check the clot at the end of 1 hour, 2 hours 3 hours and 24 hours and note if the clot has dissolved

9. Repeat steps 7.3-7,8 on normal plasma, labeling the tube as C for control

Interpretation of Results

Normal-the clot remains un-dissolved for 24 hours and remains firm clot

Abnormal- If the clot dissolves within 24 hours and if the clot has become soft

Precautions

1. **Handle all reagents with care and avoid contact with eye mouth and skin**
2. **Handle all samples as potentially infectious**
3. **Discard used reagents and sample as per disposal procedure**

DIGNOSIS OF IRON DEFICINCY ANEMIA

Anemia is the condition of decrease in number of circulating red blood cells (and hence hemoglobin) below a normal range for age and sex of the individual, resulting in decreased oxygen supply to tissues. Iron deficiency anemia is a type of microcytic hypochromic anemia, which is the most common nutritional disorder. Iron is an essential element in the synthesis of hemoglobin.

Iron deficiency anemia (IDA) may cause a problem in differential diagnosis from other hypochromic anemias like beta-thalassemia trait, alpha-thalassemia trait, HbE disease, sideroblastic anemia or anemia due to chronic diseases. This topic will discuss about laboratory investigations for the differential diagnosis of IDA from those conditions, along with some preliminary investigations.

Hematological Tests

1. Hemoglobin and Hematocrit

According to WHO, the criteria for anemia is when adult males have Hemoglobin levels <13 g/dL and adult females have <12 g/dL. As the iron deficiency worsens, both Hb and PCV decline together.

1. **Hb >12 g/dl : Not anemic**
2. **Hb 10–11 g/dl : Mild anemia**
3. **Hb 8–9 g/dl : Moderate anemia**
4. **Hb 6–7 g/dl : Marked anemia**

5. **Hb 4–5 g/dl : Severe anemia**
6. **Hb < 4 g/dl : Critical**

2. Red Cell Indices

MCV, MCH and MCHC are reduced. RDW is raised.

1. **Mean Corpuscular Volume (MCV): It is the average volume of the RBC expressed in femtoliters. It becomes <80 fL in IDA (normal 82–98 fL).**
2. **Mean Corpuscular Hemoglobin (MCH): MCH indicates the amount of Hemoglobin (weight) per RBC and is expressed as picograms. MCH will be <25 pg in IDA (normal 27–32 pg).**
3. **Mean Corpuscular Hemoglobin Concentratration (MCHC): The MCHC measures the average concentration of hemoglobin in a red blood cell. MCHC goes below 27 g/dL(normal 31–36 g/dL).**
4. **Red Cell Distribution Width (RDW): RDW is a quantitative measure of anisocytosis. In IDA, RDW is increased and >15%. It is earliest sign of iron deficiency (normal 11.5–14.5%).**

Peripheral Blood Smear

1. Red Blood Cells (RBCs)

- **Microcytosis: RBCs are usually smaller than normal. Dimorphic blood picture is seen with a dual population of red cells of which one is macrocytic and the other microcytic and hypochromic when iron deficiency is associated with severe folate or vitamin B12 deficiency.**
- **Hypochromasia: Central pallor in RBCs is more than 1/3.**
- **Poikilocytosis: Elliptical forms are common, and elongated pencil (cigar) shaped cells may be seen. Target cells and Teardrop cells may also be present in small numbers. Severe anemia shows ring/pessary cells.**

2. White Blood Cells (WBCs)

WBCs are usually normal in number, but can increase due to chronic marrow stimulation in long-standing cases. There may be associated eosinophilia if iron deficiency is secondary to hookworm infestation.

3. Platelets

Platelet count is usually normal, but may be slightly to moderately increased, especially in patients who are bleeding.

4. Reticulocyte Count

The reticulocyte count is low in relation to the degree of anemia.

Bone Marrow Examination

1. **Cellularity: moderately hypercellular.**
2. **M:E ratio: varies from 2:1 to 1:2 (normal 2:1 to 4:1).**
3. **Erythropoiesis: hyperplasia and micronormoblastic maturation.**
4. **Myelopoiesis: normal.**
5. **Megakaryopoiesis: normal.**
6. **Bone marrow iron: Absent. "Gold standard" test, demonstrated by negative Prussian blue reaction.**

Biochemical Tests

Serum iron profile studies are used to establish a differential diagnosis of microcytic, hypochromic anemia.

	Normal range	Value in IDA	Observation
Serum ferritin	15–300 µg/L	<15 µg/L	
Serum iron	50–150 µg/dL	10–15 µg/dL	
Serum transferrin saturation	30–40%	<15%	
Total plasma iron-binding capacity (TIBC)	310–340 µg/dL	350–450 µg/dL	
Serum transferrin receptor (TFR)	0.57–2.8 µg/L	3.5–7.1 µg/L	
Red cell protoporphyrin	30–50 µg/dL	>200 µg/dL	

IRON AND IRON-BINDING CAPACITY TEST

Purpose: Iron found in blood is mainly present in the hemoglobin of the RBCs. Its role in the body is mainly in the transport of oxygen and cellular oxidation. Iron is absorbed in the small intestines and bound to a globulin in the plasma, called transferrin and transported to bone marrow for the formation of hemoglobin. Increased serum levels are found in hemolytic anemias, hepatitis, and lead and iron poisoning. Decreased serum levels are found in anemias caused by iron deficiency due to insufficient intake or absorption of iron, chronic blood loss, late pregnancy and cancer. Increase in total iron-binding capacity (TIBC) is found in iron deficient anemias and pregnancy. Decrease in TIBC is found in hypoproteinemia, hemolytic/pernicious/sickle cell anemias, inflammatory diseases and cirrhosis.

Principle: Iron, bound to transferrin, is released in and acidic medium and the ferric ions are reduced to ferrous ions. The Fe (II) ions react with ferrozine to form a violet colored complex. Intensity of the complex formed is directly proportional to the amount of iron present in the sample. For TIBC, the serum is treated with excess of Fe (1) to saturate the iron binding sites of transferrin. The excess Fe (II) is adsorbed and precipitated and the iron content in the supernatant is measured to give the TIBC.

Fe (III) ----- Acid medium----------> Fe (II)

Fe (II) + Ferrozine----------> violet colored complex

Performance specifications:

- ***Linearity:*** **Up to 1000 ug/dL. If the value exceeds this limit, dilute the serum with distilled water and repeat the assay. Calculate the value using the proper dilution factor.**
- ***Measurement range:*** **This method has a measurement range 60-200 ug/dL of iron in serum.**
- ***Sensitivity:*** **The minimum detection limit by this kit is 40 ug/dL.**

Primary sample:

- **Use only serum as specimen**
- **Collect 4 mL of venous blood in a plain vacutainer tube.**
- **Allow the tube to stand for 30 min and separate the serum by centrifugation at 2500 rpm for 10 min.**
- **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 4-8° C for up to 7 days or at -20 to 25° C for up to 21 days.**
- **Sample should be collected before 11.00 am.**

Reagents:

- **L1: Iron buffer reagent 35 mL**
- **L2: Iron color reagent 35 mL**
- **S: Iron standard (100 ug/dL) 2 mL**
- **Storage/Stability: Contents are stable at 2–8°C till the expiry.**
- **The reconstituted reagent contains Arzenazo III, 8-Hydroxy quinoline 5-sulfonic acid, Phosphate buffer: Non-reactive ingredients, and stabilizers.**

Instrument: Semi-autoanalyzer/Spectrophotometer

Procedure:

- **Wavelength/filter: 570 nm (Hg 578 nm)/Yellow**
- **Temperature: RT**
- **Light path: 1 cm**
- **Iron assay: Pipette into clean dry test tubes labeled as Blank (B). Standard (S), Sample Blank (SB) and Test (T)**

Addition Sequence	*B (ml)*	*S(ml)*	*SB (ml)*	*T(ml)*
Iron buffer reagent (LI)	**1.0**	**1.0**	**1.05**	**1.0**
Milli Q H_2O	**0.2**	**--**	**--**	**--**
Iron standard (S)	**--**	**0.2**	**--**	**--**
Sample	**--**	**--**	**0.2**	**0.2**
Iron color reagent (L2)	**0.05**	**0.05**	**--**	**0.05**

Mix well and incubate at RT for 5 min. Measure the absorbance of the blank (Abs. B). standard (Abs. S), Sample Blank (Abs. SB) and Test sample (Abs.T) against DW.

- TIBC assay: Pipette into a clean dry test tube

Serum 0.5 mL

TIBC saturating reagent (LI) 10 ml

Mix well and allow to stand at RT for 10 min and add

TIBC precipitating reagent (L2) Approx. 50 mg

Mix well and allow to stand at R-T for 10 min. Centrifuge at 2500 rpm for 10 min to obtain a clear supernatant. Determine the iron content in the supernatant as above-mentioned iron assay. Calculation

Iron in pg/dL = Abs.T - (Abs. SB + Abs.B)/ Abs.S - Abs. B X 100

TIBC in ug/dl = Abs.T - (Abs. SB + Abs.B)/ (Abs. SB + Abs.B) x 300

UBIC in µg/dL = TIBC in µg/dL - iron in µg/dL

- **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.**
- **Zero with distilled water.**
- **Feed the reagent blank with each batch of patient samples and ensure the absorbance of the blank is less than 0.300 at 650 nm if the absorbance of the 'blank is more than 0.300', discard the reagent.**
- **Then feed the standard and ensure the value of the standard is 100 ug/dL. Then feed the test samples and record the values.**
- **Before processing patient samples, check whether the sample is hemolyzed, icteric before processing. If the sample is lysed collect another sample and proceed.**

Interferences: Turbid, lipemic, lysed and icteric samples interfere with the absorbance.

Reference range:

Serum Iron: Male 80–140 ug/dL

Female 80–155 ug/dL

Newborns 12-67 ug/dL

Children up to 10 yrs 30–150 ug/dL

Serum IBC: 250-400 ug/dL

UBIC: 160-360 ug/dL

Reportable interval of examination: Within 4 hours.

Critical/Alert level values: Below 50 ug/dL

Laboratory interpretation: Decreased iron and increased TIBC and UBIC suggest iron-deficiency anemia. Increase of iron suggests hemolytic anemia, hemosiderosis and hemochromatosis.

Potential sources of variability:

- **Lysed serum specimens may give falsely elevated values.**
- **As iron is a ubiquitous ion, to prevent accidental contamination, all glassware should be rinsed in dilute hydrochloric acid and water before use. Even water and glassware containing iron will react with the reagent.**

ENZYME IMMUNOASSAY FOR FERRITIN TEST SYSTEM

Intended Use: The Quantitative Determination of Circulating Ferritin Concentrations in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

Ferritin, in circulation, as measured in serum levels is a satisfactory index of body's iron storage. The iron storage is directly measured by quantitative phlebotomy, iron absorption studies, liver biopsies and microscopic examinations of bone marrow aspirates. Iron deficiency (Anemia) and iron overload (Hemochromatosis) are conditions associated with body's iron storage or lack thereof. Measurements of total iron binding capacity (TIBC) have widely been used as aids in the determination of these conditions. However, an assay of serum

Ferritin is simply more sensitive and reliable means of demonstration these disorders.

Ferritin is present in blood in very low concentrations. Normally, approximately 1% of plasma iron is contained in Ferritin. The plasma ferritin, is in equilibrium with body stores, and variations of iron storage. The plasma concentrations of ferritin decline very early in anemic conditions like development of iron deficiency, long before the changes are observed in the blood hemoglobin concentration, size of the erythrocytes and TIBC. Thus, measurements of serum ferritin can serve as an early indicator of iron deficiency that is uncomplicated by other concurrent conditions. At the same time a large number of chronic conditions can result in elevated levels of serum ferritin. These include chronic infections, chronic inflammatory diseases such as rheumatoid arthritis, heart disease and some other malignancies, especially lymphomas, leukemia, breast cancer and neuroblastoma. In patients who have these chronic disorders together with iron deficiency, serum ferritin levels are often normal. An increase in circulating ferritin is observed in patients with viral hepatitis or after a toxic liver injury as a release of ferritin from the injured liver cells. Elevated serum ferritin levels are found in patients with hemochromatosis and hemosiderosis.

Circulating ferritin levels have been used by clinicians, as an aid, in the diagnosis of several other disorders. It has proved as a valuable tool in differential diagnosis of anemia due to iron deficiency and anemias due to other disorders and, in exposing the depletion of iron reserves long before the onset of anemia. Serial determinations have been used to monitor, non-invasively, the erosion of iron storage during pregnancy and in patients undergoing dialysis. Serum ferritin is routinely used as a screen for iron deficiency for a variety of populations like blood donors and people who are receiving regular blood transfusions or iron replacement therapy.

In this method, ferritin calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal antibody (specific for ferritin) is added and the reactants mixed. Reaction results between the biotinylated ferritin antibody and native ferritin to form an immune complex that is deposited on the streptavidin coated well. The excess serum proteins are washed away via a wash step. Another ferritin specific antibody, labeled with an enzyme, is added to the wells. The enzyme labeled antibody binds to the ferritin already

immobilized on the well. Excess enzyme is washed off via a wash step. A color is generated by the addition of a substrate. The intensity of the color generation is directly proportional to the concentration of the ferritin in the sample.

The employment of several serum references of known ferritin 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 ferritin concentration.

PRINCIPLE

Immunoenzymometric sequential 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- ***ferritin*** **antibody.**

Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an antibody-antigen complex. Simultaneously the biotin attached to the antibody binds to the streptavidin coated on the microwells resulting in immobilization of the complex. After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly 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. Ferritin Calibrators

Six (6) vials of Ferritin calibrators at levels of 0(A), 10(B), 50(C), 150(D), 400(E) and 800(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 WHO 3 ^{ed} IS 94/572.

B. Ferritin Biotin Reagent

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

C. Ferritin Enzyme Reagent

One (1) vial containing Horseradish Peroxidase (HRP) labeled anti-ferritin IgG in buffer, dye and preservatives. 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 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 (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 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 and 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 for samples. 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.

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, 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 0.100 ml (100µl) of the Ferritin Biotin 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 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 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 the Ferritin Enzyme Conjugate to each well.

DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION

9. Incubate 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 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.

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

DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION

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

14. Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds.

15. 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: Always add reagents in the same order to minimize reaction time differences between wells.

EXPECTED RANGE OF VALUES

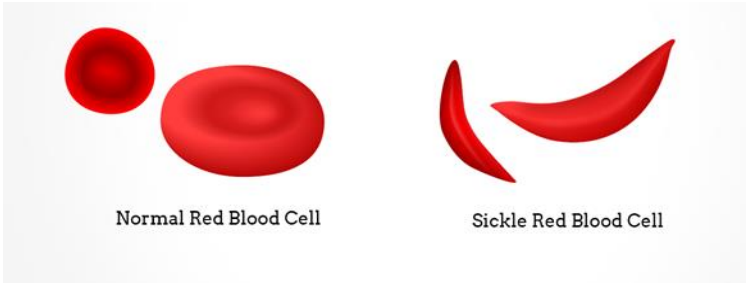
Approximate reference ranges for normal males and female adults were established by using 400 normal sera with the Ferritin ELISA test system

Males	**16-220 ng/ml**
Females	**10-124 ng/ml**

In addition to the above the following ranges were assigned based on the available literature. However, these ranges were confirmed using Ferritin Microplate Elisa Procedure with limited number of samples.

TEST FOR SICKLE CELL ANEMIA

Hemoglobin S is an inherited abnormal hemoglobin. If inherited from both parents it causes sickle-cell anemia, a serious disease. If inherited from only one parent it causes sickle-cell trait, which does not usually cause disease. Hemoglobin S occurs mainly in tropical Africa but also in the Eastern Mediterranean region and among Americans of African origin. The sickle-cell slide test does not distinguish between sickle-cell anemia and sickle-cell trait.



Principle: One drop of blood is mixed with one drop of sodium metabisulfite reagent on a slide. If the erythrocytes contain hemoglobin S, they will become sickle-shaped or half-moon-shaped. The reagent removes oxygen from the cells, allowing sickling to take place.

Materials and reagents

_ Microscope

_ Microscope slides

_ Coverslips

_ Filter-paper

_ Pasteur pipette (or dropping pipette)

_ Two small wooden sticks

_ Containers to prevent drying of the preparation, such as Petri dishes

_ Fresh sodium metabisulfite, 2% solution.

Method

1. Place a small drop of capillary blood (about 4mm diameter) in the center of a slide.

2. Add an equal-sized drop of sodium metabisulfite solution.

3. Mix carefully with the corner of a slide. Cover with a coverslip, making sure that no air bubbles form.

4. Place the slide in a Petri dish that has wet filter-paper in the bottom. Support the slide on two sticks. Wait 30 minutes before examining the slide.

***Note*: When using a reducing reagent such as sodium metabisulfite it is not necessary to seal the preparation.**

Microscopic examination

Examine the slide under the microscope using the X 40 objective.

Negative result= The erythrocytes remain round.

If the test is negative, re-examine the slide after a further 30 minutes, then after 2 hours and after 24 hours.

Positive result= The erythrocytes become sickle-shaped or banana-shaped, often with spikes. It is important to examine several parts of the preparation, as sickling can occur more quickly in one part than in another. Do not mistake normal erythrocytes lying on their side or crenate cells for sickle cells.

***Note*:**

False-negative results may occur if:

— outdated reagents are used;

— concentrations of hemoglobin S are low;

— patients have moderate or severe anemia.

If the test is positive a thin blood film should be examined. Patients with sickle-cell anemia have sickle cells, nucleated erythrocytes, target cells, marked poikilocytosis and often macrocytosis. Patients with sickle-cell trait are not usually anemic and have a normal erythrocyte morphology. Whenever possible, electrophoresis of the hemoglobin should be carried out to confirm a diagnosis of sickle-cell disease. This can be done in a reference laboratory.

Other methods

-The test can be carried out on venous blood provided it is freshly collected (within 1–2 hours of the test) or collected into an anticoagulant (EDTA dipotassium salt, 10% solution).

-The test can also be carried out using a test-tube rather than a Petri dish. Commercial reagents are available for this method.

BLOOD GROUPING AND Rh TYPING

Purpose: To determine the blood group and Rh type in human. This test is used in the identification of patient's ABO and Rh blood type prior to surgery or other procedures in which blood loss is a threat and for stored blood. It is used in the determination of ABO and Rh compatibility of donor and recipient's blood. blood. It is also used in the identification of maternal and infant ABO and Rh blood types to predict potential hemolytic disease of the newborn.

Principle: This test is based on the principle of direct hemagglutination. The erythrocytes of a person contain antigens on the surface of the members. When these antigens are allowed to react with the corresponding antigen-antibody reactions are produced. Normal erythrocyte or agglutinate when mixed with Anti A/Anti B/ Anti A and B, if they possess A/B/AB antigens respectively.

Performance Specifications

- **Presence of immune antibodies adsorbed on to the surface of the cells carrying the corresponding antigens will not produce agglutination. These blood group antigens can later be detected by the reaction anti human gamma globulin reagent.**
- **Visible agglutination reactions require proper proportion of antigen and antibody.**

Primary Sample

- **Use whole blood as specimen.**
- **Collect 2 mL of blood in 3.6 mg KEDTA vacutainer,**
- **Process the sample within 1 hour of collection**

• If a delay in testing is expected or unavoidable, red cells from come samples, EDTA/Heparinized anticoagulant samples show separated from the plasma, washed and stored in a red present solution at 2-8°C for no longer than 35 days.

• Do not use insufficient sample and sample without an ID number.

Consumables /Reagents

• Anti A, blood grouping reagent (Commercially available)

• Anti B, blood grouping reagent (Commercially available)

• Anti AB, blood grouping reagent (Commercially available)

• Anti D, blood grouping reagent (Commercially available)

• Anti A_1 lectin (Commercially available).

•Glass slides

•Applicator sticks

• 09% Isotonic saline

Procedure

Slide Agglutination Method

•Take a clean, grease free microscope slide

• Draw a line in the middle of this slide using a wax glass marking pencil and label the left portion Anti-A and the right as Anti-B

• Add a drop of Anti-A to the portion marked as Ant-A and add a drop of Anti-B to the portion marked as Ant-B

• Add one drop of well mixed 3-5% cell suspension to each side

• With a tooth pick, separate for each side. mix the cells and anti-sera well

• Gently rotate the side for mixing.

• Place the slide against a white background.

• After 2 minutes examine both macroscopically and microscopically for agglutination

Tube Agglutination Method

• Switch on the water bath and adjust the temperature to 37C

• Take two tubes of size 12 x 100 mm

• Mark one tube as anti A and the other as anti B.

• Add two drops of anti-A to the tube labelled as anti-A and two drops of anti-B to the tube labelled ant-B

• Add two drops of 3-5% cell suspension to each tube.

• Mix well and centrifuge both tubes at 1500 rpm for 1 minute.

• Remove the tubes and inspect the button of the cells in the bottom.

• With the gentle shaking or tapping of the tube observe the dislocation of the cell button

• Report - Positive if agglutination is observed

• Place the tube/tubes in which no agglutination was observed in water bath for 30 minutes.

• Remove the tubes from the water bath and centrifuge once again and serve the cell button by shaking the tube gently

• Report as positive if agglutination is observed

• Double check the results microscopically by putting one or two drops from each tube on microscopic sides. Prepare a 3-5% suspension of test red cell

• All suspensions may be prepared in autologous serum / plasma/ saline

• Place one drop of the appropriate A, B, AB blood grouping reagent on a clean, dry glass slide at room temperature

• Add one drop of the prepared 35-45% suspension of red cells to each drop of reagent on the glass slide.

• Mix the cells and reagent thoroughly over an approximate 20mm circular area, using a separate clean applicator one for each reagent-red cell mixture

• Rock or rotate slides gently and examine for macroscopic hemagglutination. Agglutination may begin within a few seconds. However, observation should not continue beyond 2 minutes.

Interpretation of Results

• If agglutination is observed with anti- A blood group reagent, then the patient's blood group is A

• If agglutination is observed with antiA_1 Lectin reagent, then the patient's blood group is A_1.

• If agglutination is observed with anti B blood group reagent, then the patient's blood group is B

• If agglutination is observed with both anti A and Anti B blood group reagent, then the patient's blood group is AB

• If agglutination is not observed with both anti A and anti B blood group reagent then the patient's blood group is O

• If agglutination is observed with anti D blood group reagent, then patient's Rh type is Positive. All Rh negative is reconfirmed with Du testing.

Precautions

• Thoroughly clean and dry the text slides before use.

• Ensure reagents and specimens are at room temperature before use.

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 Source of Variability

- **False negative or unexpectedly weak reactions may occur with blood samples of weak A or B subgroups or with cord blood red cells from newborn infants**

- **False negative or unexpectedly weak reaction may occur with red cell or anti-sera that have been subject to prolonged some inappropriate storage conditions**

Du BLOOD TYPE TESTING

Purpose: The Du factor (a variant of D antigen present in the red cells of individuals of Du-blood type) reacts with anti D but does not bring about hemagglutination that is not strong enough to be visualized. This test is done to reconfirm all Rh-negative blood groups.

Principle: Cells with Du antigen is sensitized with anti D by incubating at 37°C for 30 minutes. This results in the adsorption of anti D on the surface of the cell without producing hemagglutination. The presence of reacted on the surface of Du cells is recognized by using antihuman globulin which reacts with coated antibody and brings about hemagglutination

Primary sample

- **Use whole blood as specimen.**

- **Collect 2 ml of blood in 3.6 mg K_2 EDTA vacutainer. Mix well.**

- **Process the sample within 1 hour of collection.**

- **Do not process insufficient samples and sample without an ID number.**

Consumables Reagents

- **Slides**

- **Pipettes and tubes**

- **Anti D (Monoclonal IgM + IgG)**

- **Antihuman globulin**

• **Normal saline**

Procedure

• **Prepare 5% suspension of washed red cells with normal saline.**

• **Take 1 tube and label it as test (T)**

• **Place 2 drops of 5% cell suspension in the tube**

• **Add 1 drop of anti D in the tube labeled T**

• **Place the tube in the water bath at 37°C for 30 minutes.**

• **Remove the tubes from the water bath and wash the cells in normal saline 2-3 times.**

• **Add 2 drops of anti-human globulin to both the tubes. Mix gently**

• **Centrifuge the tubes at 1500 rpm for 1 minute and look for 1 minute and look for agglutination**

Interpretation of results

• **Du positive: if agglutination is present in the tube labeled T**

• **Du negative: if no agglutination is seen in the tube labeled T**

FLOURSENCE IMMUNOASSAY FOR D-Dimer CONCENTRATION

PURPOSE AND SCOPE

The purpose of this document is to describe the procedure for performing a D-Dimer
using the Alere Triage MeterPro analyser. The Alere Triage MeterPro analyser is to be used by trained health care professionals in accordance with a facility's policies and procedures. This document will be focusing on D-Dimer using the single Test Panel, and further information on the other parameters and multi-marker panels can be found in the appropriate product inserts.

HAZARDS

Patient Samples

All patient samples should be treated as potentially infectious and handled appropriately. Some quality control solutions contain human source material.

Personal Protective Equipment should be worn when processing samples, quality control testing and maintenance procedures.

CLINICAL SIGNIFICANCE

Venous thromboembolism is a term used for both deep venous thrombosis (DVT),

which involves clot formation in the deep veins of the body and a pulmonary embolism (PE), where the clot can break off and travel to the lungs.[1] PEs originate from proximal DVT of the leg (popliteal, femoral, or iliac veins), with upper extremity DVT less common but also may lead to PE, especially in the presence of a venous catheter.

Diagnosis of DVT is based on the Wells clinical prediction rule, which provides a reliable estimate of the pretest probability of DVT; Doppler ultrasonography; helical computed tomography (CT); contrast venography; Impedance plethysmography and D-dimer tests.

D-Dimer is a degradation product of crosslinked fibrin.[3] The D-Dimer concentration is a measure of fibrinolytic activity of plasmin in the bloodstream.[4] D-Dimer concentrations are raised in the setting of acute deep vein thrombosis (increased blood clotting and fibrinolytic activity); however, an elevated D-Dimer concentration is insufficient to establish the diagnosis of PE because such values are both nonspecific and commonly present in hospitalised patients.[2,3]

TEST PRINCIPLE

The Alere Triage D-Dimer Test is a single use fluorescence immunoassay device

designed to determine the concentration of D-Dimer in EDTA anticoagulated whole blood or plasma specimens.

The test procedure involves the addition of several drops of an EDTA anticoagulated whole blood or plasma specimen to the sample port on the Test Device. After addition of the specimen, the whole blood cells are

separated from the plasma using a filter contained in the Test Device. The specimen reacts with fluorescent antibody conjugates and flows through the Test Device by capillary action. Complexes of each fluorescent antibody conjugate are captured on a discrete zone resulting in a binding assay.
The concentration of D-Dimer in the specimen is directly proportional to the fluorescence detected therefore a greater amount of fluorescence indicates a higher D-Dimer concentration. Light from a laser hits a test device that has been inserted in the meter, which causes the fluorescent dye in the test device to give off energy.
The more energy the fluorescent dye gives off, the stronger the signal. The results are displayed on the Meter screen in approximately 20 minutes.

INSTRUMENT

SPECIMEN REQUIREMENTS
Venous whole blood or plasma collected in EDTA tubes is acceptable. Other blood collection tubes containing other additives and other blood specimen types have not been evaluated.
CARTRIDGES / REAGENTS

Storage and handling
• Perform D-Dimer test using the following Test Devices: Alere Triage D-Dimer Test, or the Alere Triage Profiler SOB Panel (CK-MB, Myoglobin, Troponin I,BNP and D-Dimer)
• Unopened Test Devices should be refrigerated at 2° - 8°C and are stable up to the expiration date.

• Once removed from refrigeration, the pouched Alere Triage D-Dimer and SOB Test Device is stable for up to 14 days at room temperature, but not beyond the expiration date printed on the pouch.
• Once equilibrated to room temperature, do not return the Test Device to refrigeration.
• Optimal results will be achieved by performing testing at temperatures between 20-24°C.
• Test Devices must be at room temperature before use (i.e. allow the unopened Test Device to sit at room temperature for at least 15 minutes)

• If a kit containing multiple Test Devices is removed from refrigeration, allow the kit to reach room temperature before use. This will take a minimum of 60 minutes.
• Keep the Test Device in the sealed pouch until ready for use. Discard after single use
• The transfer pipette should be used for one patient specimen only. Discard after single use
• Discard the Test Devices if they are past the used by date. Expired Test Devices can produce incorrect results.
• The QC Device is light sensitive and should be stored in its black opaque case when not in use.

CALIBRATION

In the self-test mode, the meter scans an Internal Calibration Chip. Each calibration chip scan is used to validate and adjust, if necessary, the meter calibration. Operator calibration is not necessary.

QUALITY CONTROL

The Alere Triage MeterPro uses the following methods for quality:

• Built-in QC Check
• Code Chip modules
• Electronic QC Device test
• Control solutions

Built-in internal QC check

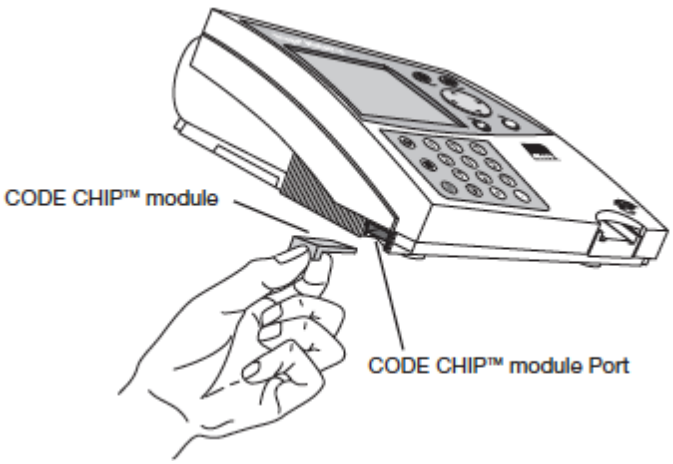
The Test Devices contains a built-in internal QC Check, which is performed

automatically by the Meter during every assay. These assay controls in each test device monitors the assay procedure and the reagent integrity. If the results are found to be in specification, the result is allowed to be used, but if the test device's
internal quality control (QC) results are unacceptable, the results for the affected analyte(s) will not appear on the screen (instead will be flagged with an exclamation mark symbol).

Installing the Reagent, QC Sample or QC Device Code Chip modules

• From the 'Main Menu' select 'Install Code Chip' using the up/down arrow keys and press 'Enter' to confirm selection.
• Use the new Code Chip module that comes with every new box of test devices.
• Compare the lot number on the code chip with the corresponding lot number on the test strip pouch.
• Insert the Code Chip module into the Code Chip module Port in the lower left front corner of the meter
• The meter will display a confirmation message that the information was installed into the meter and press the 'Enter'
• Remove the Code Chip module from the Code Chip module Port.
• Place the Code Chip module back into its original container for storage.

NOTE: The meter will direct the user to install a Code Chip module if it is attempting to run a test and does not have the data in its memory.



Electronic QC Device test

The QC Device should be run on each day of patient testing. The QC Device Code Chip Module should be performed before running a QC Device test, as stated in section 9.2

To perform a QC Device test:

• Press the 'Power' button to turn on the meter.

• Select 'Run Test' using the up/down arrow keys and press the 'Enter' key to confirm selection

• Type in your identification number (User ID) if prompted.

• Select 'QC Device' using the up/down arrow keys and press the 'Enter' key to confirm selection

• Gently insert the QC Device into the meter until you feel the QC Device catch on the pin and hear a 'click'.

• Press the 'Enter' key to start the test

NOTE: The meter will prompt the user to install the QC Device Code Chip module if the QC Device has not been run before

• The meter pulls in the QC Device and scans it. The test device may partially move in and out of the meter several times.

NOTE: After the test device has been inserted, do not push the device in further or attempt to pull it out. The device may be ejected by returning to the Main Menu and pressing the 'Exit' key.

• When the test is complete, the meter will beep, eject the device and display a Pass or Fail result on the meter's screen

• Press the 'Print' key to make a printed copy of the results

• Remove the QC Device from the meter and place in the QC Device Box. Do not discard the QC Device.

NOTE: The QC Device is light sensitive and should be stored in its black opaque case when not in use. Keep the QC Device free of contaminants, as dust, lint, fibers and other small particles may interfere with the QC Device.

• If the QC Device tests fail, wipe the QC Device clean with a lint free cloth to remove any oils, dust, fibers, or fingerprints. Do not apply any liquid to the QC Device. After cleaning the device, repeat the QC Device test.

NOTE: The QC Device does not expire.

Running control solutions

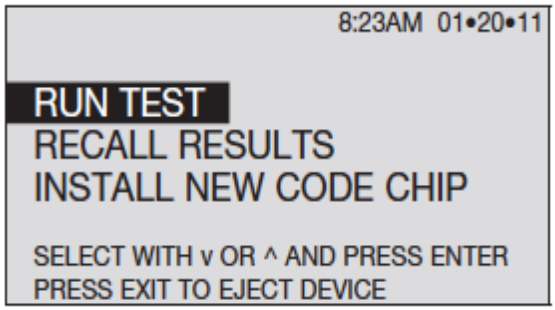
The QC Device Code Chip Module should be performed before running a QC

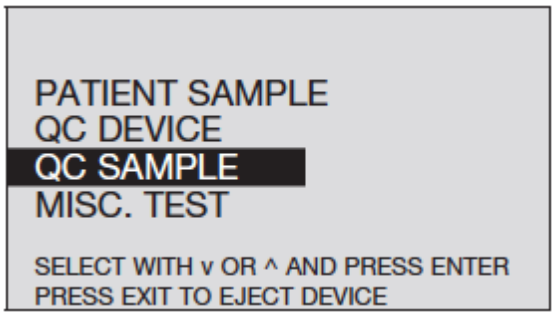
Device test, as stated in section 9.2
The control solutions have two levels:
• Alere Triage Total 5 Control 1
• Alere Triage Total 5 Control 2
Store the controls frozen at -20°C or colder in a non-defrosting freezer. Do not store near the freezer door. The reagents are stable until the date on the box.
The reagents should not be refrozen and it is recommended that each tube be used once and discarded. Frozen control material must be brought to room temperature (at least 30 minutes) prior to use.

Preparing the control solution
• Remove the control solution from the freezer and allow to thaw and warm to
room temperature (19°- 25°C) for at least 30 minutes.
NOTE: Use within 1 hour of removal from the freezer.
• Mix thoroughly by inverting the control solution tube

Preparing the meter
• Power the meter on by pressing the 'Power' key. Press the 'Enter' key to run self-test.
• Select 'Run Test' using the up/down arrow keys and press
• Type in your identification number (User ID) if prompted.
• Select 'QC Sample' using the up/down arrow keys and press the 'Enter' key to confirm selection
• Enter the QC lot number from the label on the side of the vial containing the QC

Sample and press the 'Enter' key to confirm the number.
NOTE: Only enter the four-digit numeric value of the QC lot number Do not enter the preceding alpha character
NOTE: If you enter a QC lot number for which there is no data in the meter's memory, you will see a message on the meter's screen: 'No QC Sample Data in Memory'. Press 'Enter' to continue and install Code Chip module.





Applying the control sample

NOTE: The unopened Test Devices should sit at room temperature for at least 15 minutes before performing a test.

- **Open the pouch and label the Test Device.**
- **Place the Test Device on a level, horizontal surface.**
- **Hold the tube with the tip facing upwards, ensuring that all material is at the bottom of the tube.**
- **Snap off the tab**
- **Turn the tube over and dispense the entire contents into the sample port of the Test Device.**
- **Ensure the specimen has been absorbed completely before moving the Test Device.**

Inserting the Test Device

- **Hold the Test Device by the edges and gently insert the Test Device into the meter until you feel the Test Device catch on the pin and hear a 'click'.**

NOTE: After the test device has been inserted, do not push the device in further or attempt to pull it out. The device may be ejected by returning to the Main Menu and pressing 'Exit'.

- **Press 'Enter' to start the test**
- **The meter pulls in the test device and scans it**

NOTE: The Test Device must be inserted into the Meter within 30 minutes from the time the sample was added

Results

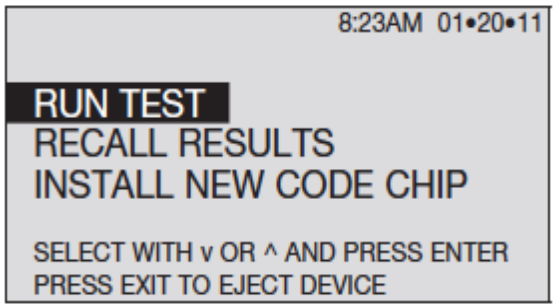
NOTE: Do not move the meter while a test is in progress.
• The results are displayed on the screen, and automatically stored in the meter's memory.
• Results should fall within the expected ranges provided on the Expected Values card provided with the D-Dimer Controls Kit
• If any of the QC Sample results are out of range, the results for that particular analyte will be displayed as light text on a dark background.
TEST PROCEDURE
To change the meter settings, insert the Supervisor CODE CHIP module and refer to the Alere Triage MeterPro User Manual for instructions.
NOTE: Prior to commencing patient testing, remove the Supervisor CODE CHIP
module from the meter, return it to the storage box and place in a safe place for future use.
Performing the Test The Reagent Code Chip Module should be performed before running a patient test, as stated in section 9.2

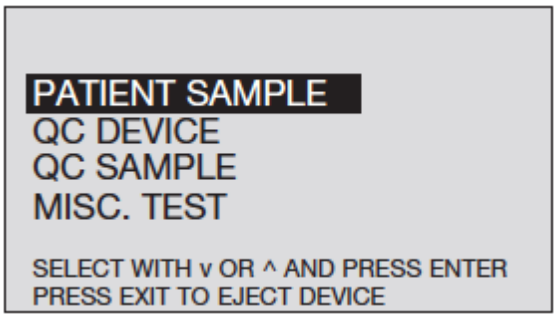
Preparing the meter

• Power the meter on by pressing the 'Power'  **key. Press the 'Enter'**  **key to run self-test.**
• Select 'Run Test' using the up/down arrow keys and press the 'Enter' key to confirm • Type in your identification number (User ID) if prompted.

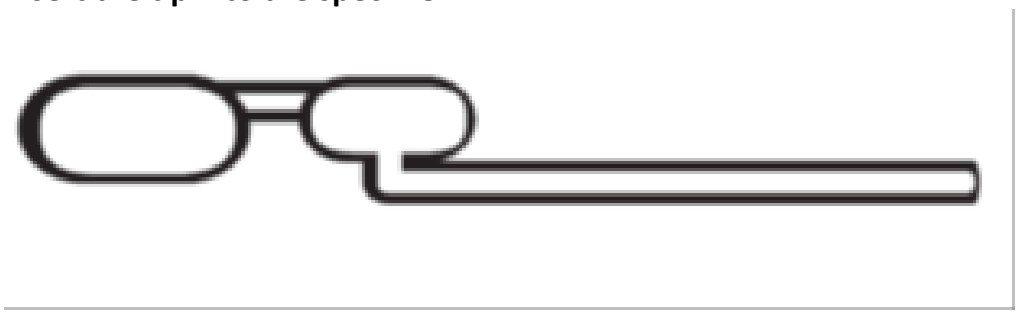
• Select ‘Patient Sample’ using the up/down arrow keys and press the ‘Enter’ key to confirm selection
• Type or (scan) the patient’s identification (Patient ID) then press the ‘Enter’ key to confirm.

NOTE: To correct the patient ID, press the ‘Delete’ key to clear the entire ID or select the incorrect character using the keys. Then type in the correct ID.



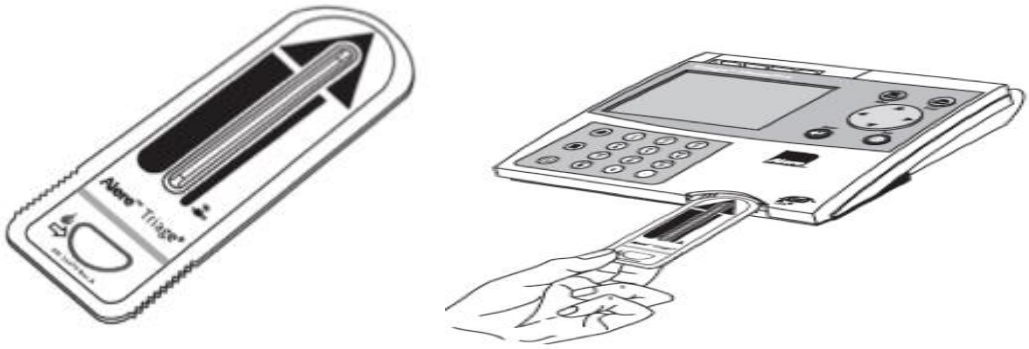


Applying the blood sample
• Open the pouch and label the Test Device with the patient identification number.
• Place the Test Device on a level, horizontal surface.
• Using the transfer pipette, squeeze the larger (top) bulb completely and insert the tip into the specimen.



Transfer pipette

• Release the bulb slowly. The transfer pipette barrel should fill completely with some fluid flowing into the smaller (lower) bulb.
• Place the tip of the transfer pipette into the sample port of the Test Device and squeeze the larger bulb completely. The entire volume of fluid in the transfer pipette barrel must flow into the sample port. The specimen in the smaller (lower) bulb will not be expelled.
• Remove the transfer pipette tip from the sample port and then release the larger (top) bulb.
• Discard the transfer pipette.
• Ensure the specimen has been absorbed completely before moving the Test Device.
NOTE: The Test Device must be inserted into the Meter within 30 minutes from the time the patient specimen was added
Inserting the Test Device
• Hold the Test Device by the edges and gently insert the Test Device into the meter until you feel the Test Device catch on the pin and hear a 'click' and press 'Enter'.
NOTE: After the test device has been inserted, do not push the device in further or attempt to pull it out. The device may be ejected by returning to the Main Menu and pressing 'Exit'.
• After the test device has been pulled into the meter, the meter will display a tick mark indicates tests which are selected and the absence of a tick mark indicates which tests have not been selected.
• The user will be prompted to select desired analyte(s) or deselect undesired analyte(s) by pressing the number to the left of the test to toggle between selected or deselected and press the 'Enter' key to confirm selection
• Press the 'Enter' key to start the test.
NOTE: If no key is pressed on the Select Tests screen, the meter waits 30 seconds then proceeds using only the selected default tests. Then, if no key is pressed on the Confirmation Screen, the test proceeds after waiting 30 seconds.



Results

NOTE: Do not move the meter while a test is in progress.

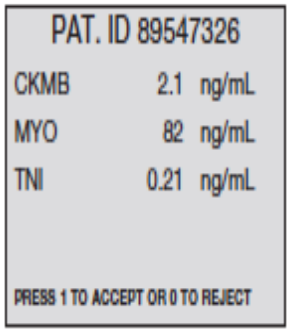
• The results are displayed on the screen, and save to the meter's memory automatically.

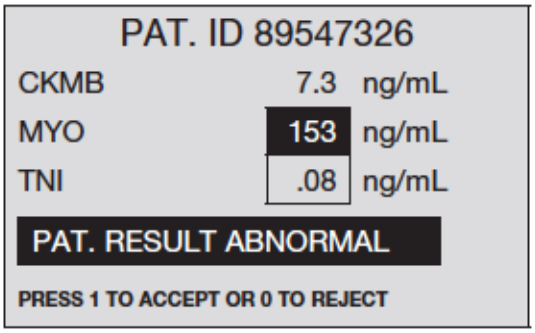
• Results displayed below the cut off levels will have no box around the result.

Results in the indeterminate range have a black border around the result. Abnormal results are in a filled black box.

• Results can be printed by pressing the 'Print' button.

NOTE: A panel with multiple analytes will still be able to report patient results on those analytes which passed QC. An analyte that failed QC will not be reported on patient tests (instead will be flagged with a # symbol).





RESULTS

Expected values

The measuring range is 100 – 5,000 ng/mL. Values above or below this range

will are displayed as >5,000 ng/mL or <100 ng/mL, respectively.

Interpretation of results

D-Dimer Concentration	Remarks
<500 ng/mL	Pulmonary embolism/ DVT unlikely. If clinical suspicion remains, follows standard investigation procedures.
> 500 ng/mL	Pulmonary embolism/ DVT cannot be ruled out. Follow standard investigation procedures for the investigation of pulmonary embolism/ DVT.

SECTION 2: MICOBIOLOGY

A- STAINING TECHNIQUES

GIEMSA STAIN

Principle: Giemsa stain used to differentiate nuclear and/or cytoplasmic morphology of RBC's, WBC's, parasites and also demonstration of some microorganisms including inclusion bodies of viruses/Chlamydia spp and presence of bacteria (coccal/ bacillary forms). The pH of the staining solution is critical and ideally should be adjusted. More pH level gives more selective chromatin staining and less cytoplasmic basophilia; less acidic pH levels give denser nuclei and increased cytoplasmic basophilia.

Reagents Required

1. Giemsa stock solution:

Giemsa powder……..1 gm

Methanol…….65 mL (51.0 gm)

Glycerin…….40 mL (51.0 gm)

**** Measure volume of Methanol and weigh the volume. Weigh an equal volume of glycerin. Use a measured volume of methanol to rinse the glycerin. Combine glycerin and methanol by shaking it slightly. Mix it well with help of glass beads or magnetic stirrer. Add giemsas powder, tighten the cap and shake well about once an hour for 3 days. When ready, filter through coarse filter***

paper for using as working solution

2. Buffer solution:

Disodium monohydrogen phosphate anhydrous …….. 6.0 gm

Potassium dihydrogen phosphate ..... 5.0 gm

**** Mix the buffer salts and weigh 1 gm unit and place in a well stoppered vial. One unit is dissolved in 1 liter of distilled water to give the buffer approximately pH.7.0***

3. Giemsa working solution:

Giemsa stock solution .....3 mL

Buffer .....97 mL

**** Never introduce pipette directly into stock solution. To avoid contamination, pour stock solution into another container and pipette required amount and discard the unused solution.***

4. Glacial acetic acid-water solution

Glacial acetic acid .....1 mL

Distilled water .....499 mL

Staining Procedure

a. Deparaffinize the slides. If necessary, treat the slides with alcoholic iodine to remove mercuric pigments.

b. Place the slides in buffer solution ..... 30 minutes

c. Working giemsa solution……..overnight

d. Quickly rinse in buffer solution

e. Glacial acetic acid water solution……1 minute

f. Absolute alcohol- 2 changes .....15 seconds each

g. Xylene 2 changes .....2 minutes each

h. Dry and mount in DPX.

Results

Malarial parasite ..... Blue

Malarial pigments ..... Black

Nuclei of tissues .... ……..Blue

SPECIAL STAINS: GRAM STAIN FOR BACTERIA

Principle: Crystal Violet is applied first and then followed by an iodine mordant forming a dye lake. At this point both Gram positive and Gram-negative organisms are stained Both types of bacteria have a cell wall composed of peptidoglycan, and the wall of Gram-positive bacteria are thicker than Gram negative organisms, and the Gram-negative bacteria also contain a layer or lipopolysaccharide external to the cell wall. The large crystal violet iodine molecular complex cannot be easily washed out of the intact peptidoglycan layer of the Gram-positive cells; however, it is easily removed from Gram negative bacteria because the acetone disrupts the outer lipopolysaccharide layer and the remaining thin peptidoglycan cell wall cannot retain the complex, Undamaged Gram positive cell walls will retain the crystal violet iodine complex. Alter decolorization, a counter stain is applied to color the Gram-negative bacteria.

REQUIREMENTS AND PREPARATION OF REAGENTS

A- Primary Stain : Crystal violet

Solution A :

- **Crystal violet = 2 gm**
- **Ethyl alcohol= 20 ml**

Solution B :

- **Ammonium oxalate = 0.8 gm**
- **Distilled water = 80 ml**

Mix solution A and B. Keep for 24 hours and filter. Store in an amber colored bottle.

B- Mordant : Gram's Iodine

- **Iodine = 1 gm**
- **Potassium iodide = 2 gm**

- **Distilled water = to 100 ml**

Mix and Store in an amber colored bottle.

C- Decolorizer : 95% Ethanol or 1:1 acetone with ethanol

- **Acetone = 50 ml**
- **Ethanol (95%) = 50ml**

D- Counterstain: safranin

- **Safranin O = 0.34 gm**
- **Absolute alcohol = 10ml**
- **Distilled water = 90ml**

Mix, filter and store in ambered colored bottle.

PROCEDURE OF GRAM STAINING

Smear preparation:

1. **Take a grease free dry slide.**
2. **Sterilize the inoculating loop on a flame of a Bunsen burner.**
3. **Transfer a loopful of culture (or the specimen) by sterile loop and make a smear at the center. Smear should not be very thin or very thick.**
4. **Allow the smear to dry in the air.**
5. **Fix the dry smear by passing the slide 3-4 times through the flame quickly with the smear side facing up.**

Gram Staining:

1. **Place the slides on the staining rods.**
2. **Cover the smear with crystal violet stain and leave for 1 minute.**
3. **Wash carefully under running tap water.**

4. **Flood the smear with Gram's iodine solution and leave for 1 minute.**
5. **Drain off the iodine Wash the slide for the again in a gentle stream of tap water.**
6. **Flood the slide with the decolorizing agent then wait for 20-30 seconds. This can also be done by adding a drop by drop to the slide until the decolorizing agent running from the slides runs clear.**
7. **Gently wash the slide under running tap water and drain completely.**
8. **Counterstain with safranin for and and wait for about 30 seconds to 1 minute.**
9. **Wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper.**
10. **Observe under microscope.**

INTERPRETATION OF GRAM STAINING
The staining results of gram stain are as follows:

- **Gram Positive: Dark purple**
- **Gram Negative: Pale to dark red**
- **Yeasts: Dark purple**
- **Epithelial cells: Pale red**

Examples of Gram-Positive Organisms

Bacillus, Nocardia, Clostridium, Propionibacterium, Actinomyces, Enterococcus, Cornyebacterium, Listria, Lactobacillus, Gardnerella, Mycoplasma, Staphylococcus, Streptomyces, Streptococcus etc

Examples of Gram-Negative Organisms

Escherichia, Helicobcater, Hemophilus, Neisseria, Klebsiella, Enterobacter, Chlamydia, Vibrio, Pseudomonas, Salmonella, Shigella

SPECIAL STAINS: AURAMINE-RHODAMINE STAINING FOR ACID FAST BACILLI

Smear microscopy is the simplest and quickest currently available procedure to detect Acid Fast Bacilli (AFB) in clinical specimens. Most common staining technique involve classic Ziehl-Neelsen (ZN) staining method or one of its variants. Now a days, these methods have been supplanted by more sensitive Auramine-Rhodamine Fluorescence staining technique, also called Truant method for acid-fast staining.

PRINCIPLE OF AURAMINE-RHODAMINE STAIN FOR AFB

Auramine and Rhodamine are nonspecific fluorochrome dyes that have an affinity for acid fast organisms. In case of Mycobacteria, the dyes can bind specifically to the mycolic acid contained in the cell wall allowing the penetration of the stain. This complex resists decolorization by acid-alcohol decolorizer solution. The counterstain, potassium permanganate helps to prevent nonspecific fluorescence, thus reducing the possibility of artifacts. When observed under the microscope with UV illumination, acid fast cells are yellow or bright orange against dark background.

REAGENTS REQUIRED

- **Primary stain (Auramine Rhodamine Stain):**
  Dissolve 1.5 g of Auramine O and 0.75 g Rhodamine B in a solution of 75 ml glycerol (glycerine), 10 ml heated phenol crystals, and 50 ml of distilled water. This solution is usually cleared by filtering through glass wool.
- **Decolorizer (Acid alcohol):**
  Carefully add 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% ethanol.
- **Counter stain (Potassium Permanganate):**
  Dissolve 0.5 g of potassium permanganate (KMn04) in 100 ml of distilled water.
-

PROCEDURE OF AURAMINE-RHODAMINE STAIN FOR AFB

1. **Make a thin smear of the material for study and heat fix by passing the slide through the flame of a Bunsen burner or use a slide warmer at 65-75 C. Do not overheat.**
2. **Flood smears with Auramine O-Rhodamine B solution and allow to stain for 15 minutes, making certain that the staining solution remains on the smear. Do not apply heat to smear.**
3. **Rinse smears with chlorine free water until no color appears in the effluent. Chlorine may interfere with fluorescence; therefore, rinse with distilled or deionized water.**
4. **Flood the smear with the decolorizing agent for 2 to 3 minutes, and then wash with distilled water.**
5. **Flood smears with acid alcohol and allow to de-stain for 2 minutes.**
6. **Rinse the slide thoroughly with distilled water and shake off excess fluid.**
7. **Flood smears with potassium permanganate and counter stain for 2 minutes. Time is critical with potassium permanganate because counter staining for a longer time may quench fluorescence of acid-fast bacilli.**
8. **Rinse thoroughly with distilled water and allow to air dry. Do not blot.**

Examine microscopically using a fluorescent microscope as soon as possible. Use a 20x or 40x objective for screening, and a 100x oil immersion objective to observe the morphology of fluorescing organisms.

If desired, the slide can be directly restained using one of the other acid-fast stains (Ziehl-Neelsen or Kinyoun Stain).

INTERPRETATION OF AURAMINE-RHODAMINE STAIN

- **Positive Test: Acid-fast organisms fluoresce yellow or bright orange against a dark background. (color may vary with the filter system used)**
- **Negative Test: Non-acid-fast organisms will not fluoresce or may appear a pale yellow, quite distinct from the bright acid-fast organisms.**

REPORTING OF AURAMINE-RHODAMINE STAINED SMEAR

The minimum number of fields to examine before reporting a smear as negative for acid-fast organisms at specific magnification are as follows:

Magnification	Number of Fields
200x	30
250x	30
400x	55
450x	70

Reporting of smears:

- **If no fluorescent rods are seen, report the smear as AFB NOT SEEN.**
- **If Fluorescent AFB are seen, report the smear as AFB positive, and give an indication of the number of bacilli present in plus signs (1+ to 4+) as follows:**

Number of AFB seen (450X Magnification)	Number of AFB seen (250X Magnification)	Reported As
0 AFB per 70 Field	0AFB per 30 Fields	AFB Not Seen
1-2 AFB per 70 Fields	1-2 AFB per 30 Fields	Doubtful; repeat with another specimen
2-18 AFB per 50 Fields	1-9 AFB per 10 Fields	1+
4-36 AFB per 10 Fields	1-9 AFB per Field	2+
4-36 AFB per Field	10-90 AFB per Field	3+
>36 AFB per Field	>90 AFB per Field	4+

LIMITATIONS OF AURAMINE-RHODAMINE STAIN

1. **A positive staining reaction provides presumptive evidence of the presence of mycobacteria. A negative staining reaction does not indicate that the specimen will be culturally negative. Therefore, cultural methods must be employed.**
2. **Most strains of rapid growers may not appear fluorescent.**

3. **It is recommended that all negative fluorescent smears be confirmed with Ziehl-Neelsen stain; at least 100 fields should be examined before being reported as negative.**
4. **Reagents like Auramine-Rhodamine are possible carcinogen, Acid – alcohol and Potassium permanganate is also strong irritant to skin, eyes and respiratory system.**
5. **Caution is required while handling and staining using such reagents.**
6. **Excessive exposure to the counterstain may result in a loss of brilliance of the fluorescing organism.**
7. **Turbidity may develop in the stain, but it will not interfere with the effectiveness of the stain. Shake the bottle before using.**
8. **Stained smears should be observed within 24 hours of staining because of the possibility of the fluorescence fading.**

SPECIAL STAINS: ZIEHL-NEELSEN STAIN

The Ziehl-Neelsen stain (ZN stain), also called the hot method of AFB staining, is a type of differential bacteriological stain used to identify acid-fast organisms, mainly *Mycobacteria*. Acid fast organisms are those which are capable of retaining the primary stain when treated with an acid (*fast=holding capacity*). Members of the Actinomycetes, genus *Nocardia* (N. *brasiliensis* and N. *asteroides* are opportunistic pathogens) are partially acid-fast. Oocysts of coccidian parasites, such as *Cryptosporidium* and *Isospora*, are also acid-fast.

PRINCIPLE OF ZIEHL-NEELSEN STAIN

Organisms such as Mycobacteria are extremely difficult to stain by ordinary methods like Gram Stain because of the high lipid content of the cell wall. The phenolic compound carbol fuchsin is used as the primary stain because it is lipid soluble and penetrates the waxy cell wall. Staining by carbol fuchsin is further enhanced by steam heating the preparation to melt the wax and allow the stain to move into the cell. Acid is used to decolorize nonacid-fast cells; acid-fast cells resist this decolorization. The ability of the bacteria to resist decolorization with

acid confers acid -fastness to the bacterium. Following decolorization, the smear is counterstained with malachite green or methylene blue which stains the background material, providing a contrast colour against which the red AFB can be seen.
Acid alcohol can also be used as decolorizing solution, resistant organisms are referred to as Acid Fast Bacilli (AFB) or Acid Alcohol Fast Bacilli (AAFB).

REQUIREMENTS

- **Primary stain: 0.3% Carbol Fuchsin – Dissolve 50g phenol in 100ml ethanol (90%) or methanol (95%). Dissolve 3g basic fuchsin in the mixture and add distilled water to bring the volume to 1 L.**
- **Decolorization solution: 25% Sulphuric acid**
- **Counter stain: 0.3% methylene blue or malachite green.**

PROCEDURE OF ZIEHL-NEELSEN STAIN

1. **Make a thin smear of the material for study and heat fix by passing the slide 3-4 times through the flame of a Bunsen burner or use a slide warmer at 65-75 C. Do not overheat.**
2. **Place the slide on staining rack and pour carbol fuschin over smear and heat gently underside of the slide by passing a flame under the rack until fumes appear (without boiling!). Do not overheat and allow it to stand for 5 minutes.**
3. **Rinse smears with water until no color appears in the effluent.**
4. **Pour 20% sulphuric acid, wait for one minute and keep on repeating this step until the slide appears light pink in color (15-20 sec).**
5. **Wash well with clean water.**
6. **Cover the smear with methylene blue or malachite green stain for 1–2 minutes.**
7. **Wash off the stain with clean water.**

8. **Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).**
9. **Examine the smear microscopically, using the 100x oil immersion objective.**

RESULTS AND INTERPRETATION

- **Acid Fast Bacilli: Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded**
- **Cells: Green (malachite green) or Blue (methylene blue)**
- **Background material: Green (malachite green) or Blue (methylene blue)**

List of Acid-Fast organisms

- ***Mycobacterium* spp: Acid Fast**
- **Cyst of *Cryptosporidium*: Acid Fast**
- **Cyst of *Isospora*: Acid Fast**
- ***Nocardia* spp: Partial Acid Fast**
- ***Rhodococcus* spp: Partial Acid Fast**
- ***Legionella micdadei*: Partially acid fast in tissue**

REPORTING THE SPUTUM SMEAR

- **When any definite red bacilli are seen:**
  Report the smear as 'AFB POSITIVE', and give an indication of the number of bacteria present as follows:

Number of AFB seen (1000X Magnification)	Reported As
0 AFB per 300 Field	AFB Not Seen
1-2 AFB per 300 Fields	Doubtful; repeat with another specimen
1-9 AFB per 100 Fields	1+
1-9 AFB per 10 Fields	2+
1-9 AFB per Field	3+
>9 AFB per Field	4+

- **When very few AFB are seen:**
  E.g. when only one or two AFB are seen, request a further specimen to examine. Tap water sometimes contain AFB that resemble tubercle bacilli, and occasionally stained scratches on a slide can be mistaken for AFB. Occasionally AFB can be transferred from one smear to another when the same piece of blotting paper is used to dry several smears.

- **When no AFB are seen after examining 300 fields:**
  Report the smear as 'AFB NOT SEEN'. Do not report 'Negative' because organisms may be present but not seen in those fields examined. Up to three specimens (one collected as an early morning specimen) may need to be examined to detect M. tuberculosis in sputum.

-

MODIFICATIONS OF ZIEHL-NEELSEN STAIN

1. **Use of alcohol as secondary decolorizer:**
   After primary decolorization with sulfuric acid, the smear may be treated with 95% alcohol as secondary decolorizer. M. tuberculosis is both acid fast and alcohol fast, while saprophytic mycobacteria are only acid fast.

2. **Use of acid-alcohol as decolorizer:**
   Instead of using 20% sulfuric acid, 3% HCl in 95% alcohol may be used. This also differentiates tubercle bacilli from saprophytic mycobacteria. It is especially used in diagnosis of renal tuberculosis.

3. **Modifications in the percentage of sulfuric acid:**
   - **<5% H_2SO_4 for M. leprae.**
   - **1% H_2SO_4 for Actinomyces in tissue.**
   - **0.5% H_2SO_4 for cultures of Nocardia.**

0.25-0.5% H_2SO_4 for spores and the oocysts of Cryptosporidium and Isospora.

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

The Wade-Fite staining technique is a less common special staining useful in Histopathology. It is the modification of Ziehl-Neelsen Staining Method to demonstrate *Mycobacterium leprae*in tissue sections, which is much less acid and alcohol fast than the tubercle bacilli.

PRINCIPLE OF WADE-FITE STAIN

***Mycobacterium leprae* in comparison to *Mycobacterium tuberculosis* are much less acid and alcohol fast. The leprosy bacilli's lipid envelope is much more affected by the fat solvents (xylene) traditionally used to dewax sections. Hence, they get frequently decolorized by the standard Ziehl-Neelsen technique. Due to these factors, a modification on the standard Ziehl-Neelsen staining technique, Wade-Fite staining technique is used for the demonstration of leprosy bacilli.**

The Wade-Fite technique combines the peanut oil with the deparafinizing solvent (xylene). This minimizes the exposure of the bacterial cell to organic solvents, thus protects the precarious acid-fastness of the organism.

REQUIREMENTS FOR WADE-FITE STAIN

- **Carbol fuchsin staining solution:**
  Basic fuchsin = 1 gm
  Absolute alcohol = 10 ml
  5% aqueous phenol = 100 ml
  (Dissolve basic fuchsin in absolute alcohol then add 5% aq. phenol, mix and filter before use.)

- **3% HCl in ethanol:**
  conc. HCl = 3 ml
  Distilled water = 2 ml
  Ethanol = 90 ml

- **Acidified Methylene Blue:**
  Methylene Blue = 0.25 gm
  acetic acid = 1 ml
  Distilled water = 99 ml

- **Dewax solution:**
  Xylene: Peanut oil = 2: 1

PROCEDURE OF WADE-FITE STAIN

1. **Slightly warm the sections and deparaffinize in a mixture of peanut oil and xylene (1:2), two changes, 6 minutes each.**
2. **Blot dry and wash in running tap water for 3 minutes. Repeat if any xyline-oil remains on the section.**
3. **Stain with carbol fuchsin at room temperature for 20-25 minutes.**
4. **Wash in running tap water for 3 minutes.**
5. **Differentiate with 3% HCl in ethanol for 2 minutes. The sections should be pale pink on rinsing in tap water.**

6. **Wash in tap water for 5 minutes.**
7. **Counterstain in acidified methylene blue for 20-30 seconds.**
8. **Wash in running tap water, do not dehydrate in alcohol.**
9. **Blot sections dry and then place in 56C oven for 5 minutes.**
10. **Once dry, one quick dip in xylene to clear.**
11. **Mount with permanent mounting media.**

RESULTS AND INTERRETATION

- ***Mycobacterium leprae*: Bright Red**
- **Nuclei and Background: Blue**

SPECIAL STAINS: WARTHIN STARRY STAINING TECHNIQUE FOR SPIROCHETES

Warthin-stary is a silver nitrate-based stain used in histopathology for the visualization of spirochetes. It is also used for *Helicobacter pylori*, Microsporedia etc.

Principle: This technique involves the argyrophilic reaction. The spirochetes are argyrophilic, which means they will absorb silver from a silver solution, but need a separate reducing agent in a solution to reduce the adsorbed silver to visible metallic silver. The stain demonstrates black spirochetes against a yellow to pale brown background.

Requirements

1. **Acetate buffer, pH 3.6**

sodium acetate = 4.1 gm
acetic acid = 6.25 gm
distilled water = 500 gm

2. **1% silver nitrate solution in pH 3.6 acetate buffer**

3. **Developer solution**

2% silver nitrate = 15 ml
5% gelatin solution = 3.75 ml
15% hydroquinone = 12 ml

Procedure

1. **Deparaffinize and bring sections to distilled water.**
2. **Place slides in 1% silver nitrate solution for 45 seconds in microwave. Let stand for 1 minute at room temperature.**
   (Note: alternatively, in a laboratory microwave, heat at 80% power, 60ºC for 5 minutes, no standing time required).
3. **Prepare and preheat developer solution in water bath.**
4. **Remove slides from silver solution. Do not rinse. Place slides horizontally on a slide rack and cover with developer. Allow sections to develop until they are light yellow to golden brown, approximately 1 minute or less.**
5. **Wash quickly and thoroughly on hot tap water.**
6. **Rinse in distilled water.**
7. **Dehydrate, clear and mount.**

Results and Interpretation

Spirochetes : black
Helicobacter pylori : black
Klebsiella : brown/black
Nuclei : brown
Background : golden yellow

SPECIAL STAINS: GROCOTTS METHENAMINE SILVER NITRATE METHOD FOR FUNGI

Principle: Polysaccharides in the fungal wall are oxidized to aldehydes by the chromic acid Chromic acid is a strong oxidant, further oxidizing many of the newly released aldehyde groups to breakdown products that will not react. Only substances that possess large quantities of polysaccharides such as the fungal walls, glycogen and mucins will remain reactive with the methenamine silver, reducing it to visible metallic silver, Methenamine gives the solution the alkaline properties necessary for proper reaction and the sodium borate acts as the buffer. Gold chloride is a toning solution and the thiosulfate removes any unreduced silver.

Reagents Required

1. 5% chromic acid

Chromium trioxide……5 gm

Distilled water……..100 ml

2. 5% silver nitrate solution

Silver nitrate……..5 gm

Distilled water……100 ml

3. 3% methenamine solution

Hexamethylene tetramine……3 gm

Distilled water……...100 ml

4. Methenamine-Silver nitrate stock solution

5% silver nitrate……...5 mL

3% methenamine.....100 ml

5. 5% borax solution

Sodium borate……5 gm

Distilled water……100 ml

6. Methenamine-Silver nitrate working solution

Methenamine Silver nitrate stock.....25 ml

Distilled water.....25 ml

5% borax.....2 mL

**** Prepare fresh prior to use, do not use if cloudy. Prewarm the solutions separately in the oven at least 40 minutes before use***

7. 1% Sodium bisulfite:

1 gm in 100 ml distilled water

8. 0.1% Gold chloride solution: 10 ml of 1% gold chloride stock solution and 90 ml of distilled water

9. 2% Sodium thiosulfate (Hypo)

Sodium thiosulfate……..2 gm

Distilled water……100 mL

10. 0.1% light green solution

0.1 gm light green in 0.1% acetic acid

Staining Procedure

a. Oxidize in freshly prepared 5% chromic acid for 1 hour, discard the solution after use

b. Rinse in tap water

c. 1% sodium bisulfite for 1 minute, discard the solution

d. Wash in tap water for 10 minutes

e. Rinse 4 times in distilled water

f. Place in working methenamine silver solution in oven at 58° to 60° for 45 minutes to 60 minutes or until sections turn a yellow-brown. Using paraffin coated or Teflon forceps, remove a control slide, rinse in the warmed distilled water and check microscopically for adequate silver impregnation. Fungi should be a dark brown. Rinse in distilled water and return to the silver solution if reaction is too pale.

g. Rinse in 6 changes of distilled water

h. Tone in 0.1% gold chloride for 2-5 minutes. Solution may be refiltered and reused for about 100 slides

i. Rinse in distilled water

j. Treat with 2% sodium thiosulfate for 2–5 minutes, discard solution

k. Wash thoroughly in tap water for 5 minutes

l. Counterstain in light green solution for 30-45 seconds

m. Dry and mount in resinous medium.

Result

Fungi……sharply delineated in black

Pneumocystis carinii ..... black

Mucin .....taupe to dark grey

Inner parts of mycelia and hyphae ..... old rose

Back ground .....pale green

SPECIAL STAINS: LACTOPHENOL COTTON BLUE FOR FUNGAL STAINING

The Lactophenol Cotton Blue (LPCB) wet mount is most widely used method for the staining and observation of fungi.

Principle: LPCB is a stain used for making semi-permanent microscopic preparation of fungi. The LPCB stain has following three components:
Phenol: Kills any live organism.
Lactic acid: Preserves fungal structures.
Cotton blue: Stains the chitin and cellulose of the fungal cell wall intensely blue.

Procedure

1. **Place a drop of 70% ethanol on a clean microscopic glass slide**
2. **Immerse the specimen in the drop of alcohol**
3. **Add one or at most two drops of the LPCB before the alcohol dries out**
4. **Holding the coverslip between the index finger and thumb, touch one edge of the drop of mountant with a coverslip edge and lower gently avoiding air bubbles**
5. **This preparation is now ready for examination**
6. **Make the initial examination using low power objective. Switch to higher power (40X) objective for more detailed examination of spores and other structures**

SECTION 2: MICROBIOLOGY

B- CULTURE AND DETACTION

CULTURING BACTERIAL PATHOGENS

The purpose of using cultural techniques in microbiology is to demonstrate the presence of organisms which may be causing disease, and when indicated, to test the susceptibility of pathogens to antimicrobial agents.

DIFFERENT TYPES OF CULTURE MEDIA

For a culture medium to be successful in growing the pathogen sought it must provide all essential nutrients, ions, and moisture, maintain the correct pH and osmotic pressure, and neutralize any toxic materials produced. It is also essential to incubate the inoculated medium in the correct atmosphere, at the optimum temperature and for an adequate period.

The main types of culture media are:

Basic media: These are simple media such as nutrient agar and nutrient broth that will support the growth of microorganisms that do not have special nutritional requirements. They are often used in the preparation of enriched media, to maintain stock cultures of control strains of bacteria, and for subculturing pathogens from differential or selective media prior to performing biochemical and serological identification tests.

Enriched media: Enriched media are required for the growth of organisms with exacting growth requirements such as *H. influenzae*, *Neisseria* species, and some *Streptococcus* species. Basic media may be enriched with whole or lyzed blood, serum, peptones, yeast extract, vitamins and other growth factors. An enriched medium increases the numbers of a pathogen by containing all the necessary ingredients to promote its growth. Such a medium is often used for specimens collected from sites which are normally sterile to ensure the rapid multiplication of a pathogen which may be present only in small numbers.

Selective media: These are solid media which contain substances (e.g. bile salts or other chemicals, dyes, antibiotics) which inhibit the growth of one organism

to allow the growth of another to be more clearly demonstrated. A selective medium is used when culturing a specimen from a site having a normal microbial flora to prevent unwanted contaminants overgrowing a pathogen. Media made selective by incorporating antibiotics are usually expensive.

Other ways to select organisms: **Incubation conditions may be used to select organisms, e.g. *P. aeruginosa* is inhibited by anaerobic conditions. Also, the pH of a medium may make it selective for a particular organism, e.g. *V. cholerae* can be isolated on an alkaline medium such as TCBS agar. Temperature may also help to select an organism e.g. *Listeria monocytogenes* can grow at 4 °C whereas other organisms are inhibited. Growth, however, is slow.**

Indicator (differential) media: These are media to which dyes or other substances are added to differentiate microorganisms. Many differential media distinguish between bacteria by incorporating an indicator which changes colour when acid is produced following fermentation of a specific carbohydrate e.g. MacConkey agar.

***Note*: Many media used to isolate pathogens are both selective and enrichment or both selective and differential.**

Transport media: These are mostly semisolid media that contain ingredients to prevent the overgrowth of commensals and ensure the survival of aerobic and anaerobic pathogens when specimens cannot be cultured immediately after collection. Their use is particularly important when transporting microbiological specimens from health centres to the district microbiology laboratory or specimens to the Regional Public Health Laboratory. Examples of transport media include Cary-Blair medium for preserving enteric pathogens and Amies transport medium for ensuring the viability of gonococci.

Identification media: These include media to which substrates or chemicals are added to help identify bacteria isolated on primary cultures. Examples include peptone water sugars, urea broth, and Kligler iron agar. Organisms are mainly identified by a change in the colour of the medium and or the production of gas. Organisms used to inoculate identification media must be first isolated in pure culture.

Quality control of commonly used culture media

Culture medium	*Recommended Control Species*
Alkaline peptone water	**Enriched: *Vibrio* species** **Inhibited controls: *Escherichia coli*, *Proteus* species**
Blood agar	***Streptococcus pyogenes, Streptococcus pneumoniae*** ***Haemophilus influenzae***
Chocolate agar	***Haemophilus influenzae***
Cooked meat medium	***Clostridium sporogenes***
Cystine lactose electrolyte deficient agar (CLED)	***Staphylococcus aureus*** ***Proteus mirabilis***
Kligler iron agar (KIA) or Triple sugar iron agar	***Citrobacter freundii*** ***Proteus vulgaris*** ***Alcaligenes faecalis***

MacConkey agar	***Escherichia coli***
	Proteus mirabilis
Modified New York City (MNYC) medium or other selective medium to isolate *N. gonorrhoeae*	**Selected: *Neisseria gonorrhoeae***
	Inhibited control: *Proteus vulgaris*
Thioglycollate broth	***Clostridium* species**
	***Bacteroides* species**
Thiosulphate citrate bile salt sucrose (TCBS) agar	**Selected: *Vibrio cholerae***
	Inhibited control: *Escherichia coli*
Columbia agar diphasic medium	***Streptococcus pyogenes, Staphylococcus aureus***
	Haemophilus influenzae
Xylose lysine deoxycholate (XLD) agar	***Salmonella* Typhimurium**
	Escherichia coli

HOW TO DISPENSE CULTURE MEDIA

Media should be dispensed in a clean draught-free room. Most fluid media are dispensed into screw capped bottles or tubes, and then sterilized by autoclaving. Sterile media must be dispensed into sterile petri dishes, tubes or bottles using an aseptic technique.

Dispensing sterile media into petri dishes

1- Lay out the sterile petri dishes on a level surface.

2- Mix the medium gently by rotating the flask or bottle. Avoid forming air bubbles. Flame sterilize the neck of the flask or bottle and pour 15–20 ml of medium into each dish (90–100 mm diameter). the surface of the medium before gelling occurs. Rotate the dish on the surface of the bench to ensure an even layer of agar.

3- When the medium has gelled and cooled, stack the plates and seal them in plastic bags to prevent loss of moisture and reduce the risk of contamination. Do not leave the plates exposed to bright light especially sunlight.

4- Store at 2–8° C.

***Note*: Agar plates should be of an even depth (not less than 4 mm) and of a firm gel. The surface of the medium should be smooth and free from bubbles.**

HOW TO INOCULATE CULTURE MEDIA

Immediately before inoculating a culture medium, check the medium for visual contamination or any change in its appearance which may indicate deterioration of the medium, e.g. darkening in colour. When inoculating, or seeding, culture media an aseptic (sterile) technique must be used. This will:

– prevent contamination of cultures and specimens,

– prevent infection of the laboratory worker and the environment.

Aseptic techniques

● **Flame sterilize wire loops, straight wires, and metal forceps before and after use. Whenever possible, use a Bunsen burner with a protective tube, e.g.** ***Bactiburner*** **to avoid particles being dispersed when flame sterilizing wire loops.**

Note**: To prevent the release of aerosols, wire loops must be well made (see later text). Aerosols can also be released when spreading inocula on media containing air bubbles.**

● **Flame the necks of specimen bottles, culture bottles, and tubes after removing and before replacing caps, bungs, or plugs.**

● **When inoculating, do not let the tops or caps of bottles and tubes touch an unsterile surface. This can be avoided by holding the top or cap in the hand.**

● **Always use racks to hold tubes and bottles containing specimens or culture media.**

● **Make slide preparations from specimens after inoculating the culture media.**

● **Decontaminate the work bench before starting the day's work and after finishing.**

Note**: Decontamination of infected material is described in Part 1 of the book.**

● **Use a safety cabinet when working with hazardous pathogens.**

● **Wear protective clothing, wash the hands after handling infected material, and never mouth pipette, eat, drink, or smoke in the laboratory.**

Inoculation of media in petri dishes

The technique used to inoculate media in petri dishes (plates) must provide single colonies for identification. It must also show whether a culture is pure or mixed, i.e. consisting of a single type of organism or several different organisms.

A pathogen must be isolated in pure culture before it can be identified and tested for antimicrobial sensitivity.

The inoculation of media in petri dishes is referred to as 'plating out' or 'looping out'. It is not necessary to use whole plates of media for every specimen. Considerable savings can be made by using a half or even a third of a plate (especially when the medium is a selective one). The area of medium used must be sufficient to give separate colonies.

Before inoculating a plate of culture medium, the surface of the medium must be dried, otherwise single colonies will not be formed. To do this, remove the lid of the plate and place this face upwards on an incubator shelf. Invert the base con taining the medium and let it rest at an angle on the lid. Usually 30–40 minutes incubation at 35–37 _C is sufficient time to dry the surface of an agar plate.

Inoculating technique

1- Using a sterile loop or swab of the specimen, apply the inoculum to a small area of the plate (the 'well').

2- Flame sterilize the loop. When cool, or using a second sterile loop, spread the inoculum. This will ensure single colony growth.

***Note*: A simplified technique of inoculating plates can be used by medical and nursing staff when culturing specimens directly from patients, e.g. urogenital specimens for the isolation of *Neisseria gonorrhoeae*. The techniques of inoculating half a plate and a third of a plate of medium.**

Inoculation of slopes

To inoculate slopes such as Dorset egg medium or Loeffler serum, use a sterile straight wire to streak the inoculum down the centre of the slope and then spread the inoculum in a zig-zag pattern.

To inoculate a slope and butt medium, such as Kligler iron agar, use a sterile straight wire to stab into the butt first and then use the same wire to streak the slope in a zig-zag pattern.

Inoculation of stab media (deeps)

Use a sterile straight wire to inoculate a stab medium. Stab through the centre of the medium taking care to withdraw the wire along the line of inoculum without making further stab lines.

Inoculation of fluid media

Broths and other fluid media are inoculated using a sterile wire loop, straight wire, or Pasteur pipette depending on whether the inoculum is a colony, a fluid culture, or a specimen.

When using a wire loop to subculture colonies, hold the bottle or tube at an angle and rub the loop against the side of the container below the level of the fluid.

When using a Pasteur pipette to inoculate a fluid culture hold the pipette.

INCUBATION OF INOCULATED MEDIA

Inoculated media should be incubated as soon as possible. A delay in incubation can affect the viability of pathogens especially anaerobes, pneumococci, meningococci, gonococci, and *Haemophilus influenzae*. It can also increase the risk of plates becoming contaminated from small insects and dust.

Uninoculated and inoculated media must be protected from sunlight. Microorganisms require incubation at the temperature and in the humidity and gaseous atmosphere most suited to their metabolism. The length of time of incubation depends on how long an organism takes to develop the cultural characteristics by which it is recognized.

CULTURING BLOOD FOR BACTERIAL PRESENCE

Blood culture is required when bacteremia (septicemia) is suspected.

Bacteremia: The presence of bacteria in the blood is called bacteremia. It is usually pathological although transitory asymptomatic bacteremia can occur during the course of many infections and following surgical procedures.

Bacteremia occurs in diseases such as typhoid fever, brucellosis, leptospirosis and endocarditis.

Septicemia: This is a clinical term used to describe severe life-threatening bacteremia in which multiplying bacteria release toxins into the blood stream and trigger the production of cytokines, causing fever, chills, toxicity, tissue anoxia, reduced blood pressure, and collapse. Septic shock is usually a complication of septicemia with Gram negative bacilli, and less frequently, Gram positive organisms. Prompt treatment is essential.

Bacteremia usually occurs when pathogens enter the bloodstream from abscesses, infected wounds or burns, or from areas of localized disease as in pneumococcal pneumonia, meningitis, pyelonephritis, osteomyelitis, cholangitis, peritonitis, enterocolitis and puerperal sepsis. There is usually a high white cell count with neutrophilia, left shift of the neutrophils, and often toxic granulation.

Possible pathogens isolated from blood cultures

- **BACTERIA**

Gram positive	*Gram negative*
Staphylococcus aureus	***Salmonella* Typhi**
Viridians streptococci	**Other *Salmonella* serovars**
Streptococcus pneumoniae	***Brucella* species**
Streptococcus pyogenes	***Hemophilus influenzae***
Enterococcus faecalis	***Pseudomonas aeruginosa***
Clostridium perfringens	***Klebsiella* strains**
Anaerobic streptococci	***Escherichia coli***
***Proteus* species**	
Bacteroides fragilis	
Neisseria meningitidis	

Yersinia pestis

Also, *Mycobacterium tuberculosis* (HIV-associated tuberculosis), *Leptospira* species, *Borrelia* species, rickettsia, and *Bartonella bacilliformis*.

- **FUNGI**

***Candida albicans* and other yeasts, e.g. *Cryptococcus neoformans*, and occasionally *Histoplasma capsulatum* and other fungi that cause systemic mycoses.**

The process

1- Collect blood and inoculate culture media

Whenever possible blood should be collected before antimicrobial treatment has started. When the patient has recurring fever, collect the blood as the temperature *begins to rise*. For other patients, collect the blood as soon as possible after receiving the request. To increase the chances of isolating a pathogen, it is usually recommended that at least two specimens (collected at different times) should be cultured. A *strict aseptic technique must be used to collect the blood* (see later text).

Choice of culture media

Media selected for the culture of blood should be capable of providing the fastest growth and isolation of as wide a range of pathogens as possible. The following media are recommended:

● **Columbia agar and Columbia broth diphasic medium with added SPS (sodium polyanethol sulphonate), also known as *Liquid*. SPS prevents the blood from clotting, neutralizes complement and other antibacterial substances in fresh blood, and has some neutralizing effect on polymyxin B, streptomycin, and gentamicin should these be present in the blood. SPS also enables a greater volume of blood to be cultured without increasing the volume of broth, i.e. up to 50% of the total volume of medium.**

Diphasic blood culture medium

A diphasic (Castaneda) medium is one that combines an agar slope with a broth medium. The blood broth is allowed to run over the slope by tipping the bottle at regular intervals. Microbial activity can be seen by growth on the slope (beginning at the broth-agar interface). This avoids the need to subculture from the bottle and therefore reduces the risk of contaminating the culture. When brucellosis is suspected, a diphasic medium is particularly recommended because *Brucella* species are slow-growing and also 'high risk' category 3 pathogens.

Use of Columbia agar and broth

Tryptone soya (tryptic soy) diphasic medium is often recommended for culturing blood but organisms such as *S. pneumoniae* and *N. meningitidis* have been shown not to grow well in this medium. Columbia agar and broth are recommended for the isolation of these pathogens and other fastidious organisms. Brucellae, however, grow well in tryptone soya diphasic medium.

● **Thioglycolate broth medium is recommended to isolate strict anaerobes should an anaerobic infection be suspected. It consists of nutrient broth to which is added thioglycolate to provide the conditions necessary for the growth of anaerobes. Because SPS is inhibitory to anaerobic streptococci, it is not added to this medium, therefore a sufficient volume of broth must be used to prevent the blood from clotting and to dilute out the blood's natural bactericidal substances.**

The blood should be diluted at least 1 in 10 with broth. *Isolation of S.* Typhi: If wishing to culture for *S.* Typhi only, the use of ox-gall medium is recommended.

Commercially produced culture media

A wide range of commercially produced culture media is available for use with manual and automated blood culture systems. Manufacturers of media for non-automated use include Oxoid, Bio-Rad Laboratories, BD Diagnostics and bioMérieux. The media are mostly tryptone soy or brain heart infusion broth with added growth factors. Some media are also available with antibiotic removing resins. A few diphasic media are available, e.g. from Bio-Rad

Laboratories (in triangular bottles), BD Diagnostics and bioMérieux. The semi-automated Oxide *Signal* Blood culture system recognizes microbial growth in the blood culture by gas production.

Aseptic blood collection and dispensing technique

Blood for culture must be collected and dispensed with great care to avoid contaminating the specimen and culture medium.

1- Using a pressure cuff, locate a suitable vein in the arm. Deflate the cuff while disinfecting the venipuncture site.

2- Wearing gloves, thoroughly disinfect the venipuncture site as follows:

– Using 70% ethanol, cleanse an area about 50 mm in diameter. Allow to air-dry.

– Using 2% tincture of iodine and a circular action, swab the area beginning at the point where the needle will enter the vein. Allow the iodine to dry on the skin for at least 1 minute.

3- Lift back the tape or remove the protective cover from the top of the culture bottle(s). Wipe the top of the bottle using an ethanol-ether swab.

Prior inspection of culture media bottles

***Do not* use a bottle of culture medium if it shows signs of contamination, i.e. broth appears turbid. Do not use a bottle of thioglycolate broth if it appears oxidized, i.e. more than a third of the top of the medium appears pink when the indicator in the medium is resazurin, or more than 20 mm down from the surface of the medium appears green-blue when the indicator is methylene blue. When oxidation has occurred, the medium must be reduced by steaming before it is used.**

4- Using a sterile syringe and needle, withdraw about 20 ml of blood from an adult* or about 2 ml from a young child.

***Note*: When an anaerobic culture is not indicated, collect 10–15 ml of blood.**

5- Insert the needle through the rubber liner of the bottle cap and dispense 10–12 ml of blood into the diphasic culture medium bottle containing 25 ml of broth.

Changing needles: Studies have shown that when dispensing the blood into the culture medium, it is not necessary to replace the needle with a sterile needle. Not changing the needle reduces the risk of accidental needle-prick injury.

When also culturing for anaerobes, dispense about 5 ml of blood into the thioglycolate culture medium containing 50 ml of broth.

Dispense the remaining approximately 2 ml of blood into a tube or bottle containing ethylenediamine tetra acetic acid (EDTA).

***EDTA sample*: This is collected to perform a total and differential white cell count, and to examine stained smears of the plasma buffy coat layer for microorganisms, when the blood is from a child or a patient with AIDS.**

6- Using a fresh ethanol-ether swab, wipe the top of each culture bottle and replace the tape or protective cover(s). Without delay, mix the blood with the broth and mix the blood in the EDTA container.

***Important*: The blood must not be allowed to clot in the culture media because any bacteria will become trapped in the clot.**

7- Clearly label each bottle with the name and number of the patient, and the date and time of collection.

8- As soon as possible, incubate the inoculated media. Protect the cultures from direct sunlight until they are incubated.

Diphasic medium

Incubate at 35–37 °C for up to 7 days, examining and sub culturing as described later. A longer incubation period should be allowed when endocarditis is suspected. When brucellosis is suspected, loosen the cap of the culture bottle (or insert a sterile needle in the cap) and incubate in a carbon dioxide enriched atmosphere for up to 4 weeks.

Thioglycolate broth

Incubate at 35–37 °C for up to 2 weeks, examining and sub culturing.

2- Examine the specimen microscopically

Centrifuge a sample of EDTA anticoagulated venous blood or heparinized capillary blood and make smears of the buffy coat layers. Stain as follows:

- ***Gram smear*: To detect Gram positive and Gram-negative bacteria, particularly when the patient is an infant or young child.**
- ***Ziehl-Neelsen smear*: To detect AFB when the patient has AIDS or suspected HIV disease.**
- ***Giemsa or rapid Field's smear*: To detect borrelia, or parasites such as trypanosomes, malaria parasites, and microfilariae.**

***Note*: Microfilariae and trypanosomes are more easily detected by their motility using a microhematocrit concentration technique.**

Allow the smears to air-dry, fix with absolute methanol for 2 minutes and stain by the appropriate staining technique.

3- Examine and report the cultures

Diphasic culture (Columbia agar and broth)

Using a hand lens, examine twice daily (up to 7 days or 4 weeks when brucellosis is suspected) for microbial growth, indicated by colonies growing on the agar slope, usually beginning at the agar-broth interface.

Colonial appearances

Colonies of staphylococci, S. Typhi, brucellae, and most coliforms can usually be seen easily, whereas colonies of *S. pneumoniae*, *Neisseria* species, *S. pyogenes*, and *Y. pestis* are less easily seen. *Pseudomonas* and *Proteus* species produce a film of growth on the agar.

When growth is present:

– Subculture on blood agar, chocolate agar, and MacConkey agar.

– Incubate the blood agar and MacConkey agar plates aerobically and the chocolate agar plate in a carbon dioxide atmosphere (candle jar).

– Examine a Gram stained smear of the colonies. Depending on the bacteria seen, test the colonies further (e.g. for coagulase, catalase, oxidase, urease, and motility).

- ***When large Gram-positive rods resembling C. perfringens are seen*: Subculture also on lactose egg yolk milk agar and incubate the plate anaerobically.**
- ***When motile, urease and oxidase negative Gram-negative rods are isolated*: Subculture the colonies on Kligler iron agar.**
- ***When catalase positive Gram-negative coccobacilli are isolated*: Suspect *Brucella* species and send the culture (securely) to a microbiology specialist laboratory for identification. Mark the culture '*High Risk*'.**

Blind subculture after overnight incubation

Because some organisms such as *Neisseria* species and *S. pneumoniae* may grow without producing easily seen colonies, it is advisable to examine a toluidine blue stained smear and subculture onto agar plates even when no microbial growth is apparent after overnight incubation.

***Important*: Always report immediately a positive blood culture, and send a preliminary report of the stained smear and other useful test**

CULTURE URINE SPECIMEN

It is *not* necessary to culture urine which is microscopically and biochemically normal, except when screening for asymptomatic bacteriuria in pregnancy. Culture is required when the urine contains bacteria (as indicated by the Gram smear), cells, casts, protein, nitrite, or has a markedly alkaline or acid reaction.

Estimating bacterial numbers

It is necessary to estimate the approximate number of bacteria in urine because normal specimens may contain small numbers of contaminating organisms, usually less than 10 000 (10^4) per ml of urine. Urine from a person with an

untreated acute urinary infection usually contains 100 000 (10^5) or more bacteria per ml.

The approximate number of bacteria per ml of urine, can be estimated by using a calibrated loop or a measured piece of filter paper. Both methods are based on accepting that a single colony represents one organism. For example, if an inoculum of 1/500 ml produces 20 colonies, the number of organisms represented in 1/500 ml of urine is 20, or 10 000 in 1 ml (500 X 20).

The calibrated loop method using quarter plates of culture media is recommended because it is inexpensive, simple to perform, and provides individual colonies that are easier to identify and remove for antimicrobial susceptibility testing.

Cystine lactose electrolyte-deficient (CLED) agar

– Mix the urine (freshly collected clean-catch specimen) by rotating the container.

– Using a sterile calibrated wire loop, e.g. one that holds ml (0.002 ml), inoculate a loopful of urine on a quarter plate of CLED agar. If microscopy shows many bacteria, use a half plate of medium.

– Incubate the plate aerobically at 35–37 °C overnight.

Cystine lactose electrolyte-deficient (CLED) agar is widely used by laboratories to isolate urinary pathogens because it gives consistent results and allows the growth of both Gram negative and Gram-positive pathogens. (The indicator in CLED agar is bromothymol blue and therefore lactose fermenting colonies appear yellow). The medium is electrolyte- deficient to prevent the swarming of *Proteus* species.

Examine and report the cultures

CLED agar culture

Look especially for colonies that could be:

- ***Escherichia coli* (perform indole and *beta*-glucuronidase tests for rapid identification**

- ***Proteus* species,**
- ***Pseudomonas aeruginosa*,**
- ***Klebsiella* strains,**
- ***Staphylococcus aureus*,**
- ***Staphylococcus saprophyticus***
- ***Enterococcus faecalis***

Appearance of some urinary pathogens on CLED agar

***E. coli*: Yellow (lactose-fermenting) opaque colonies often with slightly deeper colored center.**

***Klebsiella species*: Large mucoid yellow or yellow-white colonies.**

***Proteus species*: Translucent blue-grey colonies.**

***P. aeruginosa*: Green colonies with rough periphery (characteristic color).**

***E. faecalis*: Small yellow colonies.**

***S. aureus*: Deep yellow colonies of uniform color.**

***S. saprophyticus* and other coagulase negative staphylococci: Yellow to white colonies.**

***Note*: Contaminating organisms usually produce a few colonies of mixed growth. Most urinary infections show growth of a single type of organism although mixed infections can occur especially in chronic infections or following catheterization or gynecological surgery.**

Reporting bacterial numbers

Count the approximate number of colonies. Estimate the number of bacteria, i.e. colony-forming units (CFU) per ml of urine. Report the bacterial count as:

- Less than 10 000 organisms/ml (10^4/ml), not significant.

- 10 000–100 000/ml (10^4–10^5/ml), doubtful significance (suggest repeat specimen)

- More than 100 000/ml (10^5/ml), significant bacteriuria.

Example

If 25 *E. coli* colonies are counted and a 1/500 ml loop was used, the approximate number of CFU per ml of urine: 500 X 25 = 12 500

Such a count would be reported as:

10 000–100 000 *E. coli*/ml

Interpretation of bacterial counts: A bacterial count of 10^5 organisms/ml or more from a fresh 'clean-catch' urine specimen, indicates a urinary infection. A count of 10^4–10^5/ml, could mean infection or contamination. A repeat specimen is indicated. A count of less than 10^4/ml is nearly always due to contamination unless the urine was cultured after antimicrobial treatment had been started. It is important, however, to interpret culture counts in relation to the patient's clinical condition. UTIs with lower culture counts are often obtained from catheterized patients or those with urinary obstruction.

LOWENSTEIN JENSEN MEDIUM: ISOLATION OF MYCOBACTERIUM

Lowenstein Jensen Medium (LJ Medium) is a selective medium that is commonly used for the cultivation and isolation of Mycobacterium (notably *Mycobacterium tuberculosis*) from specimens containing mixed flora. *Mycobacterium tuberculosis* is a pathogenic bacterial species in the family Mycobacteriaceae and the causative agent of most cases of tuberculosis. The medium is light green in color with solid consistency.

Principle: Lowenstein-Jensen Medium is commonly used in the clinical laboratory to isolate acid fast organisms from sterile and non-sterile sources. LJ Medium was originally formulated by Lowenstein, containing congo red and malachite green dyes. Jensen modified Lowenstein's medium by altering the citrate and phosphate contents, eliminating the congo red dye and by increasing the malachite green concentration.

L-Asparagine and Potato Flour are sources of nitrogen and vitamins in Lowenstein-Jensen Medium. Monopotassium Phosphate and Magnesium Sulfate enhance organism growth and act as buffers. Glycerol and the Egg Suspension provide fatty acids and protein required for the metabolism of mycobacteria. When heated, the egg albumin coagulates, thus providing a solid surface for inoculation. Glycerol serves as a carbon source and is favorable to the growth of the human type tubercle bacillus while being unfavorable to the bovine type. Malachite green acts as an inhibitory agent toward microorganisms other than mycobacteria.

Composition of Lowenstein-Jensen Medium

- **LJ Medium Base :**

L-Asparagine = 3.6 g
Monopotassium Phosphate = 2.5 g
Magnesium Sulfate = 0.24 g
Sodium Citrate = 0.6 g
Malachite Green = 0.4 g
Potato Flour = 30 g

- **Supplements:**

Glycerol = 12 ml
Egg suspension = 1000 ml

Preparation

1. **Dissolve 37.3 g of the medium in 600 mL of purified water containing 12 mL of glycerol.**
2. **Heat with frequent agitation to completely dissolve the medium.**
3. **Autoclave at 121°C for 15 minutes.**
4. **Prepare 1000 mL of a uniform suspension of fresh eggs under aseptic conditions. Avoid whipping air into suspension during the collection and mixing.**
5. **Aseptically mix the 1000 mL of egg suspension with 600 mL of the sterile Lowenstein-Jensen Medium cooled to 50 – 60°C, avoiding air bubbles.**

6. **Dispense the finished medium into sterile screw-cap test tubes. Place the tubes in a slanted position and heat at 85°C for 45 minutes.**

Method of Use

1. **Inoculate the Lowenstein Jensen Media with specimen after decontamination and neutralization.**
2. **Incubate medium in a CO2 atmosphere at 35-37°C. Protect from light. Tubed media should be incubated for one week with loosened caps to allow the circulation of CO2 for the initiation of growth. Caps should be tightened after one week in order to prevent dehydration of media.**
3. **Examine the media within five to seven days, and weekly thereafter for up to eight weeks.**
4. **Examine plates under light for the appearance of macroscopic growth.**
5. **Examine tubes under light and magnifying mirror for macroscopic growth. Record and describe colony morphology on the first day growth is observed.**
6. **Consult appropriate references for recording the number of colonies and for aid in the biochemical identification of acid-fast bacilli.**

Result Interpretation

Observe for colonies that may or may not be pigmented. Colony morphology depends on the species isolated. The picture below shows Mycobacterium tuberculosis colonies on LJ Medium.

BIOCHEMICAL TESTS TO IDENTIFY BACTERIAL SPECIES

While several commercial systems for identifying bacteria are available, these are often difficult to obtain or too expensive to use in developing countries.

The following tests are described:

Test	*Purpose*
_ *Beta*-glucuronidase	To identify *E. coli*
_ Bile solubility	To differentiate *S. pneumoniae* from other *alpha*-haemolytic streptococci
_ Catalase	To differentiate staphylococci from streptococci
_ Citrate utilization	To differentiate enterobacteria
_ Coagulase	To identify *S. aureus* DNA-ase To help identify *S. aureus*
_ Indole	To differentiate Gram negative rods, particularly *E. coli*
_ Litmus milk	To help identify decolorization *Enterococcus* and some clostridia
_ Lysine decarboxylase	To assist in the identification of salmonellae and shigellae
_ Oxidase	To help identify *Neisseria*, *Pasteurella, Vibrio*, *Pseudomonas*
_ Urease	To help identify *Proteus*, *Morganella*, *Y. enterocolitica*, H. *pylori*

Bile solubility test

This helps to differentiate *S. pneumoniae*, which is soluble in bile and bile salts, from other *alpha*haemolytic streptococci (viridans streptococci) which are insoluble.

Principle: A heavy inoculum of the test organism is emulsified in physiological saline and the bile salt sodium deoxycholate is added. This dissolves *S. pneumoniae* as shown by a clearing of the turbidity within 10–15 minutes. Viridans and other streptococci are not dissolved and therefore there is no clearing of the turbidity.

Required

— Sodium deoxycholate, 100 g/l (10% w/v)

— Physiological saline (sodium chloride, 8.5 g/l)

Tube method

Although the bile solubility test can be performed by testing colonies directly on a culture plate or on a slide a tube technique is recommended because the results are easier to read.

1- Emulsify several colonies of the test organism in a tube containing 2 ml sterile physiological saline, to give a turbid suspension.

2- Divide the organism suspension between two tubes.

3- To one tube, add 2 drops of the sodium deoxycholate reagent and mix.

4- To the other tube (negative control), add 2 drops of sterile distilled water and mix.

5- Leave both tubes for 10–15 minutes at 35– 37 _C.

7- Look for a clearing of turbidity in the tube containing the sodium deoxycholate.

Results

Clearing of turbidity . . . . . Probably *S. pneumoniae*

No clearing of turbidity . . . . Organism is probably not *S. pneumoniae*

There should be no clearing of turbidity in the negative control tube to which distilled water was added.

***Note*: Some strains of *S. pneumoniae* are not dissolved by bile salts, and very occasionally some strains of viridans streptococci give a positive test.**

Controls

Bile solubility positive control: *Streptococcus pneumoniae*

Bile solubility negative control: *Enterococcus Faecalis*

Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci.

Principle: Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Required

Hydrogen peroxide, 3% H_2O_2 (10 volume solution)

Method

1- Pour 2–3 ml of the hydrogen peroxide solution into a test tube.

2- Using a sterile wooden stick or a glass rod (*not* a nichrome wire loop), remove several colonies of the test organism and immerse in the hydrogen peroxide solution.

Important: **Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur.**

3- Look for immediate bubbling

Results

Active bubbling . . . . . Positive catalase test

No bubbles . . . . . Negative catalase test

***Caution*: Performing the test on a slide is not recommended because of the risk of contamination from active bubbling. When the rapid slide technique is used, the hydrogen peroxide solution should be added to the organism suspension after placing the slide in a petri dish. The dish should then be covered *immediately*, and the preparation observed for bubbling through the lid.**

Controls

Positive catalase control: *Staphylococcus* species

Negative catalase control: *Streptococcus* species

Citrate utilization test

This test is one of several techniques used occasionally to assist in the identification of enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon.

Ways of performing a citrate test

● **Using a Rosco citrate identification tablet. This is the most economical method when only a few tests are performed. The tablets have a long shelf-life and good stability in tropical climates.**

● **Using Simmon's citrate agar but the dehydrated medium is only available in 500 g pack size from manufacturers. After being opened the medium does not have good stability in tropical climates.**

Citrate utilization using a Rosco citrate tablet

Citrate identification tablets are available from Rosco Diagnostica in a vial of 50 tablets.

1- Prepare a dense bacterial suspension of the test organism in 0.25 ml sterile physiological saline in

small tube.

2- Add a citrate tablet and stopper the tube.

3- Incubate overnight at 35–37 _C.

Results

Red color . . . . . Positive citrate test

Yellow-orange color . . . . . Negative citrate test

Controls

A positive citrate test reaction is obtained with *Klebsiella pneumoniae* and a negative reaction with *Escherichia coli*.

Citrate method using Simmon's citrate agar

1- Prepare slopes of the medium in bijou bottles as recommended by the manufacturer (store at 2–8 _C).

2- Using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt.

3- Incubate at 35 _C for 48 hours. Look for a bright blue color in the medium.

Results

Bright blue . . . . . Positive citrate test

No change in color . . . . . Negative citrate test of medium

Controls

As described above.

Coagulase test

This test is used to identify *S. aureus* which produces the enzyme coagulase.

Principle: Coagulase causes plasma to clot by converting fibrinogen to fibrin. Two types of coagulase are produced by most strains of *S. aureus*:

_ Free coagulase which converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma. Free coagulase is detected by clotting in the tube test.

_ Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor. It can be detected by the clumping of bacterial cells in the rapid slide test.

A tube test must *always* be performed when the result of a slide test is not clear, or when the slide test is negative and *Staphylococcus* has been isolated from a serious infection. A tube test may be required to detect some MRSA (methicillin resistant *S. aureus*) strains although some commercially available latex test kits to differentiate coagulase positive and coagulase negative staphylococci, overcome this. Before performing a coagulase test, examine a Gram stained smear to confirm that the organism is a Gram-positive coccus.

Required

EDTA anticoagulated human plasma (preferably pooled and previously HIV and hepatitis tested) or rabbit plasma. The plasma should be allowed to warm to room temperature before being used.

Plasma: Oxalate or heparin plasma can also be used. Do not use citrated plasma because citrate-utilizing bacteria e.g. enterococci, *Pseudomonas* and *Serratia* may cause clotting of the plasma (in tube test). Occasionally, human plasma may contain inhibitory substances which can interfere with coagulase testing. It is therefore essential to test the plasma using a known coagulase positive *S. aureus*. The plasma can be stored frozen in amounts ready for use.

Slide test method (detects bound coagulase)

1- Place a drop of distilled water on each end of a slide or on two separate slides.

2- Emulsify a colony of the test organism (previously checked by Gram staining) in each of the drops to make two thick suspensions.

Note: **Colonies from a mannitol salt agar culture are not suitable for coagulase testing. The organism must first be cultured on nutrient agar or blood agar.**

3- Add a loopful (not more) of plasma to one of the suspensions, and mix gently. Look for clumping of the organisms within 10 seconds.

No **plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping.**

Results

Clumping within 10 secs . . . . . *S. aureus*

No clumping within 10 secs . . . . . No bound coagulase

Note**: Virulent strains of *Yersinia pestis* are also coagulase positive.**

Controls

Positive coagulase control: *Staphylococcus aureus*

Negative coagulase control: *Escherichia coli* or *Staphylococcus epidermidis*

Tube test method (detects free coagulase)

1- Take three small test tubes and label:

T = Test organism (18–24 h broth culture)*

Pos = Positive control (18–24 h *S. aureus* broth culture)*

Neg = Negative control (sterile broth)*

***Nutrient broth is suitable (see No. 63). Do not use glucose broth.**

2- Pipette 0.2 ml of plasma into each tube.

3- Add 0.8 ml of the test broth culture to tube T.

Add 0.8 ml of the *S. aureus* culture to the tube labelled 'Pos'.

Add 0.8 ml of sterile broth to the tube labelled 'Neg'.

4- After mixing gently, incubate the three tubes at 35–37 _C. Examine for clotting after 1 hour. If no clotting has occurred, examine after 3 hours. If the test is still negative, leave the tube at room temperature overnight and examine again.

***Note*: When looking for clotting, tilt each tube gently.**

Results

Clotting of tube contents or fibrin clot in tube . . . . . *S. aureus*

No clotting or fibrin clot . . . . . Negative test

***Note*: There should be no clotting in the negative control tube.**

Commercially produced agglutination tests to identify *S. aureus*

Several latex agglutination test kits have been developed to identify *S. aureus* based on the detection of clumping factor, and, or protein A.

DNA-ase test

This test is used to help in the identification of *S. aureus* which produces deoxyribonuclease (DNAase) enzymes. The DNA-ase test is particularly useful when plasma is not available to perform a coagulase test or when the results of a coagulase test are difficult to interpret.

Principle: Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA). The test organism is cultured on a medium which contains DNA. After overnight incubation, the colonies are tested for DNA-ase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolyzed DNA. DNA-ase-producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Required

— DNA-ase agar plate

Up to six organisms may be tested on the same plate.

— Hydrochloric acid Reagent 1 mol/1 (1 N)

Method

1- Divide a DNA-ase plate into the required number of strips by marking the underside of the plate.

2- Using a sterile loop or swab, spot-inoculate the test and control organisms. Make sure each test area is labelled clearly.

3- Incubate the plate at 35–37 _C overnight.

4- Cover the surface of the plate with 1 mol/l hydrochloric acid solution. Tip off the excess acid.

5- Look for clearing around the colonies within 5 minutes of adding the acid, as shown in Plate 7.10.

Results

Clearing around the colonies . . . . . DNA-ase positive strain

No clearing around the colonies . . . . . DNA-ase negative strain

***Note*: Some methicillin resistant *S. aureus* (MRSA) strains give a negative DNA-ase test. Some coagulase negative staphylococci are weakly positive. Also, *S. pyogenes*, *Moraxella* and *Serratia* species frequently give a positive DNA-ase test.**

Controls

Positive DNA-ase control: *Staphylococcus aureus*

Negative DNA-ase control: *Staphylococcus epidermidis*

Indole test

Testing for indole production is important in the identification of enterobacteria. Most strains of *E. coli*, *P. vulgaris*, *P. rettgeri*, *M. morganii*, and *Providencia* species break down the amino acid tryptophan with the release of indole.

Principle: The test organism is cultured in a medium which contains tryptophan. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylaminobenzaldehyde. This reacts with the indole to produce a red coloured compound. Kovac's reagent is recommended in preference to Ehrlich's reagent for the detection of indole from enterobacteria.

Ways of performing an indole test

An indole test can be performed:

- **As a single test using tryptone water and Kovac's reagent.**
- **As a combined *beta*-glucuronidase-indole test using a Rosco PGUA/Indole identification tablet and Kovac's reagent. This is useful when identifying *E. coli*.**
- **As a combined lysine decarboxylase-indole test using a Rosco LDC/Indole identification tablet.**

This is useful in helping to identify salmonellae and shigellae.

Detecting indole using tryptone water

1- Inoculate the test organism in a bijou bottle containing 3 ml of sterile tryptone water.

2- Incubate at 35–37 _C for up to 48 h.

3- Test for indole by adding 0.5 ml of Kovac's, reagent. Shake gently. Examine for a red colour in the *surface layer* within 10 minutes.

Kovac's indole reagent: This can be economically purchased as a ready-made reagent from Merck. It is available in a 100 ml bottle, code 1.09293.0100 or in a 30 ml dropper bottle, code 1.11350.0001. It has a long shelf-life when stored at 4–8 _C.

Results

Red surface layer . . . . . Positive indole test

No red surface layer . . . . . Negative indole test

Detecting indole using Rosco PGUA/Indole

tablet

PGUA/Indole tablets are available from ROSCO Diagnostica, in a vial of 50 tablets. They have a long shelf life (3–4 y).

1- Prepare a *dense* suspension of the test organism in 0.25 ml physiological saline in a small tube.

2- Add a PGUA/Indole tablet and close the tube. Incubate at 35–37 _C for 3–4 hours (or overnight).

3- First read the *beta*-glucuronidase (PGUA) reaction:

Results

Yellow colour . . . . . Positive PGUA test

Colourless . . . . . Negative PGUA test

4- Add 3 drops of Kovac's reagent and shake.

4- **Wait 3 minutes before reading the indole reaction. Examine the colour of the *surface layer*.**

Results

Red surface layer . . . . . Positive indole test

Yellow surface layer . . . . . Negative indole test

***Note*: About 94% of *E. coli* strains are PGUA positive and 99% are indole positive.**

Detecting indole using Rosco LDC/Indole tablet

LDC/Indole tablets are available from Rosco Diagnostica in a vial of 50 tablets. They have a long shelf-life (3–4 y).

1- Prepare a *dense* suspension of the test organism in 0.25 ml physiological saline in a small tube.

2- Add an LDC/Indole tablet. Add 3 drops of paraffin oil* and close the tube.

***The oil overlayer provides the anaerobic conditions required for the LDC reaction.**

3- Incubate at 35–37 _C for 3–4 hours (or overnight).

4- First read the lysine decarboxylase (LDC) reaction:

Results

Blue/violet colour* . . . . . Positive LDC test

Yellow, green or grey colour . . Negative LDC test

***If examining after overnight incubation, a positive test is indicated by a *strong* blue or violet colour.**

5- Add 3 drops of Kovac's reagent and shake.

6- Wait 3 minutes before reading the indole reaction. Examine the colour of the *surface layer*.

Results

Red surface layer . . . . . Positive indole test

Yellow surface layer . . . . . Negative indole test

Litmus milk decolorization test

This test is a rapid inexpensive technique to assist in the identification of enterococci. It is based on the ability of most strains of *Enterococcus* species to reduce litmus milk by enzyme action as shown by decolorization of the litmus.

***Note*: Enterococci can also be identified using an aesculin hydrolysis test (see later text).**

Principle: A heavy inoculum of the test organism is incubated for up to 4 hours in a tube containing litmus milk. Reduction of the litmus milk is indicated by a change in colour of the medium from mauve to white or pale yellow.

Required

Litmus milk medium

Method

1- Using a sterile loop, inoculate 0.5 ml of sterile litmus milk medium with the test organism.

***Important*: A *heavy* inoculum of the test organism must be used. Scraping the loop three times across an area of heavy growth is recommended.**

2- Incubate at 35–37 _C for up to 4 hours, examining at half hour intervals for a reduction reaction as shown by a change in colour from mauve to white or pale yellow (compare with the positive control.)

***Note*: The incubation time should not be more than 4 hours because some strains of viridans streptococci will reduce litmus milk with prolonged incubation.**

Results

White or pale yellow-pink colour . . . . Suggestive of *Enterococcus*

No change or a pink colour . . . . . Probably not *Enterococcus*

Controls

Positive control: *Enterococcus* species

Negative control: Viridans streptococci

Note: The work of Schierl and Blazevic (1981)* demonstrated that up to 83% of *Enterococcus* could be identified by the rapid litmus milk reduction test. No false positive reactions were observed. A negative result can be checked by culturing the organism in aesculin broth and examining daily for up to 7 days for aesculin hydrolysis as shown by a blackening in the medium, Enterococci hydrolyze aesculin.

Aesculin hydrolysis test to identify enterococci

This test can be economically performed using a Rosco bile aesculin tablet. The tablets are available from Rosco Diagnostica in a vial of 25 tablets. They have good stability (3–4 y).

The test can be performed by placing a tablet on a blood agar plate inoculated with the test organism and incubating it at 35–37 _C overnight. A positive test is indicated by the tablet and colonies around it turning black/grey. A negative test is shown by the tablet remaining white and no change in colour of the colonies. A zone of inhibition may appear around the tablet. Alternatively, the test can be performed by making a *dense* suspension of the test organism in 0.25 ml of physiological saline in a small tube, adding a tablet, and incubating at 35–37 _C for 4 hours (or overnight). A positive reaction is shown by a black/grey colour in the medium.

***Note*: An aesculin hydrolysis can also be performed by incubating the test organism on bile aesculin agar but this medium is expensive.**

Oxidase test (Cytochrome oxidase test)

The oxidase test is used to assist in the identification of *Pseudomonas*, *Neisseria*, *Vibrio*, *Brucella*, and *Pasteurella* species, all of which produce the enzyme cytochrome oxidase.

Principle: A piece of filter paper is soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper. Alternatively,

an oxidase reagent strip can be used. When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.

Occasionally the test is performed by flooding the culture plate with oxidase reagent but this technique is not recommended for routine use because the reagent rapidly kills bacteria. It can however be useful when attempting to isolate *N. gonorrhoeae* colonies from mixed cultures in the absence of a selective medium. The oxidase positive colonies *must* be removed and subcultures within 30 seconds of flooding the plate.

***Important*: Acidity inhibits oxidase enzyme activity; therefore, the oxidase test must *not* be performed on colonies that produce fermentation on carbohydratecontaining media such as TCBS or MacConkey agar. Subinoculation on nutrient agar is required before the oxidase test can be performed. Colonies tested from a medium that contains nitrate may give unreliable oxidase test results.**

Oxidase reagent *freshly* prepared or use an oxidase reagent strip

***Note*: Fresh oxidase reagent is easily oxidized. When oxidized it appears blue and must not be used.**

Stable oxidase reagent strips

These can be purchased from Merck in a pack of 50 strips (code 1.13300.0001). The strips have a 5-year shelf-life when stored at 2–8 _C.

Method (fresh reagent)

1- Place a piece of filter paper in a clean petri dish and add 2 or 3 drops of *freshly* prepared oxidase reagent.

2- Using a piece of stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.

3- Look for the development of a blue-purple colour within a few seconds.

Results

Blue-purple colour . . . . . Positive oxidase test (within 10 seconds)

No blue-purple colour . . . . . Negative oxidase test (within 10 seconds)

***Note*: Ignore any blue-purple colour that develops after 10 seconds.**

Method using an oxidase reagent strip

1- Moisten the strip with a drop of sterile water.

2- Using a piece of stick or glass rod (not an oxidized wire loop) remove a colony of the test organism and rub it on the strip.

3- Look for a red-purple colour within 20 seconds.

Red-purple colour . . . . . positive oxidase test.

***Note:* When using a Merck reagent strip, follow exactly the manufacturer's instructions on how to perform the test.**

Controls

Positive oxidase control: *Pseudomonas aeruginosa*

Negative oxidase control: *Escherichia coli*

Urease test

Testing for urease enzyme activity is important in differentiating enterobacteria. *Proteus* strains are strong urease producers. *Y. enterocolitica* also shows urease activity (weakly at 35–37 _C). Salmonellae and shigella do not produce urease.

Principle: The test organism is cultured in a medium which contains urea and the indicator phenol red. When the strain is urea reproducing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red.

Ways of performing a urease test

- **Using a Rosco urease identification tablet.**
- **Using modified Christensen's urea broth.**

Urease test using a Rosco urease tablet

Urease identification tablets are available from Rosco Diagnostica in a vial of 50 tablets. The tablets have a long shelf-life (3–4 y).

1- Prepare a dense 'milky' suspension of the test organism in 0.25 ml physiological saline in a small tube.

2- Add a urease tablet, close the tube and incubate at 35–37 _C (preferably in a water bath for a quicker result) for up to 4 hours or overnight.

***Proteus* and *M. morganii* organism give a positive reaction within 4 hours.**

Results

Red/purple colour . . . . . Positive urease test

Yellow/orange . . . . . Negative urease test

Urease test using Christensen's (modified) urea broth

1- Inoculate heavily the test organism in a bijou bottle containing 3 ml sterile Christensen's modified urea broth.

2- Incubate at 35–37 _C for 3–12 h (preferably in a water bath for a quicker result).

3- Look for a pink colour in the medium.

Results

Pink colour . . . . . Positive urease test

No pink colour . . . . . Negative urease test

DIFFERENTIATION OF ENTERIC BACTERIAL SPECIES (Enterobacteriaceae)

Enterobacteriaceae includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia*

***pestis*, *Klebsiella*, and *Shigella*. Other disease-causing bacteria in this family include *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*.**

Methyl Red (MR) test is a biochemical test performed on bacterial species to detect the ability of an organism to produce stable acids end products (Mixed-acid fermentation) from supplied glucose. It is used as the part of the IMViC tests, a set of four useful reactions that are commonly designed for the differentiation of enteric (members of family Enterobacteriaceae). The IMViC series includes the following four tests:

- **Methyl red test**
- **Indole test**
- **Voges-Proskauer test**
- **Citrate test**
    - **Principle of Methyl Red Test**

Some bacteria have ability to perform mixed acid fermentation of glucose in MR-VP medium. The products of mixed-acid fermentation are a complex mixture of acids, particularly lactate, acetate, succinate and format as well as ethanol and equal amounts of H_2 and CO_2. This causes the medium to acquire an acidic pH. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

Procedure

1. **By using sterile inoculating loop, inoculate the unknown microorganism into the fresh, sterile medium.**
2. **Leave the other broth uninoculated (this will be a control).**
3. **Incubate the inoculated tube at 35-37C for two to five days.**
4. **After incubation, obtain the broths from the incubator and add 5 drops of Methyl Red reagent to the broth**
5. **Observe the color.**

Interpretation

-Development of red color is taken as Positive.

-Development of yellow color (No color change) after addition of reagent is taken as Negative.

Examples

- **MR Positive Organisms: *Escherichia, Salmonella, Proteus***
- **MR Negative Organisms: *Enterobacter, Klebsiella***

***Salmonella* is a genus of rod-shaped (bacillus) Gram-negative bacteria of the family Enterobacteriaceae. They usually invade only the gastrointestinal tract and cause food poisoning, typhoid fever(*Salmonella* Typhi, also known as *Salmonella enterica* serotype Typhi, and paratyphoid fever (caused by *Salmonella* serotypes Paratyphi A, Paratyphi B, and Paratyphi C).**

Three species of *P. vulgaris*, *P. mirabilis*, and *P. penneri* are opportunistic human pathogens. *Proteus* includes pathogens responsible for many human urinary tract infections.

- **Indole Test**

Principle of Indole Test

Some bacteria split amino acid tryptophan into indole and pyruvic acid using the enzyme called tryptophanase. Indole can be detected with Kovac's reagent or Ehrlich's reagent. Indole reacts with the aldehyde in the reagent to give a red color which concentrates as a ring at the top.

Procedure

1. **Bacterium to be tested is inoculated in tryptone broth.**
2. **Incubate overnight at 37°C.**
3. **Add few drops of Kovac's reagent. Ehrlich's reagent is more sensitive in detecting indole production in anaerobes and non-fermenters.**
4. **Do not shake the tube and observe the result.**

Interpretation

Formation of red or pink colored ring at the top is taken as Positive. No color change after addition of reagent is taken as Negative.

Most strains of *E. coli, P. vulgaris, M. morganii and Providenica* are indole positive.

Importance

Along with differentiation of enteric, Indole test can also be used for species differentiation.

- **Proteus Species: To differentiate *Proteus mirabilis* (indole negative) from all other Proteus species (indole positive)**
- **Klebsiella species: To differentiate *Klebssiella pneumoniae* (indole negative) from *Klebsiella oxytoca* (indole positive)**
- **Citrobacter species: To differentiate *Citrobacter freundii* (indole negative) from *Citrobacter koseri* (indole positive)**

Additional Notes

Preparation of reagent:

Tryptone broth:
Tryptone = 10gm
Sodium chloride = 5gm
Dissolve the ingredients in 1 liter of sterile water. Dispense 4 ml per tube. Cap tube and autoclave at 121°C under 15 psi pressure for 15 minutes. Store the tubes in the refrigerator at 4 to 10°C.

Kovac's reagent:
Amyl alcohol: 150ml
p-dimethylaminobenzaldehyde (DMAB): 10gm
conc. HCl: 50ml
(Dissolve DMAB in the alcohol. Slowly add the acid to the aldehyde-alcohol mixture.)

ANTIMICOBIAL SUCEPTIBILITY TESTING

Antimicrobial agents include naturally occurring antibiotics, synthetic derivatives of naturally occurring antibiotics (semi-synthetic antibiotics) and chemical antimicrobial compounds (chemotherapeutic agents). Generally, however, the term 'antibiotic' is used to describe antimicrobial agents (usually antibacterial) that can be used to treat infection.

Compared with antibacterial agents, fewer antiviral and antifungal agents have been developed. Many antiviral agents have serious side-effects e.g. those used to treat HIV infection.

Antimicrobial activity

Not all antimicrobials, at the concentration required to be effective are completely non-toxic to human cells. Most, however, show sufficient selective toxicity to be of value in the treatment of microbial diseases. Antibacterial agents can be grouped by their mode of action, i.e. their ability to inhibit the synthesis of the cell wall, cell membrane, proteins, and nucleic acids of bacteria.

Mode of action of antimicrobial agents

INHIBITORS OF BACTERIAL CELL WALL SYNTHESIS

INHIBITORS OF PROTEIN SYNTHESIS

INHIBITORS OF BACTERIAL NUCLEIC ACID SYNTHESIS

ANTIMICROBIAL SUSCEPTIBILITY TESTING

In the treatment and control of infectious diseases, especially when caused by pathogens that are often drug resistant, susceptibility (sensitivity) testing is used to select effective antimicrobial drugs. Susceptibility testing is not usually indicated when the susceptibility reactions of a pathogen can be predicted, for example:

– *Proteus* species are generally resistant to nitrofurantoin and tetracyclines,

– *S. pyogenes* is usually susceptible to penicillin,

– *K. pneumoniae* is generally ampicillin resistant,

– Anaerobes are susceptible to metronidazole.

Susceptibility tests must never be performed on commensal organisms or contaminants because this would mislead the clinician and could result in the patient receiving ineffective and unnecessary antimicrobial therapy, causing possible side effects and resistance to other potentially pathogenic organisms.

Susceptibility testing techniques

Laboratory antimicrobial susceptibility testing can be performed using:

– A dilution technique

– A disc diffusion technique.

***Dilution susceptibility tests*: Manual or semi-automated dilution susceptibility tests are performed in Microbiology Reference Laboratories for epidemiological purposes or when a patient does not respond to treatment thought to be adequate, relapses while being treated, or when there is immunosuppression. Dilution techniques measure the minimum inhibitory concentration (MIC). They can also be used to measure the minimum bactericidal concentration (MBC) which is the lowest concentration of antimicrobial required to kill bacteria.**

A dilution test is carried out by adding dilutions of an antimicrobial to a broth or agar medium. A standardized inoculum of the test organism is then added. After overnight incubation, the MIC is reported as the lowest concentration of antimicrobial required to prevent visible growth. By comparing the MIC value with known concentrations of the drug obtainable in serum or other body fluids, the likely clinical response can be assessed.

***Disc diffusion susceptibility tests*: Disc diffusion techniques are used by most laboratories to test routinely for antimicrobial susceptibility. A disc of blotting paper is impregnated with a known volume and appropriate concentration of an antimicrobial, and this is placed on a plate of susceptibility testing agar uniformly inoculated with the test organism. The antimicrobial diffuses from the disc into the medium and the growth of the test organism is inhibited at a distance from the disc that is related (among other factors) to the susceptibility of the organism. Strains susceptible to the antimicrobial are inhibited at a distance from the disc whereas resistant strains have smaller zones of inhibition**

or grow up to edge of the disc. For clinical and surveillance purposes and to promote reproducibility and comparability of results between laboratories, WHO recommends the (NCCLS*) modified Kirby-Bauer disc diffusion technique.

National Committee for Clinical Laboratory Standards. *Kirby-Bauer NCCLS modified disc diffusion technique

The validity of this carefully standardized technique depends on, for each defined species, using discs of correct antimicrobial content, an inoculum which gives confluent growth, and a reliable Mueller Hinton agar. The test method must be followed exactly in every detail. After incubation at 35 _C for 16–18 hours, zone sizes are measured and interpreted using NCCLS standards. These are derived from the correlation which exists between zone sizes and MICs. *

***An approximately linear relationship exists between log MIC as measured by the dilution test, and the inhibition zone diameter in the diffusion test. A regression line expressing this relationship can be obtained by testing a large number of strains by both techniques. This has been done and enables zone diameter sizes to be correlated to MIC values in the NCCLS modified Kirby-Bauer technique.**

The NCCLS Kirby-Bauer technique should only be used for well-evaluated bacterial species. It is not suitable for bacteria that are slow-growing, need special nutrients, or require CO2 or anaerobic incubation.

***Stokes disc diffusion technique*: In this disc technique both the test and control organisms are inoculated on the same plate. The zone sizes of the test organism are compared directly with that of the control. This method is not as highly standardized as the Kirby-Bauer technique and is used in laboratories particularly when the exact amount of antimicrobial in a disc cannot be guaranteed due to difficulties in obtaining discs and storing them correctly or when the other conditions required for the Kirby-Bauer technique cannot be met.**

One-way laboratories in developing countries performing the Stokes technique could change to a technique comparable to the WHO recommended Kirby-Bauer technique is to use highly stable Rosco Diagnostica antibiotic tablets (*Neo-Sensitabs*) instead of less stable paper discs.

Rosco *Neo-Sensitabs* susceptibility testing

Neo-Sensitabs **antimicrobial tablets are standardized according to the 2004 MIC-breakpoints recommended by the NCCLS. The tablets are 9 mm in diameter and color-coded. The formulae used to produce the tablets gives them a shelf life of about 4 years and many *Neo-Sensitabs* can be stored at room temperature.**

The same principles and quality control as used in the modified Kirby-Bauer method apply when using *Neo- Sensitabs*. An excellent 2004 booklet *User's Guide- Neo-Sensitabs Susceptibility Testing* is available from Rosco Diagnostica. This describes the principles and how to perform susceptibility testing and exactly how to measure and interpret zone sizes. The cost and local availability of *Neo- Sensitabs* can be obtained from Rosco Diagnostica.

Control strains

Control strains are used to test the performance of the method. The following strains of bacterial species are recommended.

- ***Staphylococcus aureus*** **ATCC 25923.**
- ***Escherichia coli*** **ATCC 25922.**
- ***Pseudomonas aeruginosa*** **ATCC 27853.**

Sources of control strains

Reference Laboratories should supply local laboratories. The control strains should be grown on slopes of nutrient agar or tryptone soya agar and stored refrigerated at 2–8 _C. They should be subcultured every 3–6 months. At the beginning of each week a nutrient broth or agar culture should be prepared for daily use.

Method

1- Using a sterile wire loop, touch 3–5 well-isolated colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth.

2- In a good light match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use). When comparing turbidities, it is easier to view against a printed card or sheet of paper.

3- Using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension. Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60_ to ensure even distribution.

4- With the petri dish lid in place, allow 3–5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.

5- Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate. Using a template as shown in Plate 7.26 will help to ensure the discs are correctly placed.

***Note*: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish). Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place. 6 Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35 _C for 16–18 h (temperatures over 35 _C invalidate results for oxacillin).**

7- After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate measure the diameter of each zone of inhibition in mm. The endpoint of inhibition is where growth starts. *Sulphonamides and co-trimoxazole* Ignore any slight growth within the inhibition area.

Beta-lactamase producing staphylococci

A zone of inhibition can be formed by penicillin resistant staphylococci when the amount of *beta*-lactamase (penicillinase) is insufficient to inactivate the penicillin close to the disc. Such a zone, however, has a heaped up clearly defined edge with no gradual fading away of growth towards the disc as seen with susceptible strains. Colonies may sometimes be seen growing within the

inhibition zone. Report all strains showing a heaped-up zone edge, regardless of the size of the inhibition zone, as '*Resistant*'.

Proteus strains

Some *Proteus* strains may swarm into the area of inhibition but the actual zone of inhibition is usually clearly outlined.

Interpretation of zone sizes

Using the Interpretative Chart, interpret the zones sizes of each antimicrobial, reporting the organism as 'Resistant', 'Intermediate/Moderately susceptible', 'Susceptible'.

***Resistant*: A pathogen reported as 'resistant' implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.**

***Intermediate*: A pathogen reported as intermediately susceptible suggests that the infection it has caused is likely to respond to treatment when the drug is used in larger doses than normal or when the drug is concentrated at the site of infection, e.g. in the urinary tract. Consideration should be given to using other drugs that may provide more optimal therapy.**

***Susceptible*: A pathogen reported as susceptible suggests that the infection it has caused is likely to respond to treatment when the drug to which it is susceptible is used in normal recommended doses and administered by an appropriate route.**

***Note*: It is usually only necessary to report the first and second choice antibiotics for a patient's infection, unless the strain is resistant. Always use generic names of antibiotics, not trade names, in laboratory reports.**

Summarized procedure

1-Removing colonies from a primary culture plate to make a suspension of the test organism.

2-Checking the turbidity of the test suspension against the turbidity of a chemical standard.

3-Avoiding using too much inoculum by pressing and rotating the swab against the side of the tube.

4- Swabbing the surface of the susceptibility testing agar. The plate is swabbed in three directions, rotating the plate approximately 60° to ensure even distribution.

5-Placing antimicrobial discs on the inoculated plate.

6- Measuring the zones of inhibition in mm. The end of inhibition is where growth end.

MANTOUX TEST: SCREEN IN VIVO SENSETIZATION BY M. TUBERCULOSIS

The Mantoux test is a qualitative, skin test to screen in vivo sensitization by *Mycobacterium tuberculosis* either due to active infection or past infection. It is also used to check the prophylaxis and efficacy of BCG vaccination. Mantoux test is a routine screening procedure for children, healthcare workers, individuals at high risk of being infected and individuals who are suspected of being infected with Tuberculosis.

Mantoux Test doesn't distinguish between an active and a latent infection; nor does it provide a definitive diagnosis. If positive reaction occurs, additional tests such as sputum smear, culture and chest X-rays etc are necessary to establish a diagnosis of an active TB infection.

Principle of Mantoux Test

Mantoux test is based on a delayed type hypersensitivity reaction (Type IV) to test for individual cell mediated immunity against Mycobacterium tuberculosis. Mycobacterial antigen is available in the form of Purified Protein Derivative (PPD). 5 units of PPD (0.1 ml) is injected intradermally with 26,27 or 30 gauze needles. The results are read in 48-72 hours for induration (elevated hardened area). Erythema (redness) is not significant.

After injection, cytokines are released by memory Th1 cells to attract macrophages and granulocytes which cause induration and erythema. Delayed hypersensitivity reaction begin after 5-6 hours and reach peak at 48-74 hours.

Requirements

1. **Graduated 1ml syringe**
2. **PPD or Tuberculin**
3. **Spirit swab**

Procedure of Mantoux Test

1. **Bring PPD reagent to room temperature.**
2. **The preferred site for injection is dorsal surface of the forearm, about 4cm below the elbow joint. Select an area free of barriers (e.g. scars, sores).**
3. **Disinfect the site of injection and allow to dry.**
4. **Draw up just over 0.1 ml of PPD by using 1 ml syringe. Remove excess PPD to make exactly 0.1 ml and remove air from the syringe if present.**
5. **Using 27 g needle to inject the PPD intradermally to make the deposition wheel, in the diameter of 6 to 8 mm which will rise up to the point of needle.**
6. **Mark the area of injection with indicator.**
7. **Read the result after 48-72 hours for induration.**

Results and Interpretation

After 48-72 hours of administration of PPD, reaction should be measured in millimeters of induration (elevated hardened area). Erythema (redness) is not significant, it is thus not measured.

According to Center for Disease Control (CDC), interpretation of Mantoux test depends on two factors:

- **Measurement in millimeters (mm) of the induration**

- **Person's risk of being infected with TB and progression to disease if infected**

Induration of ≥5 mm is considered positive in

1. **Human immunodeficiency virus (HIV)-infected persons**
2. **Recent contacts of TB case patients**
3. **Persons with fibrotic changes on chest radiograph consistent with prior TB**
4. **Patients with organ transplants and other immunosuppressed patients**

Induration of ≥10 mm is considered positive in

1. **Recent immigrants (i.e., within the last 5 years) from countries with a high prevalence of TB**
2. **Injection drug users**
3. **Residents and employees of the high-risk congregate settings like; prisons and jails, nursing, hospitals and other health care facilities, residential facilities for patients with AIDS and homeless shelters**
4. **Mycobacteriology laboratory personnel**
5. **Persons with the clinical conditions that place them at high risk; silicosis, diabetes mellitus, chronic renal failure, some hematologic disorders (e.g., leukemias and lymphomas), other specific malignancies (e.g., carcinoma of the head, neck, or lung)**
6. **Infants, children, and adolescents exposed to adults at high risk for developing active TB**

Induration of ≥15 mm is considered positive in

1. **Persons with no known risk factors for TB**

Limitations of Mantoux Test

Mantoux Test doesn't distinguish between an active and a latent infection. If positive reaction occurs, additional tests such as sputum smear, culture and chest X-rays etc are necessary to establish a diagnosis of an active TB infection. Several factors can lead to false-positive or false-negative skin test reactions.

False Positive Reactions

Due to the test's low specificity, most positive reactions in low-risk individuals are false positives. Some major causes of false positive Mantoux Test are:

- **Infection with nontuberculous mycobacteria (NTM)**
- **BCG vaccination.**
- **Administration of incorrect antigen.**
- **Incorrect interpretataion of results.**

False Negative Reactions

Some people have a negative reaction to the TST even though they have been infected with M. tuberculosis. A false-negative reaction can be caused by many things:

- **Concurrent viral infection (e.g., measles, mumps, chicken pox, HIV)**
- **Concurrent bacterial infection (e.g., typhoid fever, brucellosis, typhus, leprosy, pertussis)**
- **Concurrent fungal infection**
- **Chronic renal failure**
- **Low protein states (e.g., severe protein depletion, afibrinogenemia)**
- **Diseases affecting lymphoid organs (e.g., Hodgkin's disease, lymphoma, chronic leukemia, sarcoidosis)**
- **Immunosuppressive drugs (e.g., medical steroids)**
- **Children aged 6 months or less or elderly patients (i.e., immature or waning immunity)**
- **Stress (e.g., surgery, burns, mental illness, graft-versus-host reactions)**
- **Incorrect storage or handling of antigen or results that are not measured or interpreted properly**
- **Vaccinations using live virus; or**
- **Recent TB infection.**

COAGULASE TEST: DETECTION OF S. AUREUS

Coagulase is an enzyme that works in conjugation with normal plasma components to convert fibrinogen to finbrin and causes clotting of plasma. This enzyme is a good indicator of pathogenic potential of *Staphylococcus aureus*. As 97% of the strains of *S. aureus* have proven to be coagulase positive, the Coagulse test is useful for differentiating S. aureus from other Gram positive, Catalase positive cocci.

Principle

***S. aureus* produces two types of coagulases: bound and free. Slide coagulase test is done to detect bound coagulase, whereas tube coagulase test is done to detect free coagulase. Both tests utilize rabbit plasma treated with anticoagulant to interrupt the normal clotting mechanism.**

Bound Coagulase

It is also known as clumping factor. It is attached to bacterial cell wall and reacts directly with fibrinogen. This is shown by formation of visible mass. it doesn't require coagulase reacting factor (CRF).

Free Coagulase

It is an extracellular enzyme (released from the cell). It converts fibrinogen to fibrin by activity of coagulase reacting factor (CRF) in plasma. This is detected by appearance of fibrin clot in the tube coagulase test. It is usaually recommended to do tube coagulase test on all 'slide-coagulase-negative' staphylococci.

Procedure

Slide Test

1. **Place two separate drops of saline on a slide.**
2. **Using a sterile inoculating loop, emulsify one or two colonies of organism in one drop to make thick suspension of bacteria.**
3. **Add a loopful of plasma to both the suspension and saline drop and mix gently.**

4. **Look for immediate coarse clumping of the mixture within 10-15 seconds.**

Tube Test

1. **Dilute the plasma 1:10 with saline.**
2. **Take 2 test tubes and add 0.5 ml of diluted plasma to each.**
3. **Inoculate a tube with bacterial colonies to make a cloudy suspension. Alternatively, add about 5 drops of thick 18-24 hours broth cultures.**
4. **Incubate both tubes at 35-degree Celsius for 1 to 4 hours in water bath.**
5. **Afterward, examine both tubes for presence or absence of clots.**

Results and Interpretation

Slide Coagulase Test: The formation of clumps within 10-15 seconds is positive test result. Saline and plasma mixture should show no clumping.

Tube Coagulase Test: A positive coagulase test is represented by any degree of clotting, from a loose clot suspended in plasma to a solid clot. If negative, the plasma remains a liquid.

Positive coagulase test is shown by: *Staphylococcus aureus, S. pseudintermedius, S. intermedius, S. schleiferi, S. delphini, S. hyicus, S. lutrae* etc.

Negative coagulase test is shown by: *Staphylococcus epidermidis, S. saprophyticus, S. warneri, S. hominis, S. caprae* etc.

DETECTION OF MALARIA, PLASMODIUM SPP.

Malaria, which is caused by infection with protozoa of the genus *Plasmodium*, is the most important parasitic disease in tropical countries. It is transmitted to humans through the inoculation of *Plasmodium* sporozoites by female *Anopheles* mosquitoes or by blood transfusion. The sporozoites travel through the blood to the liver, where they transform into large tissue schizonts containing considerable numbers of merozoites (tissue schizogony). These begin to rupture after 5–20 days, according to the species, and the released

merozoites invade circulating erythrocytes. The replication cycle is repeated at regular intervals.

Identification of *Plasmodium* spp. in blood films

Preparation of a thick and a thin blood film on the same slide

For routine malaria microscopy, a thin and a thick film are made on the same slide. The thick film is used for the detection of parasites, while the thin film is used in identifying the species of parasite.

Materials and reagents

_ Microscope

_ Clean glass microscope slides

_ Sterile blood lancets

_ Cotton wool

_ Grease pencil

_ Methanol

_ 70% Ethanol.

Method

Blood to be examined for malaria parasites is usually collected at a health center. The most suitable time for collection is at the height of an episode of fever, when the parasites are most numerous in the blood. Blood specimens should always be collected *before* antimalarial drugs are given.

1. With the patient's left-hand palm upwards, select the third or fourth finger. (The big toe can be used with infants. The thumb should never be used for adults or children.) Use cotton wool lightly soaked in ethanol to clean the finger — using firm strokes to remove dirt and grease from the ball of the finger. Dry the finger with a clean piece of cotton wool (or lint).

2. With a sterile lancet, puncture the ball of the finger, using a quick rolling action. By applying gentle pressure to the finger, express the first drop of blood

and wipe it away with dry cotton wool. Make sure that no strands of cotton wool remain on the finger.

3. Working quickly and handling clean slides only by the edges, collect the blood as follows:

• Apply gentle pressure to the finger and collect a single small drop of blood, about this size _, on to the middle of the slide. This is for the thin film.

• Apply further pressure to express more blood and collect two or three larger drops, about this size _, on to the slide about 1 cm from the drop intended for the thin film. Wipe the remaining blood away with cotton wool.

4. *Thin film*. Using another clean slide as a "spreader", and with the slide with the blood drops resting on a flat, firm surface, touch the small drop with the spreader and allow the blood to run along its edge. Firmly push the spreader along the slide, away from the largest drops, keeping the spreader at an angle of 45°. Make sure that the spreader is in even contact with the surface of the slide all the time the blood is being spread.

5. *Thick film*. Always handle slides by the edges, or by a corner, to make the thick film as follows: Using the corner of the spreader, quickly join the larger drops of blood and spread them to make an even, thick film.

6. Allow the thick film to dry in a flat, level position protected from flies, dust and extreme heat. Label the dry film with a grease pencil by writing across the thicker portion of the thin film the patient's name or number and date.

Staining blood films with Giemsa stain

Principle

During staining of the blood film, the hemoglobin in the erythrocytes dissolves (hemoglobinization) and is removed by the water in the staining solution. All that remain are the parasites and the leukocytes, which can be seen under the microscope.

Materials and reagents

_ Microscope

_ Measuring cylinders, 10, 50 and 100ml

_ Beakers, 50 and 250ml

_ Staining troughs

_ Glass rods

_ Wash bottle

_ Slide forceps

_ Slide racks

_ Timer

_ Giemsa stain

_ Methanol in a drop bottle

_ Buffered water, pH 7.2 or distilled water.

Routine method for staining thick and thin blood films Ideally, for optimum staining, thick and thin films should be made on separate slides. This is often not possible and thick and thin films are generally made on the same slide. When this is done, good-quality staining of the thick film is of primary importance. Best results are obtained if the blood films have dried overnight. This method is suitable for staining 20 or more slides.

1. Fix the thin film by adding three drops of methanol, or by dipping it into a container of methanol for a few seconds. With prolonged fixation it may be difficult to detect Schüffner's dots and Maurer's clefts. To permit hemoglobinization, the thick film should not be fixed; therefore, avoid exposure of the thick film to methanol or methanol vapor.

In some laboratories with limited supplies the diluted Giemsa stain is reused; in such cases it must be used on the same day.

2. Using forceps, remove the slides one by one. Place them in a slide rack to drain and dry, film side downwards, making sure that the film does not touch the slide rack.

Rapid method for staining thick and thin blood films

This method is suitable for rapid staining of thick films when urgent results are required. It uses much more stain than the regular method.

1. Using forceps, place the slides back to back in a staining trough.

2. Prepare a 3% Giemsa solution in buffered or distilled water, pH 7.2, in sufficient quantity to fill the number of staining troughs being used. Mix the stain well.

3. Pour the stain gently into the staining trough, until all the slides are totally covered. Stain for 30–45 minutes out of sunlight.

4. Pour clean water gently into the trough to remove the deposit on the surface of the staining solution.

5. Gently pour off the remaining stain, and rinse again in clean water for a few seconds. Pour the water off.

1. Allow the thick film to dry thoroughly; if results are required urgently, drying may be hastened by fanning, or briefly exposing the slide to gentle heat such as that from a microscope lamp. Care should be taken to avoid overheating, otherwise the thick film will be heat-fixed.

2. Fix the thin film by adding three drops of methanol, or by dipping it into a container of methanol for a few seconds. To permit hemoglobinization, the thick film should not be fixed; therefore, avoid exposure of the thick film to methanol or methanol vapor.

3. Prepare a 10% Giemsa solution in buffered or distilled water, pH 7.2; if a small quantity is being used, three drops of stain per ml of buffered water will give the correct concentration of Giemsa solution. One slide requires about 3ml of made up stain. Mix the stain well with a glass rod.

4. Gently pour the stain on to the slides or use a pipette. Stain for 5–10 minutes.

5. Gently flush the stain off the slides by adding drops of clean water. Do not tip off the stain and then wash, as this will leave a deposit of scum over the smears.

6. Place the slides in the slide rack to drain and dry, film side downwards, making sure that the film does not touch the slide rack.

Staining blood films with Field stain

Staining with Field stain allows rapid detection of malaria parasites (but it does not always stain Schüffner's dots).

Materials and reagents

_ Microscope

_ Glass jars

_ Slide racks

_ Methanol

_ Field stain

_ Buffered water, pH 7.2.

Method for staining thick films

1. Dip the unfixed film into a jar containing Field stain A solution for 3 seconds.

2. Wash gently by dipping (once) into a jar of clean water for 5 seconds.

3. Dip the slide into a jar containing Field stain B solution for 3 seconds.

4. Wash the slide gently as in step 2.

5. Place the slide upright in a slide rack to air-dry.

Method for staining thin films

1. Fix the film in methanol for 1 minute.

2. Wash off the methanol with buffered water.

3. Using a pipette, cover the film with diluted Field stain B (one volume of stain plus four volumes of buffered water).

4. Immediately add an equal volume of Field stain A solution and mix well by tilting the slide.

5. Allow to stain for 1 minute.

6. Wash off the stain with clean water.

7. Place the slide upright in a slide rack to air-dry.

Dipstick test for falciparum malaria

Dipstick tests are also available for the detection of the malarial parasite *Plasmodium falciparum*. The test described here is based on the detection by monoclonal antibodies of the species-specific histidine-rich protein II (HRP-II) which is expressed by the asexual blood stages and possibly early gametocyte stages of the parasite.

Materials and reagents

_ Capillary tubes and rubber bulbs

_ Test-tubes

_ Test-tube rack

_ Reaction stand

_ Commercially available test kit containing dipsticks, test cards, reagents and controls.

The dipstick is pretreated with a mouse monoclonal antibody against HRP-II which is applied in a line across the stick about 1 cm from its base. A second dotted line of HRP-II antigen is incorporated into the dipstick about 2–3mm above the line of monoclonal antibody as a positive reagent control.

Method

1. Collect a finger-prick sample of blood from the patient.

2. Place one drop of blood into a test-tube containing three drops of lysing reagent.

3. Place one drop of the lysed blood sample into one of the wells of the test card in the reaction stand.

4. Place the dipstick in the lysed blood until all the blood has been absorbed.

5. Apply one drop of detection reagent to the base of the dipstick. This reagent consists of a suspension of micelles (phospholipid vesicles) containing sulfo-rhodamine B as a marker coupled to rabbit antibody raised against HRP-II.

6. When the reagent has been absorbed, apply two drops of washing reagent to clear the lysed blood.

If the result is positive a thin red line will be left across the dipstick with a broken line (the reagent control) above it.

If the result is negative only the broken line is seen. The whole test takes less than 10 minutes. Current studies indicate that the test has a sensitivity and a specificity of 86–95% when compared with standard light microscopy carried out by experienced technicians. A similar test for *P. vivax* is under development.

INDIRECT DETECTION OF LEISHMANIASIS: SERUM GAMMA GLOBULIN

Leishmaniasis is a group of diseases caused by infection with parasitic protozoa of the genus *Leishmania*. It can affect the skin (cutaneous leishmaniasis), mucous membranes (mucocutaneous leishmaniasis) and the reticuloendothelial system (visceral leishmaniasis or kala-azar).

Formol- gel test for visceral leishmaniasis

This test is a non-specific indicator for the increased serum levels of gamma globulin that are seen in most patients with visceral leishmaniasis.

Materials and reagents

_ Test-tubes

_ Test-tube rack

_ Centrifuge

_ Centrifuge tubes

_ Formalin (37% formaldehyde).

Method

1. Collect 2–5ml of blood into a centrifuge tube and allow it to clot.

2. Separate the serum by centrifuging the tube for 3 minutes at 5000*g* or by leaving the tube overnight in a refrigerator or on the bench.

3. Pipette 1 ml of clear serum into a test-tube.

4. Add two or three drops of formalin to the serum. Allow the tube to stand for 30 minutes.

Results

A positive result is shown by gelling of the serum — it becomes solid and turns white, usually after about 5 minutes. A negative result is recorded when there is no gelling or whitening of the serum.

***Note*: Increased gamma globulin concentrations in serum are also seen following hepatitis B infection and in certain malignant diseases, such as multiple myeloma and Waldenström macroglobulinemia.**

DETECTION AND IDENTIFICATION OF MICROFILARIAE IN BLOOD

***Wuchereria bancrofti* is a human parasitic Filariform that is the major cause of lymphatic filariasis. It is one of the three parasitic worms, together with *Brugia malayi* and *B. timori*, that infect the lymphatic system to cause lymphatic filariasis.**

Microscopic examination of capillary blood

Materials and reagents

_ Microscope

_ Microscope slides

_ Coverslips

_ Blood lancets

_ Cotton wool swabs

_ Sodium chloride, 0.85% solution

_ 70% Ethanol.

Method

1. Sterilize the third finger with ethanol. Dry well. Prick with the lancet.

2. Collect the *first drop* of blood that appears (it contains most microfilariae) directly on to the middle of the slide.

3. Add an equal-sized drop of sodium chloride solution to the slide.

4. Mix the blood and sodium chloride solution using the corner of a slide. Cover the preparation with a coverslip.

5. Examine the smear systematically under the microscope using the X 10 objective with the condenser aperture reduced. The first sign of the presence of microfilariae is rapid movement among the erythrocytes.

6. To identify the species of microfilariae, prepare two smears on another slide using two more drops of blood and stain them.

Microscopic examination of venous blood concentrated by centrifugation

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Syringes (5 ml or 10ml)**
- **Needles for venipuncture**
- **Centrifuge or microhematocrit centrifuge**

- **Conical centrifuge tubes or microhematocrit capillary tubes**
- **Plastic modelling clay**
- **Adhesive tape**
- **Anticoagulant: trisodium citrate, 2% solution in saline**
- **Formalin, 2% solution (prepared by diluting 37% formaldehyde solution 1:50 with distilled water) or saponin, 1% solution**
- **Ether**
- **70% Ethanol.**

Method

1. Collect 4 ml of venous blood. Expel into a bottle containing 1 ml of trisodium citrate solution. Mix.

2. Measure into a conical centrifuge tube 10 ml of 2% formaldehyde solution. Add 1ml of citrated blood. Mix. Wait 5 minutes for the erythrocytes to lyse.

3. Centrifuge for 5 minutes at 10000*g*. Pour off the supernatant fluid. Tap the tube to mix the deposit.

4. Place one drop of the deposit on a slide. Spread the drop to form a thin smear and leave to air-dry.

5. Fix the smear using a 1:1 mixture of ethanol and ether. Leave to dry for 2 minutes, then stain immediately as described on page 170 to identify the species of microfilaria.

Alternative method using a microhematocrit centrifuge

1. Collect 4 ml of venous blood. Expel into a bottle containing 1 ml of trisodium citrate solution. Mix.

2. Three-quarters fill a microhematocrit capillary tube with the citrated blood. Seal one end of the tube with plastic modelling clay or by heating.

3. Centrifuge in a microhematocrit centrifuge at 10000*g* for 2 minutes.

4. Lay the capillary tube on a slide and secure the two ends with adhesive tape.

5. Examine the dividing line between the blood cells and the plasma under the microscope, using the X10 objective with the condenser aperture reduced.

Alternative method using saponin lysing solution

1. Add 10ml of citrated blood (see above) to 10 ml of saponin lysing solution.

2. Mix the blood gently and leave for 15 minutes to allow the erythrocytes to lyse.

3. Centrifuge at 2000*g* for 15 minutes.

4. Remove the supernatant with a pipette and discard it into a dish containing disinfectant.

5. Transfer the deposit to a slide and cover with a coverslip.

6. Examine the entire deposit for motile microfilariae using the X 10 objective. (Microfilariae will still be motile in a "night blood" sample examined the following morning.)

7. Count the number of microfilariae in the preparation and divide by 10 to give the number of microfilariae per ml of blood.

Microscopic examination of venous blood concentrated by filtration

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Syringe,15ml**
- **Swinnex-type filter holder**
- **Polycarbonate membrane filter (25mm diameter, 5mm pore size)**
- **Filter-paper pad (25mm diameter)**
- **Shallow dish, 15 ml, with lid**
- **Blunt forceps**

- **Sodium chloride, 0.85% solution**
- **Absolute methanol**
- **Distilled water.**

Method

1. Draw up 10ml of distilled water into a syringe.

2. Draw 1ml of fresh blood or citrated blood into the syringe. Rotate gently to mix the contents. Wait for 2–3 minutes, for the erythrocytes to lyse.

3. Moisten the filter-paper pad with a few drops of distilled water and cover with the membrane filter. Place the filter on the filter holder.

4. Connect the syringe to the filter holder. Gently push the blood through the filter into a dish containing disinfectant solution.

5. Remove the syringe from the filter holder (taking care to avoid disturbing the filter) and draw up 10ml of distilled water.

6. Reconnect the syringe to the filter holder and gently push the water through the filter into the dish containing disinfectant solution, to remove the debris from the filter.

***In areas endemic for *Mansonella perstans*, a membrane filter with a pore size of 3mm should be used.**

7. Remove the syringe from the filter holder and draw up approximately 5 ml of air.

8. Reconnect the syringe to the filter holder and push the air through the filter over the dish containing disinfectant, to remove excess water from the filter. Discard the disinfectant solution into a sink.

9. Remove the syringe from the filter holder. Dismantle the filter holder and remove the membrane filter using forceps.

10. Place the membrane filter, top side facing up, on a slide. Add a drop of sodium chloride solution and cover with a coverslip.

11. Examine the entire membrane for motile microfilariae, using the X 10 objective. (Microfilariae will still be motile in a "night blood" specimen examined the following morning.

12. Count the number of microfilariae in the preparation and divide by 10 to give the approximate number of microfilariae per ml of blood.

To prepare a stained preparation, follow the method described above, with the following modifications:

8. Reconnect the syringe to the filter holder and push the air through the filter over the dish containing disinfectant, to remove excess water from the filter.

9. Remove the syringe from the filter holder and draw up approximately 7 ml of air and 3 ml of methanol.

10. Reconnect the syringe to the filter holder and push the methanol and air through the filter over the dish containing disinfectant, to fix the microfilariae and remove excess methanol from the filter, respectively.

11. Remove the syringe from the filter holder. Dismantle the filter holder and remove the membrane filter using forceps.

12. Place the membrane filter, top side facing up, on a slide. Allow to air-dry.

13. Stain with Giemsa stain as for thick films and examine the entire filter membrane using the X10 objective.

Technique for staining microfilariae

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Giemsa stain**
- **Delafield's hematoxylin stain - Methanol**
- **Buffered water.**

Method

1. Prepare a thick blood smear of the deposit as described on page 174. Allow the smear to air-dry.

2. Fix in methanol for 1 minute.

3. Stain with Giemsa stain (diluted 1 in 20 with buffered water, pH 6.8) for 30 minutes.

4. Examine the preparation under the microscope using the X10 objective. If it is difficult to distinguish the nuclei of the microfilariae, return the slide to the Giemsa stain solution for another 5–10 seconds.

5. Stain with Delafield's hematoxylin stain (diluted 1 in 10 with buffered water, pH 6.8) for 5 minutes. Wash in buffered water, pH 6.8. (This second stain is required because Giemsa stain alone does not stain the sheath of *Loa loa* very well.)

6. Examine the preparation under the microscope. Use the X 10 objective first to locate the microfilariae; then identify the filarial species using the X 40 and X100 objectives.

MICROSCOPIC DETECTION OF BACTERIA IN URINE

In healthy persons the urine contains practically no organisms. Bacteria may be found in patients who have an infection of some part of the urinary tract (e.g. urethritis, cystitis or nephritis), or where bacteria from an infection elsewhere in the body are excreted in the urine. The urine is centrifuged at high speed and the resulting deposit is examined under the microscope. This is the most important part of the analysis. However, the deposit may also be used to make smears that are stained by Gram and Ziehl–Neelsen stains and examined under the microscope. Culture is always essential for precise determination of the identity of the organisms found and the quantity present.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Sterile 250-ml Erlenmeyer flask with stopper**
- **Centrifuge**
- **Sterile conical centrifuge tubes with stoppers**
- **Inoculating loop**
- **Bunsen burner or spirit lamp**
- **70% Ethanol**
- **Reagents needed for Gram staining and Ziehl–Neelsen staining.**

Method

Collection of specimens

The genitals should be cleansed beforehand, using soap and water. Collect a midstream specimen in the sterile flask. Examine as quickly as possible. (Another way is to collect the urine in a conical tube rinsed only in boiling water and to examine it immediately.)

Preparation of slides

1. Pour 10 ml of fresh urine into a sterile centrifuge tube. Seal the tube with either a screw-cap or a plug of sterile cotton wool fixed with gauze and string.

2. Centrifuge the specimen at 1500*g* for 10 minutes. If tuberculosis is suspected, centrifuge a further 10-ml specimen at 5000*g* for 20 minutes.

3. Pour off the supernatant from the two tubes. Using an inoculating loop (sterilized by flaming), mix the deposit with distilled water until it forms a homogeneous suspension.

4. Using an inoculating loop (sterilized by flaming), prepare a smear from each of the two suspensions. Leave the slides to air-dry.

5. Fix the slides by flooding with ethanol and flaming or by heating.

6. Stain the first slide with Gram stain and the second with Ziehl–Neelsen stain.

Microscopic examination of cultured specimen

Examine the slides under the microscope using the X 100 objective.

Examine the slide stained with Gram stain for the following:

- **pus (many leukocytes stained red by Gram stain)**
- **Gram-negative bacilli**
- **Gram-positive cocci**
- **Gram-positive diphtheroid bacilli**
- **Gram-positive fungi.**

Examine the slide stained with Ziehl–Neelsen stain for tubercle bacilli. Tubercle bacilli appear dark red and are arranged in rows.

Reporting the results

State whether pus or leukocytes are present. Give a precise description of the organisms found.

Example

Organisms found:

— many leukocytes

— a few erythrocytes

— a few epithelial cells

— many Gram-positive cocci in clusters. or

Organisms found:

— a few leukocytes

— occasional erythrocytes

— a few epithelial cells

— a few Gram-negative bacilli.

Gonococci

Never diagnose a gonococcal infection on the basis of an examination of a urinary deposit. Look for gonococci in urethral pus.

Urine dipsticks to detect Bacteria in urine

Bacteria in urine may also be detected using urine dipsticks. A commercially available dipstick with reagents for the detection of nitrite (which is produced by certain pathogenic bacteria) and leukocyte esterase has been shown to have a high specificity and a high sensitivity for the detection of bacteria in urine.

The nitrite test is a rapid, indirect method for the early detection of significant and asymptomatic bacteriuria. Common organisms that can cause urinary tract infections, such as *Escherichia coli*, Enterobacter, Citrobacter, Klebsiella, and Proteus species, produce enzymes that reduce urinary nitrate to nitrite.

Reagent strips for the detection of nitrite in the urine commonly use *p*-arsanilic acid and a quinoline compound. Nitrite results are read at 30 or 60 seconds, depending on the manufacturer. Any degree of uniform pink color should be interpreted as a positive nitrite test suggesting the presence of 105 or more organisms per milliliter. The test is reported as positive or negative.

Fig. Nitrate color chart.

EXAMINATION OF UROGENITAL SPECIMENS FOR GONORRHEA

The urogenital sinus is a part of the human body only present in the development of the urinary and reproductive organs. It is the ventral part of the cloaca, formed after the cloaca separates from the anal canal during the fourth to seventh weeks of development.

***Neisseria gonorrhoeae*, also known as *gonococcus* (singular), or *gonococci* (plural) is a species of gram-negative diplococci bacteria isolated by Albert Neisser in 1879. It causes the sexually transmitted genitourinary infection gonorrhea as well as other forms of gonococcal disease including disseminated gonococcemia, septic arthritis, and gonococcal ophthalmia neonatorum.**

Materials and reagents

- **Microscope-**
- **Microscope slides**
- **Bottle, 100ml**
- **Pasteur pipette**
- **Cotton wool- amides transport medium.**

Method

Collection of specimens

From male patients

1. If possible, collect the specimen first thing in the morning before the patient passes urine.

2. Clean around the urethral opening with sterile saline.

3. Apply gentle pressure on the penis so that a drop of pus appears on the meatus; if no pus appears, gently massage the urethra from above downwards.

4. Collect a sample of the pus using a sterile cotton wool swab on a stick. Insert the swab into a small bottle containing Amides transport medium. Cut the stick to allow the top to be tightened.

5. Use another swab to collect a drop of the pus for Gram staining.

From female patients

The specimen should be taken from the cervical canal by a physician or specialist nurse. In cases of chronic gonorrhea, the specimen should be taken just before or just after the menstrual period.

Preparation of slides

Prepare a smear from each of the specimens. Leave the smears to air-dry and then stain with Gram stain.

Microscopic examination

Microscopic examination is of great value in the diagnosis of gonorrhea in males: it is much less so in females. Culture is therefore necessary to isolate and identify the gonococci in specimens from females. Examine the slides using the X100 oil-immersion objective. Pay particular attention to the edges of the smears, where the elements are spread more thinly and are easier to see and the stain is less concentrated.

Pus cells have a pink nucleus and a colorless cytoplasm. The nucleus may appear degenerated.

Gonococci appear as Gram-negative diplococci (in pairs (a)). Cocci appear oval and kidney-shaped. Extracellular Gram-negative diplococci ((b)) should also be reported. A presumptive diagnosis of gonorrhea can be made if Gram-negative intracellular diplococci are seen in smears from male patients. Extracellular Gram-negative diplococci may be seen if the pus cells are damaged.

Report as:

— Gram-negative intracellular diplococci present;

— Gram-negative extracellular diplococci present;

— no Gram-negative diplococci found.

Other bacteria that cause infections in male patients

Numbers of the following may occasionally be seen in smears of urethral pus:

— Gram-positive cocci (e.g. staphylococci);

— Gram-positive bacilli (e.g. diphtheria bacilli);

— Gram-negative bacilli (e.g. coliform bacilli).

Other bacteria that cause infections in female patients

All kinds of organisms are found in the smears, particularly:

— Gram-positive bacilli;

— Gram-negative cocci (saprophytes).

Dispatch of specimens for culture

Using Stuart transport medium

Sending the specimen in Stuart transport medium, modified is the best method, if the medium can be obtained from a specialized laboratory. It is usually supplied in 30-ml flat bottles that contain 8 ml of solid medium (along one side of the bottle) and are filled with a mixture of air (90%) and carbon dioxide (10%). Round bottles may also be used. The bottle should remain open for as short a time as possible to prevent the escape of gas.

Method

1. Place the bottle of medium upright. Collect the pus specimen on a swab. Unscrew the bottle cap.

2. Holding the bottle as upright as possible (to prevent the gas escaping), rub the swab of pus over the whole surface of the solid medium, from one side of the bottle to the other, starting from the bottom.

3. Replace the cap on the bottle at once. Dispatch the bottle (at ambient temperature) immediately.

Maximum preservation time: 3 days, but the shorter the delay the better. This transport medium is also suitable for meningococci.

Using a Pasteur pipette

Method

1. Collect the pus specimen on a sterile cotton wool swab.

2. Draw the pus specimen into a sterile Pasteur pipette plugged with cotton wool.

3. Place the pipette in a sterile test-tube, padded and plugged with cotton wool.

Maximum preservation time: 6 hours (at ambient temperature).

MICROSCOPIC DETECTION OF PARASITES FOUND IN URINE

Parasites may occasionally be found in the urine, either because they are indigenous to the urinary tract or as the result of vaginal or fecal contamination. Chemical analysis does not detect parasites in urine. Microscopic evaluation of urinary sediment is important if parasitic infections are suspected. Chemical analysis may reveal the presence of leukocytes, if present during these infections.

TRICHOMONAS VAGINALIS

***Trichomonas vaginalis* is the most frequently occurring parasite in the urine. It is a flagellate organism that is about the same size as a large white cell. In the unstained wet mount, the organism should not be reported unless it is motile. Sometimes when bacteria are next to a white cell, the cell may be mistaken for Trichomonas, which is why motility is the diagnostic feature. This organism may be found in males, although it is more common in females. *T. vaginalis* is frequently accompanied by WBCs and epithelial cells.**

ENTEROBIUS VERMICULARIS

***Enterobius vermicularis* (pinworm) ova and occasionally also the female adult may be found in the urine, perhaps even more frequently than was once believed. Occasionally the eggs of *Enterobius vermicularis* are found in urine, especially from young girls when the eggs are washed off the external genitalia when urine is being passed. The ova are very characteristic in shape, having one flat and one rounded side. The developing larva can usually be observed through the transparent shell of the egg. If the urine is found to contain many ova, examination of the original urine container may reveal the adult worm**

SCHISTOSOMA HAEMATOBIUM

In countries where schistosomiasis is endemic, urine specimens are examined for eggs of *Schistosoma haematobium*. Trophozoites of *Trichomonas vaginalis*

may also be seen. Microfilariae of *Washeteria bancrofti* and *Onchocerca volvulus* may also be found in the centrifuged sediment of urine from patients in countries where filariasis is endemic. The first indirect evidence of *Schistosoma haematobium* infection is hematuria and/ or proteinuria, which is detectable using a urine dipstick. Gross hematuria indicates heavy infection.

The two methods used for detection of ova of *Schistosoma haematobium* are sedimentation and filtration. The sedimentation method is less sensitive but is cheaper and simpler to perform. The filtration technique is used when quantitative information is required for epidemiological surveillance purposes.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Centrifuge (sedimentation method)**
- **Conical centrifuge tubes, 15 ml (sedimentation method)**
- **Filter holder, 13 or 25mm diameter (filtration method)**
- **Membrane filter, 12–20mm pore size (nylon or polycarbonate) or Whatman No. 541 (or equivalent) filter-paper (filtration method)**
- **Conical flask for urine collection**
- **Pasteur pipettes (sedimentation method)**
- **Plastic syringe, 10 ml (filtration method)**
- **Lugol iodine, 0.5% solution (filtration method)**
- **Formaldehyde, 37% solution.**

Method

Collection of urine specimens

The number of ova in the urine varies throughout the day; it is highest in urine obtained between 10:00 and 14:00. The specimen should therefore be collected between these times and should consist of a single terminal urine specimen of at least 10 ml. Alternatively, a 24-hour collection of terminal urine can be made.

The whole specimen must be examined, as it may contain only a few ova. Ask the patient to collect the urine in a clean flask or bottle. Examine the specimen at once. If the urine cannot be examined for an hour or longer, add 1 ml of

undiluted formalin (37% formaldehyde solution) to each 100 ml of urine. This will preserve any eggs that might be present.

***Note*: If formalin is not available, 2 ml of ordinary household bleach can be added to each 100 ml of urine.**

***Warning*: Formalin and bleach are corrosive and must not be swallowed.**

Sedimentation method

1. Shake the urine specimen well and pour into the conical flask.

2. Allow the urine to sediment for 1 hour. Remove the supernatant and transfer the sediment into a centrifuge tube. Centrifuge at 2000*g* for 2 minutes.

3. Examine the deposit under the microscope for the presence of ova. Do not increase the centrifugation time and do not exceed 2000*g* as this may disrupt the ova and release miracidia.

***Important*:**

— process the specimen as soon as possible;

— shake the container before pouring the urine specimen into the conical flask;

— label slides and tubes carefully.

Filtration method

1. Place a filter in the filter holder.

2. Agitate the urine sample gently and draw 10ml into the syringe. Attach the syringe to the filter holder.

3. Expel the urine from the syringe through the filter over a bucket or sink.

4. Disconnect the syringe from the filter holder. Draw air into the syringe, reattach the syringe to the filter holder and expel the air through the filter.

5. Disconnect the syringe from the filter holder. Using forceps, carefully remove the membrane filter or filter-paper and place it on a microscope slide. The nylon

membrane and filter-paper should be placed face-up, while the polycarbonate membrane should be placed face-down.

6. Add one drop of Lugol iodine solution to improve the visibility of the eggs.

7. Examine the entire filter under the microscope at X10 or X 40. Record the results as the number of eggs per 10 ml of urine.

Reuse of filters

If you have used a plastic filter, remove it immediately after use and soak it overnight in a 1% hypochlorite solution (domestic bleach). After soaking the filter, wash it thoroughly with detergent solution, then rinse it several times with clean water. Check the filter under the microscope to ensure that it is free of parasites before reusing it.

Microscopic examination

The eggs of *Schistosoma haematobium* are large, about 120–150mm long, and have a terminal spine at one end. An embryo (the miracidium) can be seen inside the egg. Sometimes it is necessary to determine whether the eggs are viable. This can be done if the specimen is fresh and no preservatives have been added. Look carefully at the eggs to see if the embryos are moving. This is the best indication of viability. If no movement is seen, look for the "flame cells". There are four flame cells, one at each corner of the embryo. Use a X100 objective with slightly reduced illumination to look for the rapid movement of cilia (short hairs) in the flame cells.

Reporting the results

When the syringe filtration technique is used, the results may be reported according to egg count categories:

_ Light infection: 1–49 eggs per 10 ml of urine.

_ Heavy infection: > 50 eggs per 10 ml of urine.

A third category, such as >500 eggs per 10 ml of urine, or >1000 eggs per 10 ml of urine, may be appropriate in areas where the intensity of infection frequently reaches this level (i.e. in more than 10% of cases).

Other parasites that may be found in urine

Very occasionally the microfilariae of *Wuchereria bancrofti* can be found in urine. This happens when a urogenital lymphatic vessel rupture. The urine appears milky-white or reddish-pink (Chile mixed with blood). The microfilariae are large (225–300 X10 µm), motile, and sheathed. No nuclei are present in the tail (feature looked for in a Giemsa stained preparation).

Microfilariae of *Onchocerca volvulus* may be found in the urine in onchocerciasis, especially in heavy infections. The larvae are large (280–330X 7 µm), unsheathed, with a slightly enlarged head-end and a tail which is sharply pointed and contains no nuclei

In addition, urine sample may contain Spermatozoa

Occasionally found in the urine of men, they can be easily recognized by their head and long thread-like tail. They may be motile in fresh urine.

MICROSCOPIC DETECTION OF MOST COMMON INTESTINAL PROTOZOA FOUND IN STOOLS

Protozoa are microorganisms consisting of a single cell. Intestinal protozoa may be found in stools in their motile form (trophozoites) or as cysts. Some intestinal protozoa are pathogenic (see Table); others are harmless. All these protozoa are found throughout the world.

Table: Pathogenicity of intestinal protozoa

Species	Pathogenicity
Amoebae	
Entamoeba histolytica	**Only amoeba that is commonly pathogenic to humans. May cause dysentery or abscesses**
Entamoeba coli	**Non-pathogenic, but very common**
Entamoeba hartmanni, Endolimax nanus, Iodamoeba butschlii, Dientamoeba fragilis	**Non-pathogenic. Differentiation is difficult but not really necessary; it is enough to be able to distinguish these species from *Entamoeba histolytica***
Flagellates	
Giardia intestinalis	**Pathogenic**
Trichomonas hominis	**Non-pathogenic**
Chilomastix mesnili	**Non-pathogenic**
Ciliates	
Balantidium coli	**Pathogenic**

EXAMINATION OF STOOL SPECIMENS FOR PARASITES

Collection of specimens

Collect approximately 100 g of faces in a clean, dry container without preservatives. A screw-top container is most suitable. Make sure that any adult worms or segments passed are included.

Visual examination

Fecal samples are best described by their color, consistency and presence or absence of macroscopic blood or exudate.

Color

The color can be described as:

— black (occult blood)

— brown, pale yellow (fat)

— white (obstructive jaundice).

Consistency

The consistency can be described as:

— formed (normal shape)

— soft formed

— unformed or liquid (watery).

The presence of external blood or mucus, usually seen as streaks of red or white, should be noted. Blood may be present in certain medical conditions (e.g. ulcerative colitis, schistosomiasis).

Microscopic examination

Direct microscopic examination of faces in saline or iodine suspension is useful for the following reasons:

—to detect motile trophozoites;

—to detect ova and cysts present in moderate numbers;

—to detect erythrocytes, cellular debris or excess fat.

Select unformed or liquid faces when using direct microscopy for detection of trophozoites. Formed stools rarely contain motile trophozoites. Also perform a direct examination of any external blood or mucus.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Wooden applicators or wire loops (0.45mm, nickel–chromium alloy wire)**
- **Grease pencils**
- **Sodium chloride, 0.85% solution**
- **Lugol's iodine, 0.5% solution**
- **Acetic acid, 50% solution, diluted 1:1 with distilled water**
- **Methylene blue solution**
- **Eosin, 2% solution in saline.**

Method

1. Prepare a 1:1 mixture of Lugol's iodine solution and acetic acid solution (diluted as above). Dilute the mixture with four volumes of distilled water and stir.

2. Take a dry microscope slide and label it with the name or number of the patient.

3. Put:

— one drop of sodium chloride solution warmed to 37°C in the middle of the left half of the slide; and

— one drop of the iodine–acetic acid solution in the middle of the right half of the slide.

4. Using an applicator or wire loop, take a small portion (about 2–3mm diameter) of the stool.

(a) If the stools are formed, take the portion from the center of the sample and from the surface to look for parasite eggs.

(b) If the stools contain mucus or are liquid, take the portion from the mucus on the surface or from the surface of the liquid to look for amoebae.

5. Mix the sample with the drop of sodium chloride solution on the slide.

6. Using the applicator or wire loop, take a second portion of stool from the specimen as described above and mix it with the drop of iodine–acetic acid solution.

Discard the applicator (or flame the wire loop) after use.

7. Place a coverslip over each drop (apply the coverslips in a way to avoid the formation of air bubbles).

8. Examine the preparations under the microscope. For the saline preparation use the X 10 and X 40 objectives and a X 5 eyepiece. As the eggs and cysts are colorless, reduce the amount of light using the condenser aperture or lower the condenser to increase the contrast.

Examine the first preparation with the X10 objective, starting at the top left-hand corner Focus on the edge of one coverslip using the X10 objective and examine the whole area under each coverslip for the presence of ova and larvae of *Strongyloides stercoralis*. Then switch to the X40 objective and again examine the whole area of the coverslip over the saline for motile trophozoites and the area of the coverslip over the iodine for cysts.

9. Lugol's iodine – acetic acid solution causes the trophozoite forms to become nonmotile. The nucleus is clearly stained but it may be difficult to distinguish between trophozoite and cystic forms.

10. Using a fine Pasteur pipette, allow a drop of methylene blue solution to run under the coverslip over the saline preparation. This will stain the nuclei of any cells present and distinguish the lobed nuclei of polymorphs from the large single nuclei of mucosal cells.

11. If a drop of eosin solution is added, the whole field becomes stained except for the protozoa (particularly amoebae), which remain colorless and are thus easily recognized.

Dispatch of stools for detection of parasites

Stools may be sent to a specialized laboratory for the identification of rare parasites that are difficult to recognize. In such cases a preservative should be added to the specimens before they are dispatched for examination.

The following preservatives are used:

— formaldehyde, 10% solution, for wet mounting;

— Lugol's iodine, 0.5% solution;

— polyvinyl alcohol (PVA) fixative;

— thiomersal–iodine–formaldehyde (TIF) fixative, for wet mounting.

1. Prepare a mixture containing about one part of stool to three parts of formaldehyde solution.

2. Crush the stool thoroughly with a glass rod. Formaldehyde solution preserves eggs and cysts of parasites indefinitely if the specimen container is tightly closed. It does not preserve vegetative forms of protozoa, which are destroyed after a few days. Using polyvinyl alcohol fixative

In a bottle

1. Pour about 30 ml of PVA fixative into a 40-ml bottle.

2. Add enough *fresh* stools to fill the last quarter of the bottle.

3. Mix thoroughly with a glass rod.

PVA fixative preserves all forms of parasites indefinitely.

On a slide

1. To examine for amoebae and flagellates, place a small portion of the stool on one end of the slide.

2. Add three drops of PVA fixative to the stool.

3. Using a glass rod, carefully spread the specimen over about half of the slide. Leave to dry for 12 hours (preferably at 37 °C).

Specimens preserved in this way can be kept for about 3 months. They can be stained on arrival at the specialized laboratory.

Using thiomersal1–iodine–formaldehyde fixative

1. Just before dispatch, mix 4.7 ml of TIF fixative and 0.3 ml of lughole iodine solution in a tube or a small bottle.

2. Add approximately 2 ml (2cm^3) of stool and crush well with a glass rod.

The above-mentioned mixture preserves all forms of parasites indefinitely, including vegetative forms of amoebae (those of flagellates deteriorate slightly).

Rapid Field stain for fecal trophozoites

A trophozoite is the activated, feeding stage in the life cycle of certain protozoan parasites such as in the malaria-causing *Plasmodium falciparum* and those of the *Giardia* group.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Slide rack**
- **Field stain:**
  -Field stain A (undiluted) is methylene blue and Azure 1 dissolved in phosphate buffer solution

  -Field stain B (diluted one part of stain in four parts of distilled water) is Eosin Y in buffer solution.

- **Sodium chloride, 0.85% solution**

- **Methanol.**

Method

1. Prepare a thin fecal smear in sodium chloride solution on a clean slide.

2. Once the smear is dry, fix it by covering the slide with methanol for 3 minutes.

3. Tip off the methanol.

4. Pipette 1 ml of diluted Field stain B onto the slide, followed by 1 ml of undiluted Field stain A.

5. Mix well by tilting the slide and allow to stain for 1 minute.

6. Wash the slide in water and allow it to air-dry.

7. Examine the slide using the X100 oil-immersion objective. Scan the smear, particularly around the edges

The cytoplasm and flagella of trophozoites of *Giardia intestinalis* stain blue and their nuclei stain red. Cysts of *G. intestinalis* also stain blue and their nuclei stain red.

***Note*:**

_ Leave freshly prepared stains for 3 days before use.

_ Use rainwater to prepare the stains if the local well-water supply is too salty.

_ Cover the jars containing the staining solutions to prevent evaporation and absorption of dust.

_ Avoid carrying over one staining solution to another.

Eosin stain for fecal trophozoites and cysts

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Slide rack**
- **Coverslips**
- **Eosin, 1% solution.**

Method

1. Emulsify a small portion of stool in 1% eosin solution on a clean slide. Spread over an area of approximately 2cm X 1cm.

2. Put a coverslip on the slide and place it on the microscope stage.

3. Use the X10 objective to examine the smear systematically for unstained trophozoites and cysts. Examine in more detail with the X40 objective.

The eosin stain provides a pink background against which unstained trophozoites and cysts are clearly visible.

***Note*: If 1% eosin solution is not available, use a drop of Field stain B (see above).**

Microscopic examination of cysts

Preparation in saline wet mount

Cysts can be seen as transparent shiny globules standing out clearly against a grey background. They have well-defined shells. Using the X 40 objective, look for shiny round objects with a diameter roughly equal to 1–3 erythrocytes.

Chromatid bodies

Look also for chromatid bodies (rod-shaped structures). Chromatid bodies are more distinct in saline mounts than in iodine mounts. These bodies are characteristic in appearance and occur in cysts of *Entamoeba histolytica* and *E. coli*. The rod-shaped chromatid bodies of *E. histolytica* have blunt rounded ends; those of *E. coli* have pointed Chromatid bodies ends. These chromatid bodies are seen less frequently in cysts of *E. coli* than in those of *E. histolytica*.

Nuclei

Nuclei are not easily visible in saline mounts but are clearly seen in iodine mounts. The appearance of the nucleus is important in differentiating between species of amoeba. Therefore, if cysts (or cyst-like bodies) are seen in the saline mount, examine an iodine mount.

Measurement

Accurate measurement of cysts is essential for their correct identification. Measure any cysts you find; if possible, use a calibrated graticule in the eyepiece.

Preparation in iodine wet mount

Iodine mounts are used to detect cysts of amoebae and flagellates. Cysts can be detected with the X10

objective. Use the X40 objective to see the characteristics of the cysts and measure them to ensure correct identification. Iodine stains the cytoplasm of the cysts yellow or light brown; nuclei are stained dark brown. When cysts of *Entamoeba* spp. are stained with iodine, the arrangement of the peripheral chromatin and the position of the karyosome can be seen. (If the peripheral chromatin is absent, the cyst is not a species of *Entamoeba*.) These peripheral chromatid bodies stain light yellow and may not be very clear. Sometimes, young cysts contain glycogen; this stain dark brown with iodine. Staining flagellate cysts with iodine enables the fibrils (filaments) to be seen. Cysts of several different species may be found in the same stool specimen.

Concentration

If necessary, use the formaldehyde–ether sedimentation technique to examine a larger number of cysts for more certain identification.

Saline and eosin preparations to detect *E. histolytica* and other parasites

– Place a drop of fresh physiological saline on one end of a slide and a drop of eosin stain (Reagent No. 36) on the other. Using a piece of stick or wire loop, mix a small amount of *fresh* specimen (especially mucus and blood) with each drop. Cover each preparation with a cover glass.

***Important*: The eosin preparation must not be too thick otherwise it will not be possible to see amoebae or cysts.**

– Examine the preparations using the 10X and 40X objectives with the condenser iris closed sufficiently to give good contrast.

– Look especially for motile *E. histolytica* trophozoites containing red cells, motile *G. lamblia* trophozoites, motile *Strongyloides* larvae, and the eggs and cysts of parasitic pathogens.

ADDITIONAL

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

Testing for Cryptosporidiosis causes diarrhea

Modified Ziehl–Neelsen technique for staining oocysts of *Cryptosporidium* spp.

***Cryptosporidium* is a genus of apicomplexan parasitic alveolates. Infections with *Cryptosporidium* spp. cause fever, abdominal cramps, diarrhea and weight loss with an associated eosinophilia. In severe cases, a malabsorption syndrome may develop. Cryptosporidiosis causes self-limiting diarrhea in children. It is a recognized cause of chronic diarrhea in adults with lowered immunity, e.g. patients with acquired immunodeficiency syndrome (AIDS). Cryptosporidiosis should be suspected in patients with chronic diarrhea and weight loss, for which no other cause can be found.**

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Slide rack**
- **Petri disk**
- **Cotton wool**
- **Sodium chloride, 0.85% solution**

- **Formaldehyde, 37% solution (formalin)**
- **Carbol fuchsin for Ziehl–Neelsen stain**
- **Acid–ethanol for Ziehl–Neelsen stain**
- **Malachite green, 1% solution**
- **Methanol.**

Method

1. Emulsify a small amount of faces in saline on a clean slide. Spread over an area of approximately 2cm X 1cm.

2. Allow the smear to dry before fixing in absolute methanol for 5 minutes. If the patient is known to be, or suspected of being, positive for human immunodeficiency virus (HIV), fix the smear in formalin vapor for 15 minutes by placing the slide in a Petri dish with a cotton wool ball soaked in formalin.

3. Flood the slide with carbol fuchsin for 5 minutes. Wash the stain off with water.

4. Flood the slide with acid–ethanol solution to decolorize until faint pink. Wash the slide in water.

5. Counterstain the slide with malachite green solution for 2 minutes. Wash in water and place in a slide rack to drain and dry.

Examine the slide under the microscope using the X40 objective.

Oocysts of *Cryptosporidium* spp. stained by this method may show a variety of stain reactions from pale pink to deep red. The oocysts measure 4–6mm. The sporozoites within the oocysts have an outer rim of deep stained material with a pale center. This differentiates oocysts from some yeasts which may stain red but have a homogeneous smooth appearance. The oocysts of *Cryptosporidium* spp. are highly resistant to disinfecting agents.

***Note*: *Cryptosporidium* spp. belong to a group of parasites called coccidia. Other parasites of this group are: *Isospora belli, Toxoplasma gondii* and *Plasmodium* spp.**

Features not to be mistaken for cysts: **Fungi, *Blastocystis hominis* (yeast), *Leukocytes* (white blood cells). The presence of pus should be reported as it is a sign of infection.**

MICRESCOPIC DIGNOSIS OF INTESTINAL HELMINTHS

Helminth infections cause a variety of clinical symptoms including abdominal cramps, fever, weight loss, vomiting, appendicitis, blood loss, anemia and eosinophilia.

There are three groups of medically important helminths:

— nematodes (roundworms)

— cestodes (tapeworms)

— trematodes (flukes).

Helminth infections are usually diagnosed by detecting eggs and larvae. Less frequently, infections are diagnosed by detecting adult worms (e.g. *Ascaris lumbricoides* and *Enterobius vermicularis*) or proglottids (segments) of adult worms (e.g. *Taenia saginata* and *T. solium*). However, for most helminth infections, eggs are used for identification.

Technique for the collection and examination of eggs

Principle: The eggs of *Enterobius vermicularis* (pinworm) are usually collected (particularly in children) from the folds of skin around the anus. They rarely appear in the stools.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Test-tubes**
- **Pasteur pipette**
- **Adhesive cellophane tape**
- **Spoon 10cm long or, better, a wooden tongue depressor**
- **Cotton wool**
- **Sodium chloride, 0.85% solution.**

Method 1

1. Place a strip of cellophane tape, sticky side down, on a slide.

2. Place the spoon handle against the underside of the slide.

3. Gently pull the tape away from the slide and loop it over the end of the spoon handle.

4. Hold the completed tape swab in your right hand, pressing the slide firmly against the spoon.

5. Separate the patient's buttocks with your left hand. Press the end of the spoon covered with tape against the skin round the anus in several places.

6. Take the slide and fold the tape back on to it, sticky side down.

7. Make sure that the tape is firmly stuck flat to the slide by pressing it with a piece of cotton wool.

8. Examine under the microscope with the condenser aperture reduced, using the X10 objective. Look for eggs of *E. vermicularis*.

Alternative method

1. If no cellophane tape is available, use a cotton wool swab to wipe around (but not inside) the anus.

2. Dip the swab into a test-tube containing about 0.5ml (10 drops) of sodium chloride solution. Rinse the swab well in the solution.

3. Draw up the liquid with a Pasteur pipette. Transfer it to a slide, cover with a coverslip and examine under the microscope as described in step 8 above.

Cellophane fecal thick-smear technique for diagnosis of *Schistosoma mansoni* infection (Kato–Katz technique)

The Kato–Katz technique has proved to be an efficient means of diagnosing *S. mansoni* and certain other intestinal helminth infections. The technique is not suitable for diagnosing strongyloidiasis or infections with *Enterobius vermicularis* or protozoa.

Materials and reagents

- **Flat-sided applicator stick, wooden**
- **Screen, stainless steel, nylon or plastic, 60–105 mesh**
- **Template, stainless steel, plastic or cardboard**
- **Microscope**
- **Microscope slides**
- **Cellophane, 40–50mm thick, in strips 25mm X 30mm or 25mm X 35mm**
- **Flat-bottomed jar**
- **Forceps**
- **Toilet paper or absorbent tissue**
- **Scrap paper (e.g. newspaper)**
- **Glycerol–malachite green solution or methylene blue solution.**

Method

***Important*: Care must be taken to avoid contamination during collection of stool specimens. Always wear gloves.**

1. Soak the cellophane strips in the glycerol–malachite green (or methylene blue) solution for at least 24 hours before use.

2. Transfer a small amount (approximately 0.5 g) of faces on to a piece of scrap paper (newspaper is ideal).

3. Press the screen on top of the fecal sample.

4. Using the applicator stick, scrape across the upper surface of the screen to sieve the fecal sample.

5. Place the template on a clean microscope slide. Transfer the sieved fecal material into the hole of the template and level with the applicator stick.

6. Remove the template carefully so that all the fecal material is left on the slide and none is left sticking to the template.

7. Cover the fecal sample on the slide with a glycerol-soaked cellophane strip.

8. If any glycerol is present on the upper surface of the cellophane, wipe it off with a small piece of absorbent tissue.

9. Invert the microscope slide and press the fecal sample against the cellophane on a smooth surface (a piece of tile or flat stone is ideal) to spread the sample evenly.

10. Do not lift the slide straight up or it may separate from the cellophane. Gently slide the microscope slide sideways while holding the cellophane.

Preparation of the slide is now complete. Wipe off any excess glycerol with a piece of absorbent tissue to ensure that the cellophane stays fixed. With practice you can obtain perfect preparations.

Identification of adult helminths

Adult helminths brought to the laboratory for identification may have been found in stools, in clothing or bed linen, or during a surgical operation.

What to examine:

— their length

— their shape

— whether they are flat or segmented

— whether they are cylindrical (round)

— their internal structure.

Examination

Materials and reagents

- **Microscope or magnifying glass**
- **Microscope slides**
- **Petri dish**
- **Forceps.**

Method

- **Examine a chain of segments to observe the arrangement of the lateral pores.**
- **Examine a single segment gently flattened between two slides.**

Hold the slide against the light to observe and count the uterine branches with the naked eye.

To examine the head (scolex):

1. Place the whole worm in a Petri dish (or on a plate) filled with water.

2. Using forceps, transfer the worm little by little into another dish; untangle it, starting with the thicker end.

3. If at the end of a very narrow section (the neck) you find a swelling the size of a small pinhead, examine it under the microscope with the X 10 objective or with a magnifying glass. (The head is rarely found.)

Techniques for concentrating parasites

Concentration techniques are used when the number of helminthic ova or larvae, or protozoal cysts or trophozoites, is small. Four different concentration techniques are described in this book:

— the flotation technique using sodium chloride solution (Willis)

— the formaldehyde–ether sedimentation technique (Allen & Ridley)

— the formaldehyde–detergent sedimentation technique

— the sedimentation technique for larvae of *Strongyloides stercoralis* (Harada–Mori).

***Important*: Always make a direct microscopic examination of stools before preparing a concentration. (Motile forms of protozoa are not found in concentrated preparations.)**

Flotation technique using sodium chloride solution (Willis)

This method is recommended for the detection of eggs of *Ancylostoma duodenale* and *Necator americanus* (best method), *Ascaris lumbricoides*, *Hymenolepis nana*, *Taenia* spp. and *Trichuris trichiura*. It is not suitable for the detection of eggs of flukes and *Schistosoma* spp., larvae of *Strongyloides stercoralis*, or protozoal cysts or trophozoites.

Principle: The stool sample is mixed with a saturated solution of sodium chloride (increasing the specific gravity). The eggs are lighter in weight and float to the surface where they can be collected.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Wide-mouth bottle, 10ml**
- **Wooden applicators**
- **Gauze**
- **Petri dishes**
- **95% Ethanol**
- **Ether**
- **Willis solution**
- **Petroleum jelly**
- **Wax.**

Concentration of parasites

1. Place approximately 0.5 g of stool in a wide-mouth bottle. Fill the bottle to the 2.5-ml mark with Willis solution.

2. Using an applicator, crush the portion of stool and mix it well with the solution. Then fill the bottle to the top with Willis solution; the suspension should be completely uniform.

3. Place a coverslip carefully over the mouth of the bottle.

4. Check that the coverslip is in contact with the liquid, with no air bubbles. Leave for 10 minutes.

5. Remove the coverslip with care; a drop of liquid should remain on it. Place the coverslip on a slide and examine under the microscope (using the X10 objective) at once because the preparation dries very quickly. Otherwise seal the coverslip with petroleum jelly and wax. Use the fine adjustment of the microscope to examine every object in the field (eggs tend to stick to the coverslip and are not immediately distinct).

Formaldehyde–ether sedimentation technique (Allen & Ridley)

Principle: The stool specimen is treated with formaldehyde, which preserves any parasites present. Lumpy residues are removed by filtration. Fatty elements of the fecal suspension are separated by extraction with ether (or ethyl acetate), followed by centrifugation, which sediments any parasites present.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Centrifuge**
- **Test-tubes**
- **Test-tube rack**
- **Centrifuge tubes**
- **Wooden applicators**
- **Brass wire filter, 40 mesh (425mm), 7.2 cm diameter (nylon coffee strainers provide an inexpensive alternative)**
- **Small porcelain or stainless-steel dish or beaker**
- **Pasteur pipette**

Method

Preparation of grease-free coverslips

1. Mix in a cylinder: 10 ml of 95% ethanol and 10 ml of ether.

2. Pour into a Petri dish and in it place 30 coverslips, one by one; shake and leave for 10 minutes.

3. Take the coverslips out one by one and dry them with gauze.

4. Keep them in a dry Petri dish.

_ Formalin, 10% solution (100 ml of formaldehyde, 37% solution in 900 ml of distilled water)

_ Ether (or ethyl acetate).

Method

1. Using a wooden applicator, remove a small amount (approximately 0.5 g) of faces from both the surface and the inside of the stool specimen.

2. Place the sample in a centrifuge tube containing 7 ml of 10% formalin.

3. Emulsify the faces in the formalin and filter into the dish.

4. Wash the filter (with soapy water) and discard the lumpy residue.

5. Transfer the filtrate to a large test-tube. Add 3ml of ether (or ethyl acetate).

6. Stopper the tube and mix well.

7. Transfer the resulting suspension back to the centrifuge tube and centrifuge at 2000*g* for 1 minute.

8. Loosen the fatty plug with an applicator and pour the supernatant away by quickly inverting the tube.

9. Allow the fluid remaining on the sides of the tube to drain on to the deposit and then mix well. Using the pipette, transfer a drop on to the slide and cover with a coverslip.

10. Use the X10 and X 40 objectives to examine the whole of the coverslip for ova and cysts. It is now common practice to perform all the above steps in a biological safety cabinet. If the extraction system of the cabinet is not fireproof, the steps involving ether should be done outside the cabinet. Ethyl acetate provides a less flammable alternative to ether.

Formaldehyde–detergent sedimentation technique

Principle: The formaldehyde–detergent sedimentation technique is an inexpensive, safe and simple quantitative sedimentation method in which a measured amount of faces is mixed in formaldehyde–detergent solution of low specific gravity. The suspension is sieved and is then left undisturbed to allow the ova to sediment under their own weight. The detergent "clears" the fecal debris in a short time. Following sedimentation and clearing, the small amount

of fine sediment which forms is examined under the microscope for ova and the eggs are counted to give a quantitative result.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Commercial test kit, consisting of a conical-based container, a plastic strainer, a Pasteur pipette, a beaker and a commercial detergent, diluted 1:50 with distilled water**
- **Formalin, 2% solution (prepared by diluting formaldehyde, 37% solution 1:50 with distilled water).**

Method

Details of the method as supplied with the kit are as follows:

1. Fill the conical-based container to the 10-ml mark with 2% detergent in 2% formalin.

2. Using the spoon attached to the lid of the container, transfer approximately 350mg of faces to the container and mix well in the formaldehyde–detergent solution.

3. Using the plastic strainer, strain the suspension into the beaker supplied with the kit. Rinse the container and then add the filtrate.

4. Stand the container upright in the rack provided and leave for 1 hour (do not centrifuge). Under field conditions, the emulsified faces can be transported back to the laboratory for examination. The schistosome eggs are fixed and will not become distorted.

5. Carefully remove and discard the supernatant fluid, taking care not to disturb the sediment which has formed in the base of the container.

6. Add 10ml of the formaldehyde–detergent solution; mix and allow to sediment for a further 1 hour. Further clearing of the fecal debris will take place.

7. Remove and discard the supernatant fluid, leaving approximately 0.5 ml of fine sediment.

8. Using the Pasteur pipette, transfer the entire sediment to a slide and cover with a 22mm X 40mm coverslip (supplied with the kit).

9. Examine the entire preparation under the microscope, using the X 10 objective with the condenser iris closed sufficiently to give good contrast.

Count all the ova present and multiply the number by 3 to give the approximate number per gram of faces.

***Note*: If the supernatant fluid is not removed after 1 hour, but instead a further 10ml of reagent is added and the suspension is remixed and allowed to sediment overnight, ova, cysts and larvae of other parasites will be sedimented. The technique is of particular value in laboratories without the facilities to perform the formaldehyde–ether sedimentation technique. The formalin preserves the parasites without distorting their morphology.**

Sedimentation technique for larvae of *Strongyloides stercoralis* (Harada–Mori)

Principle: A strip of filter-paper is partially submerged in a test-tube containing water. Any larvae of *Strongyloides stercoralis* present in the specimen migrate against the current of water that rises by capillary action and accumulate at the bottom of the tube.

Materials and reagents

- **Microscope**
- **Cellophane tape**
- **Test-tubes**
- **Test-tube rack**
- **Strips of filter-paper (30mm X 150mm)**
- **Spatula**
- **Lugol iodine, 0.5% solution.**

Method

1. Use the spatula to spread a small quantity of the fecal specimen along a strip of filter-paper (previously folded lengthwise to keep it straight), but leave the last 4 or 5 cm clean to be put into water.

2. Put the strip of filter-paper, clean end first, into a test-tube containing filtered or boiled water 2.5–3.0cm deep; fold the strip at the top so that the bottom does not touch the bottom of the tube.

3. Record the serial number or name of the patient indelibly on the tube.

4. Plug the tube with cotton wool or, preferably, seal with cellophane tape and keep for 7–8 days at room temperature.

5. Look for the larvae at the bottom of the tube. Stain with iodine solution for 1 minute and then examine under the microscope, using the ¥10 objective.

The larvae usually seen in fresh stool specimens are the rhabditiform (first-stage) larvae of *S. stercoralis*. However, if the stool was passed more than 12 hours earlier, the larvae may have hatched into filariform (infective-stage) larvae. These must be differentiated from larvae of *Ancylostoma duodenale* and *Necator americanus* (hookworm), which may also hatch in stools 12–24 hours after passage. The appearance of filariform larvae of *S. stercoralis* may indicate a systemic hyperinfection.

The genital primordium will be more visible in preparations stained with iodine. The iodine kills the larvae and makes the features easier to see. You will need to use the X 40 objective to see these structures.

- **If you see a larva with a short mouth opening and a prominent (clearly visible) genital primordium, it is *S. stercoralis*.**
- **If you see a larva with a long mouth opening and do not see a genital primordium, it is *A. duodenale* or *N. americanus.***

The main distinguishing features of *S. stercoralis* and *A. duodenale* or *N. americanus* larvae are summarized in Table.

Table. Characteristics of larvae of *Strongyloides stercoralis* and *Ancylostoma duodenale* or *Necator americanus*

Larval stage	*S. stercoralis*	*A. duodenale* or *N. americanus*
Rhabditiform	Buccal cavity short (4 mm) Esophagus one-third of body length with 2 swellings	Buccal cavity long (15 mm) Esophagus one-third of body length with 2 swellings
Genital primordium	large (22 mm) Anal pore 50 mm from posterior end	Genital primordium small (7 mm) Anal pore 80 mm from posterior end
Filariform	Size 200–500 µmX15–20 µm Unsheathed Tail forked or blunt Esophagus half of body length with no swelling length	Size 200–500 µm X14–20 µm Sheathed Tail tapered Esophagus one-third of body with no swelling

MICRESCOPIC EXAMINATION OF WATERY STOOL SPECIMENS FOR VIBRIO CHOLERAE

Some strains of *V. cholerae* cause the disease cholera Dark-field microscopy is used to identify *Vibrio cholerae* and *Campylobacter* spp., in watery stool specimens.

Materials and reagents

- **Microscope with dark-field attachment**
- **Microscope slides**
- **Coverslips**
- **Inoculating loop**
- **Sodium chloride, 0.85% solution.**

Method

1. Suspend 0.2g of stool in 5 ml of sodium chloride solution. Allow the large particles to sediment.

2. Using an inoculating loop (sterilized by flaming), prepare a very thin smear on a slide. Carefully remove any large particles.

3. Cover with a coverslip. Place the slide on the microscope stage.

4. Open the iris diaphragm fully and place the dark-field attachment in position.

Microscopic examination

Use the X10 objective for focusing. The background appears black, and all objects suspended in the saline solution appear bright. Use the X40 objective to search for bacteria with characteristic shapes and motility. *Vibrio cholerae* appears as motile rods, which may be short, curved, straight or involuted.

Dispatch of specimens for culture

It is often necessary to send stool specimens to a bacteriology laboratory for culture:

— for the detection of *Cholera vibrios*

— for the detection of other bacteria that cause dysentery (species of *Salmonella*, *Shigella*, etc.).

Using Cary–Blair transport medium

Cary–Blair transport medium will preserve many kinds of enteric bacteria (*Cholera vibrios*, other vibrios, *Salmonella*, *Shigella*, etc.) for up to 4 weeks. The uninoculated medium may be stored in a sealed bottle at room temperature for 8–12 weeks.

1. Dip a sterile cotton wool swab in the stool specimen.

2. For infants or other patients who cannot produce a stool specimen, take a rectal swab. Moisten the swab with sodium chloride solution and introduce the swab into the rectum. Turn the swab several times with a circular movement.

3. Place the swab in a bottle containing Cary–Blair medium (three-quarters full) and send it to the bacteriology laboratory. If you cannot send the swab immediately, store it at room temperature.

***Important*:**

-Never store the swab in the incubator.

-Never store the swab in the refrigerator.

Using buffered glycerol saline

When specimens are to be sent for culture of enteric organisms other than cholera vibrios and Cary–Blair transport medium is not available, buffered glycerol saline may be used.

***Note*: If the buffered glycerol saline has changed color from pink to yellow, discard it and prepare a fresh solution.**

1. A bijou bottle with a capacity of 7.5 ml is recommended. Fill it to within 2cm of the top with buffered glycerol saline.

2. Place the stool swab or rectal swab in the bottle and send it directly to the bacteriology laboratory.

Basic fuchsin smear to detect campylobacters

Campylobacter enteritis is a common cause of intestinal infection. These bacteria are also one of the many causes of traveler's diarrhea or food poisoning. People most often get infected by eating or drinking food or water that contains the bacteria

Prepare when the specimen is unformed and, or, contains mucus, pus, or blood and is from a child under 2 years.

– Make a *thin* smear of the specimen on a slide. When dry, *gently* heat-fix. Stain by covering the smear with 10 g/l basic fuchsin* for 10–20 seconds. Wash well with water and allow to airdry.

***Dissolve 1 g basic fuchsin in 100 ml of water, and filter.**

– Examine the smear for campylobacters using the 100X oil immersion objective.

***Campylobacter organisms*: Look for abundant small, delicate, spiral curved bacteria (often likened to gull wings), S-shapes, and short spirochetal forms.**

***Note*: Examination of stained fecal smears for campylobacters has been shown to be a sensitive method for the presumptive diagnosis of *Campylobacter enteritis*.**

Motility test and Gram stained smear when cholera is suspected

Examine an alkaline peptone water culture (sample from the surface of the culture) for vibrios showing a rapid and darting motility. The preparation is best examined using dark-field microscopy but the vibrios can also be seen using transmitted light. Experience is required to identify the characteristic motility of *V. cholerae*. Examine also a Gram-stained smear of the culture for Gram negative vibrios (use 1 in 10 dilution of carbol fuchsin as the counterstain instead of neutral red).

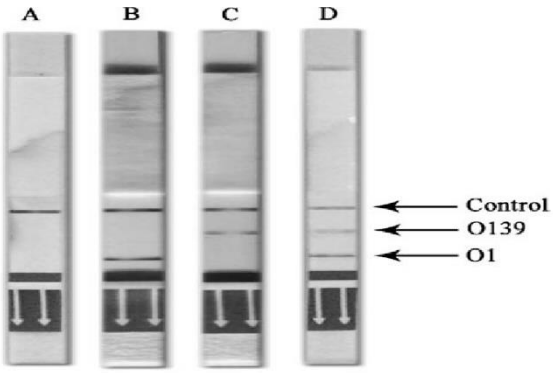
Use of *V. cholerae* 0 (group 1) antiserum to immobilize vibrios

Cholera can be caused by serogroups 01 and 0139 and therefore a negative test cannot exclude cholera when *V. cholerae* 0 group 1 antiserum only is used in an

immobilization test. Some workers have also found the test to be unreliable because the vibrios can be immobilized by preservative in the antiserum.

***Antigen detection*: A rapid, simple to perform dipstick test to detect *V. cholerae* 01 and 0139 in faces has been developed.**

Rapid dipstick test to detect V. cholerae 01 and 0139 in fecal specimens



An easy to perform low cost immunochromatographic dipstick (strip test) has been developed recently by the Institute Pasteur for the rapid detection of *V. cholerae* 01 and 0139 in endemic areas directly from faces or from a rectal swab that has been first incubated in alkaline peptone water. The dipsticks are packaged individually and can be transported and stored at room temperature (4–25 ºC). They must be protected from direct sunlight.

Method using faces

1- Centrifuge fluid faces* at 1500 rpm for 5minutes.

***Emulsify formed faces in physiological saline or distilled water prior to centrifuging.**

2- Transfer 200 µl (0.2 ml) of supernatant fluid to a small test tube.

3- Immediately before use, remove a dipstick from its moisture-proof plastic bag and place it in the tube ensuring the absorbent pad of the dipstick is below the level of the fluid.

4- Read the result within 15 minutes.

***Positive test for V. cholerae 01*: Two pink red lines, one in T1 test area, and another in area C (Control line).**

***Positive test for V. cholerae 0139*: Two pink red lines, one in T2 test area, and another in area C (Control line).**

***Negative test**: Pink-red line in area C (Control line) only**

******Note*: A negative test result will also occur when the quantity of antigen in the sample is below the sensitivity level of the dipstick (i.e. below 107 CFU *V. cholerae* 01 or 0139/ml).**

Method using a rectal swab

1- Incubate the swab in alkaline peptone water at 37 ºC for 6 hours.

2- Transfer 200 µl (0.2 ml) of alkaline peptone water culture to a small test tube.

3- Continue as described in the previous Method.

Interpret the results as described previously.

Sensitivity and specificity of V. cholerae 01 and 0139 dipstick

When compared to culture, the sensitivity of the *V. cholerae* dipstick in cholera endemic areas has been reported as 77–96% and the specificity as 97–99%.

Availability: The *V. cholerae* 01 and 0139 dipstick is manufactured and marketed by Span Diagnostics.

MICROSCOPIC EXAMINATION OF CSF FOR PARASITES, BACTERIA AND FUNGI DETERMINATION

Includes:

— examination of a wet preparation for blood cells;

— examination of a wet preparation for trypanosomes in areas where African trypanosomiasis occurs;

— examination of a Gram-stained smear for organisms that cause meningitis, such as *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (see Table);

— examination of a Ziehl–Neelsen-stained smear if tuberculous meningitis is suspected;

— examination for fungi such as *Cryptococcus neoformans* and *Candida albicans*, if suspected.

The above examinations are made using the deposit from centrifuged CSF.

Wet preparation for trypanosomes

Method

Place one drop of CSF deposit on a slide and cover with a coverslip. Examine the preparation under the microscope using the X40 objective.

Use of the improved Neubauer counting chamber

If you are using an improved Neubauer chamber, count the cells within the entire ruled area, which is 9mm^3. If undiluted CSF is used, multiply the number of cells counted by 10 and divide by nine to give the number of cells per mm^3 of CSF. If a 1 in 20 dilution of CSF is used, multiply the number of cells counted by 20 and divide by nine to give the number of cells per mm^3 of CSF.

Results

Normal CSF contains less than 5 X 10^6 leukocytes per liter (less than 5 per mm^3). An increased number of leukocytes can be found in:

-Bacterial meningitis (meningococcal, *Haemophilus influenzae*, pneumococcal): mostly neutrophils

Report any organisms seen in the Gram-stained smear by their:

-Gram reaction: positive or negative

-morphology: cocci, diplococci, bacilli, etc.

-numbers found.

A definite species identification cannot be made from a Gram-stained smear only. Culture of the organisms is necessary. The organisms that commonly because meningitis is described on the following.

Neisseria meningitidis (meningococci)

_ Gram-negative

_ Diplococci, lying side by side

_ Intracellular, inside the neutrophils.

***Note*: Diplococci may occasionally be seen outside the cells and are usually few in number.**

Streptococcus pneumoniae (pneumococci)

_ Gram-positive

_ Diplococci, lying end to end

African trypanosomiasis

The finding of motile trypanosomes in the CSF means that the later stage of trypanosomiasis has been reached, in which the central nervous system is infected. The protein concentration of the CSF is raised and the Pandy test is positive. The fluid also contains an increased number of white blood cells. In a wet preparation stained with Romanowsky stain, the leukocytes can be identified as lymphocytes (L), and Mott cells (M) can often be seen. These are large cells containing vacuoles and large amounts of immunoglobulin M (IgM) that stain dark with the eosin part of Romanowsky stains.

Gram-stained smear for meningitis

Method

Make a smear of the CSF deposit and allow it to dry in the air. Stain the smear with Gram stain.

-Surrounded by a capsule, which is not visible with Gram stain-

- Not intracellular

-Usually many in number.

Haemophilus influenzae (especially in young children)

_ Gram-negative

_ Small bacilli (coccobacilli)

_ Not intracellular

_ Often numerous.

In all the above-mentioned forms of meningitis the leukocytes present are neutrophils.

Gram-positive bacilli

Very rarely found. May belong to the *Listeria* group. Culture is essential.

Ziehl–Neelsen-stained smear for tuberculous meningitis

Tuberculous Meningitis Image

Method

If tuberculous meningitis is suspected, the CSF should be left to stand. If a clot forms, it should be removed, spread on a slide and stained with Ziehl–Neelsen stain. If organisms are seen, report the smear as "acid-fast bacilli present".

Fungi in the CSF

Very rarely, fungi (*Cryptococcus neoformans* and *Candida albicans*) may be observed in a smear stained with Gram stain. *Cryptococcus neoformans* may be found in cloudy CSF with lymphocytes.

Method

Mix on a microscope slide:

— one drop of CSF deposit

— one drop of Indian ink.

Examine the mixture between a slide and a coverslip.

Cryptococcus neoformans appears as follows:

— round budding spores containing greyish granulations;

— each group of 1–3 spores is surrounded by a colorless capsule.

***Candida albicans* may be found in an unstained wet preparation of CSF deposit. It appears as follows:**

— oval budding spores

— short mycelium filaments.

Table: Typical findings on examination of CSF

Disease or condition	Appearance	Blood cell concentration	Protein concentration	Glucose concentration	Other findings
Purulent meningitis	Cloudy, yellowish	>3000 cells/µl, mainly granulocytes	Highly elevated, 1– 10g/l	Greatly reduced	Bacteria
Tuberculous meningitis	Clear or almost clear	30–300 cells/µl, mainly lymphocytes	Elevated	Greatly reduced	Bacteria, clotted proteins
Viral meningitis	Clear	10–300 cells/µl, mainly lymphocytes	Normal or slightly elevated	Normal	
Malaria	Slightly cloudy	Elevated, mainly	Elevated	Reduced	Granulocytes

African trypanosomiasis	**Clear or slightly cloudy**	**>5 cells/ml, mainly lymphocytes**	**Elevated**	**Reduced Mott cells**	**Trypanosomes,**

MICROBIOLOGICAL EXAMINATION OF SYNOVIAL FLUID

Infectious agents that can enter the synovial fluid include bacteria, fungi, Mycobacteria, and viruses, with bacteria being the most common. Bacteria and other microorganisms enter the synovial capsule through the bloodstream, deep penetrating wounds, and rupture of osteomyelitis into the joint. In addition, bacteria may be introduced during procedures such as arthroscopy, intra-articular steroid injections, and prosthetic joint surgery.

Gram stain is performed on synovial fluid smears prepared by centrifugation or cytocentrifugation. Diluting synovial fluid with saline helps separate cells that tend to cluster. Even if Gram staining does not suggest the presence of infectious agents, both aerobic and anaerobic cultures should be performed. Synovial fluid Gram stains are positive in only 50% of cases with joint sepsis.

MICROBIOLOGIC EXAMINATION OF SEMAN CULTURE

Urogenital infections, caused by various microorganisms, are responsible for about 15% cases of male infertility. Microorganisms that may lead to antisperm antibody production include *Ureaplasma urealyticum, Mycoplasma hominis, Chlamydia trachomatis, Herpes simplex.* Urogenital infections with *Candida albicans* impair sperm motility by agglutinating with spermatozoa heads. Each of these microorganisms has specific media and growth requirements.

Semen Culture is essential when the man suddenly presents with symptoms of the lower urinary tract (LUTS) and genital system -such as painful ejaculation, haemospermia (presence of blood in the semen)- and also in cases of urinary frequency or urgency, dysuria etc. Paricularly in the last cases, semen culture has to be combined with urine culture, for sometimes the pathogenic bacterium may be detected only in the urine or only in the semen.

How is Semen Culture performed?

It is essential to avoid contamination by microorganisms from other sources than semen, for example from the skin or an already contaminated container.

In most cases, results are ready within 48 hours, that is the time it takes for common pathogenic microorganisms to incubate. Semen Culture may be found to be:

- **Negative: no pathogenic microorganism is found.**
- **Positive: some pathogenic microorganism is found. In this case, an antibiogram follows with a list of antibiotic agents to which the microorganism is sensitive. With some pathogenic bacteria, such as mycoplasma and ureoplasm, the antibiogram is not necessary, as the therapy is very specific. However, the man has to consult his physician again for determining the proper dosage and duration of treatment.**

Superinfection: Three or more bacterial strains are found in the sample, meaning either that sampling was not conducted under sterile conditions or the sample was contaminated by other external sources. In this case, semen culture has to be repeated.

EXAMINATION OF VAGINAL SECREATION

Bacterial Vaginosis

Bacterial vaginosis is the most common vaginal infection in women. In bacterial vaginosis, the vaginal flora is altered. Normally, *Lactobacillus* predominates in the healthy vaginal flora. In vaginosis, other bacteria such as *Gardnerella vaginalis*, or *Mobiluncus* species, or the anaerobic *Prevotella* species predominate.

Examination of vaginal discharge

Vaginal discharge is examined by microscopy to exclude infections with gonococci, *Candida albicans* and *Trichomonas vaginalis*, which cause bacterial vaginosis, vulvovaginal candidiasis and trichomoniasis, respectively.

Materials and reagents-

- **Microscope**
- **Microscope slides**
- **Coverslips**
- **Sodium chloride, 0.85% solution.**

Method

Collection of specimens

The specimen should be collected by a physician or specialist nurse.

Preparation of slides

1. Make a smear of the discharge on a slide and allow it to air-dry. Stain the smear with Gram stain and examine for *Candida albicans*.

2. Transfer a small sample of discharge to a second slide, add a drop of saline solution and cover with a coverslip. Look for gonococci and *Trichomonas vaginalis* trophozoites in this preparation.

Microscopic examination

Examine the Gram-stained slide using the X 40 objective and the X 100 oil immersion objective. *Candida albicans* appears as large Gram-positive yeasts, often with budding or short lengths of mycelium. Examine the saline preparation as soon as possible using the X10 and the X40 objectives. Use a microscope with the iris diaphragm closed to give good contrast. Do not allow the specimen to dry out.

***Trichomonas vaginalis* trophozoites appear as highly motile flagellates measuring 8–20mm, with an undulating membrane and a prominent nucleus.**

Germ Tube Test for the identification of *Candida albicans*

The Germ Tube Test, also known as Reynold's Brande phenomena is a screening procedure which is used for the identification and differentiation of *Candida albicans* from other yeasts.

Principle of the Germ Tube Test

This test allows the detection of the Germ Tubes which are the initial stage of hyphae formation. These are the short, non-septate germinating hyphae which are one half the width and three to four times the length of the yeast from which they originate. Approximately 95-97% of *Candida albicans* isolate develop germ tube when incubated in a proteinaceous media at 35C for 2.5-3 hours.

Materials required

- **Sheep serum or pooled human serum**
- **Test tube**
- **Wooden applicator stick**
- **Microscopic glass slide**
- **Cover slip**
- **Pasteur pipette**
- **Micropipette**
- **Incubator**

Procedure of germ tube test

1. **Put about 0.5 ml of sheep serum or pooled human serum in a test tube.**
2. **With a sterile wooden applicator stick, lightly touch a yeast colony (1-2 large colonies or 3-4 small colonies) and then place the stick into the serum.**
3. **Suspend the yeast in serum and discard the stick.**
4. **Incubate the tube for 2-3 hours in a 35-37C incubator.**
5. **After incubation, place a drop of suspension on a clean microscopic slide and coverslip it.**
6. **Examine the wet mount microscopically using low power objective. Use high power objective to confirm the presence or absence of germ tubes.**

Result and Interpretation

Presence of germ tube: *Candida albicans*
Absence of germ tube: other yeasts

GROSS VAGINAL SECRETION EXAMINATION

pH

Normally, vaginal secretions have a pH of 3.8–4.5, due to the growth of Lactobacillus species and its acidic byproducts. With the alterations of bacterial flora that occur in bacterial vaginosis and in Trichomonas, the pH goes up and Lactobacillus numbers decrease. In candidiasis, the pH is largely unchanged, between 3.8 and 4.5.

KOH Preparation and Amine Test

A KOH preparation is a slide prepared for examination by adding one drop of 10% KOH to vaginal secretions with a coverslip. As the KOH slide is prepared, if the microbial flora of the vaginal secretions is altered due to bacterial vaginosis, a foul-smelling trimethylamine odor is given off when the KOH is added and the pH of the sample changes.

EXAMINATION 0F BRONCHOALVEOLAR LAVAGE AND BRONCHIAL WASHINGS FOR INFECTIOUS AGENTS

Bronchoalveolar lavage (BAL) and bronchial washings are body fluids that are generally collected to assess the cellular composition and to detect any infectious agents present in the lower respiratory tract.

SPECIMEN COLLECTION

These specimens are obtained in surgery. A lighted optical instrument, the bronchoscope, is used to examine the tracheobronchial tree and can help detect obstructions, pneumonia, carcinoma, hemoptysis, foreign bodies, or abscesses. These instruments can be equipped with suction catheters, brushes, or biopsy attachments for specimen collection. For washings, 20–60 mL of saline is infused and then recollected by aspiration. Bronchial washings obtain material from the

more proximal areas of the bronchoalveolar tree. The BAL is used at more distal sites to retrieve material more representative of the alveoli and to obtain more cellular alveolar material.

DISEASE CORRELATIONS

These specimens are obtained for routine bacterial, fungal, and mycobacterial examination and culture, and for cytological studies. Cell counts are performed with a hemocytometer. As with most body fluids, cytocentrifugation gives the best cellular preparations for staining for cellular differentiation. Cytological and microbiological stains are used on these specimens. Cells seen in bronchial washings and BAL include macrophages, lymphocytes, neutrophils, eosinophils, ciliated columnar epithelial cells, and squamous epithelial cells. A variety of microorganisms, bacteria, fungi, and mycobacteria, can be found in these samples in lower respiratory tract infections.

TESTING

Culture, stains, wet mounts, and molecular tests are used to look for infectious organisms. A variety of histological stains can also be performed in the pathology laboratory to find these organisms as well. Wet Mounts, Calcofluor White Stain, and Other Stains Wet mounts are useful to detect fungal elements and cells that may be present in these samples. Stains can be used along with wet mounts or stains can be used on smears.

A technique that is particularly helpful to detect *P. jiroveci*, *C. albicans*, and other fungi is the calcofluor white wet preparation. The calcofluor white stain is a fluorescent stain that has increased sensitivity in the detection of these organisms and detection of fungi. It can be combined with KOH to dissolve cells in order to see fungal structures more. Histologic stains such as Gomori methenamine silver are also helpful in detecting these organisms.

Procedure of Calcofluor White Staining

1. **Carefully put the specimen on a clean glass slide**
2. **Add a drop of Calcofluor White Stain to produce an intense fluorescence**
3. **Add one drop of 10% Potassium Hydroxide**

4. **Cover the specimen with a coverslip and leave it to absorb the stain for 1 minute**
5. **Remove excess dye with a dry paper towel by gently pressing on the stain**
6. **Observe the stain under ultraViolet rays at x100-x400 magnification.**

Results

Fungi, Pneumocystis cysts, and parasites appear brilliant apple-green under UV fluorescent microscope, Violet and Blue light.

MICROSCOPIC EXAMINATION OF SPUTUM SPECIMENS AND THROAT SWABS

The presence of pathogenic organisms is revealed by microscopic examination of sputum specimens and throat swabs. The organisms include:

-Bacteria: Gram-positive and Gram-negative acid-fast bacilli.

-Fungi or yeasts: filaments of mycelium with or without pores. They may be pathogenic or saprophytes that have multiplied in the sample after collection (correct identification by a specialized laboratory necessary).

-Actinomycetes: granules.

-Parasites: eggs of pulmonary flukes and, very rarely, eggs of schistosomes and adult worms of *Mammomonogamus laryngeus*.

Culture is often necessary for the identification of the infective agents.

Materials and reagents

- **Microscope**
- **Microscope slides**
- **Wide-necked, leakproof containers for sputum specimens, such as jars or stiff paper boxes**
- **Sterile cotton wool swabs**
- **Tongue depressor or spatula**

- **Test-tubes- Sodium chloride crystals**
- ***N*-cetylpyridinium chloride**
- **Distilled water.**

If possible, sterile cotton wool swabs should be prepared at a central-level laboratory; otherwise, the following technique may be used.

1. Prepare some thin sticks of wood (or aluminum wire), 18 cm long and 2mm in diameter. Prepare strips of cotton wool, 6 cm long by 3cm wide and as thin as possible.

2. Roll the cotton wool round one end of the stick (or wire).

3. Mold the swab into a conical shape.

4. Place in a glass test-tube. Plug with non-absorbent cotton wool. Sterilize.

Method

Collection of *Sputum specimens*

Sputum specimens should be collected early in the morning.

1. Ask the patient to take a deep breath and then cough deeply, spitting what he or she brings up into the container. Secure the top and label the container with the name and number of the patient. Check that a sufficient amount of sputum has been produced.

2. If the specimen is to be dispatched to a laboratory for culture of *Mycobacterium tuberculosis*, ask the patient to expectorate directly into a wide-mouthed, screw-topped jar containing 25 ml of the following solution:

***N*-cetylpyridinium chloride 5 g**

Sodium chloride 10g

Distilled water to 1000ml.

Screw on the top and label the jar with the patient's name and the date of collection of the specimen.

***Important*: Liquid frothy saliva and secretions from the nose and pharynx are not suitable for bacteriological examination. Ask the patient to produce another specimen.**

Throat specimens

1. Using a tongue depressor or a spatula to press the tongue down, examine the back of the throat.

2. Look carefully for signs of inflammation and any exudate, pus, membranous deposits or ulcers.

3. Use a sterile cotton wool swab to swab the infected area. Take care not to contaminate the swab with saliva. Return the swab to the sterile test-tube.

Preparation of slides

Prepare two smears from each of the specimens. Stain one smear with Albert stain and the other with Ziehl–Neelsen stain.

Microscopic examination

Examine the sputum with the naked eye and then by microscopy. The sputum of a person suffering from a bacterial infection usually contains:

— thick mucus with air bubbles

— threads of fibrin

— patches of pus

— occasional brownish streaks of blood.

After visual inspection report the appearance of the sputum as:

— purulent: greenish, containing pus;

— mucopurulent: greenish, containing both pus and mucus;

— mucoid: containing mostly mucus;

— mucosalivary: containing mucus with a small amount of saliva.

If there is blood present, this must also be reported. A sputum sample composed mostly of saliva will not be useful either for culture or for direct examination. Examine the smear stained with Albert stain. If green rods containing green-black volutin granules are seen, report as "*Corynebacterium diphtheriae* present".

Examine the smear stained with Ziehl–Neelsen stain. If red bacilli can be seen, report as "acid-fast bacilli present". Report the numbers of acid-fast bacilli present. If no acid-fast bacilli are seen, report as "no acid-fast bacilli found".

Detection of diphtheria, *Corynebacterium diphtheriae*

If diphtheria (Diphtheria is an infection caused by the bacterium *Corynebacterium diphtheriae*), is suspected a sputum smear should be stained with Albert stain. This stain is used to show the dark-staining volutin granules that appear in *Corynebacterium diphtheriae* bacilli.

Materials and reagents

- **Microscope**
- **Slide rack**
- **Albert stain.**

Method

1. Fix the smear.

2. Cover the smear with Albert stain for 3–5 minutes.

3. Wash off the stain with clean water and place the slide upright in a slide rack to drain and air-dry.

Microscopic examination

First examine the slide using the X40 objective to see how the smear is distributed and then use the X100 oil-immersion objective. *Corynebacterium diphtheriae* appears as green rods containing green– black volutin granules. The

rods may be arranged in rows (a) or in V-formation (b), or joined at angles, giving the appearance of Chinese characters (c).

The presence of slender rods containing volutin granules is sufficient evidence for starting treatment for diphtheria. If diphtheria is suspected, a specimen should be sent to the bacteriology laboratory for culture.

Culture the specimen

Blood agar

– Inoculate the swab on a plate of blood agar. Use the loop to make also a few stabs in the agar (well area). Colonies of *S. pyogenes* growing below the surface will show more distinct zones of hemolysis because of the anaerobic conditions provided.

– When a swab is received in silica gel (e.g. from a health center), moisten it first with sterile nutrient broth and then inoculate the plate.

– Add a 0.05-unit bacitracin disc to the plate. This will help in the identification of *S. pyogenes. Some* workers also add a co-trimoxazole disc (as used for susceptibility testing) which prevents the growth

of other bacteria, making it easier to see *beta*-hemolytic *S. pyogenes* colonies.

– Incubate the plate preferably anaerobically or, when this is not possible, in a carbon dioxide enriched atmosphere overnight at 35–37˚C. Candle jar incubation will detect most *beta*haemolytic streptococci.

***Note*: *Beta*-hemolytic streptococci produce larger zones of hemolysis when incubated anaerobically. A minority of Group A *Streptococcus* strains will only grow anaerobically.**

Examine the specimen microscopically

Gram smear

Make an evenly spread smear of the specimen on a slide. Allow the smear to air-dry in a safe place. Fix and stain by the Gram technique. Use dilute carbol fuchsin as the counterstain in preference to safranin or neutral red (stains

Vincent's organisms better). Examine the smear for pus cells and Vincent's organisms:

Vincent's organisms: **These are seen as Gram negative spirochetes (*B. vincenti*) and Gram-negative fusiform rods.**

Examine and report the cultures

Blood agar culture

Look for *beta*-hemolytic colonies that could be *Streptococcus pyogenes* (Lancefield Group A *Streptococcus*). Most strains are sensitive to bacitracin. However, bacitracin sensitivity cannot be completely relied on to identify *S. pyogenes*. The organism should be tested serologically to confirm that it belongs to Lancefield Group A or tested biochemically using the PYR test.

Isolation and identification of C. diphtheriae

When colonies suspected of being *C. diphtheriae* are isolated, identify as follows:

– Examine a Gram stained smear for variable staining pleomorphic rods

– Sub inoculate two slopes of Dorset egg medium or Loeffler serum agar. Incubate at 35–37˚C for 6 hours or until sufficient growth is obtained.

Examine an Albert stained smear of the subculture for pleomorphic rods containing volutin granules. Examine a Gram stained smear to check that the subculture is a pure growth.

– Identify the isolate biochemically

– Using the growth from the other subculture, test the strain for toxin production using the Elek precipitation technique

EXAMINATION OF PUS, ULCER MATERIAL AND SKIN SPECIMENS

Pus Examination

Possible pathogens*

***It is impossible to list all the pathogens that may be found in pus. Those listed are the more commonly isolated pathogens from wounds, abscesses, burns, and draining sinuses.**

- ***BACTERIA***

Gram positive	*Gram negative*
Staphylococcus aureus	***Pseudonomas aeruginosa***
Streptococcus pyogenes	***Proteus* species**
***Enterococcus* species**	***Escherichia coli***
Anaerobic streptococci	***Bacteriodes* species**
Other streptococci	***Klebsiella* species**
Clostridium perfringens	***Pasteurella* species**
and other clostridia	
Actinomycetes	
Actinomyces israeli	
Also, *Mycobacterium tuberculosis*	

- **FUNGI**

***Histoplasma c. duboisii*,**

***Candida albicans*,**

- **PARASITES**

***Entamoeba histolytica* (in pus aspirated from an amoebic liver abscess),**

ULCER MATERIAL AND SKIN SPECIMENS

Possible pathogens

- **BACTERIA**

Gram positive	*Gram negative*
Staphylococcus aureus	***Escherichia coli***
Streptococcus pyogenes	***Proteus***
***Enterococcus* species**	***Pseudomonas aeruginosa***
Anaerobic streptococci	***Yersinia pestis***
Erysipelothrix rhusiopathiae	**Vincent's organisms**
Bacillus anthracis	

Also, *Mycobacterium leprae*, *Mycobacterium ulcerans*, *Treponema carateum*, and *Treponema pertenue*.

- **VIRUSES**

Poxviruses and herpes viruses

- **FUNGI**

Dermatophytes (ringworm fungi)

***Malassezia furfur*,**

Fungi that cause chromoblastomycosis,

***Candida albicans*,**

- **PARASITES**

Leishmania* species

Onchocerca volvulus*

Dracunculus medinensis*

Commensal organisms that may be found on the skin include:

Gram positive	Gram negative
Staphylococci	***Escherichia coli***
Micrococci	**and other coliforms**
Anaerobic cocci	
Viridans streptococci	
Enterococci	
Diphtheroids	
Propionibacterium acnes	

COLLECTION AND TRANSPORT OF PUS, ULCER MATERIAL, SKIN SPECIMENS

1. Collect the specimen using a sterile cotton-wool swab. Insert it in a container of Amie's transport medium, breaking off the swab stick to allow the bottle top to be replaced tightly. When the material is aspirated fluid from a pustule, transfer the fluid to a sterile, leak-proof container. Stopper, and seal in a leak-proof plastic or metal container.

Note: **It is not possible to transport exudate from a suspected treponemal ulcer because the treponemes remain motile for only a short time.**

2. Make a smear of the material on a clean slide (for Gram staining) and allow to air-dry in a safe place. Heat-fix the smear.

Caution: **Do *not* make a smear for transporting when the specimen is from a patient with suspected anthrax or bubonic plague.**

LABORATORY EXAMINATION OF PUS, ULCER AND SKIN SPECIMENS

1- Describe the appearance of the specimen

When from a patient with suspected mycetoma or actinomycosis, report the appearance of the specimen and whether it contains granule

2- Examine the specimen microscopically

Note: **When a swab has been used to collect the pus, inoculate the culture media *first* before using the swab to make smears.**

Gram smear

Make an evenly spread smear of the specimen on a slide. Allow the smear to air-dry in a safe place. Fix and stain by the Gram technique

Examine the smear for bacteria among the pus cells using the 40X and 100Xobjectives. Look especially for:

- Gram positive cocci that could be *S. aureus* or streptococci that could be *S. pyogenes* or other *beta*-hemolytic streptococci, anaerobic streptococci, or enterococci.

- Gram negative rods that could be *Proteus* species, *E. coli* or other coliforms, *P. aeruginosa* or *Bacteroides* species.

- Gram positive large rods with square ends that could be *C. perfringens* or *B. anthracis*

- Large numbers of pleomorphic bacteria (streptococci, Gram positive and Gram-negative rods of various size and fusiform bacteria), associated with anaerobic infections.

- Gram positive yeast cells with pseudohyphae, suggestive of *Candida albicans*

- Vincent's organisms if tropical ulcer is suspected. These appear as Gram negative spirochetes (*B. vincenti*) and Gram-negative fusiform rods

3- Culture the specimen

Blood agar MacConkey agar, cooked meat medium (or thioglycollate broth)

Inoculate the specimen:

- On blood agar to isolate *S. aureus* and streptococci. Add a bacitracin disc if streptococci are seen in the Gram smear.

- On MacConkey agar to isolate Gram negative rods.

- Into cooked meat medium or thioglycolate broth.

Cooked meat medium: This is an enrichment medium for aerobes and anaerobes. The glucose in the medium helps to produce a rapid growth of anaerobes (at the bottom of the medium).

– Incubate the inoculated blood agar plate at 35–37˚C in a carbon dioxide atmosphere (candle

jar) and the MacConkey agar plate aerobically. Incubate the inoculated cooked meat medium at 35–37˚C for up to 72 hours. Subculture at 24 h, and if indicated at 48 h and 72 h.

Anaerobic culture

When an anaerobic infection is suspected (specimen is often foul-smelling), or the Gram smear shows an 'anaerobic mixed flora', inoculate a second blood agar plate and incubate it anaerobically for up to 48 hours. The anaerobic blood agar plate may be made selective by adding neomycin to it. At a final neomycin concentration of 50–70 µg/ml, the majority of facultative anaerobic Gram-negative rods will be inhibited. To aid detection of anaerobes, a metronidazole disc (5 µg) may be added to the anerobic blood plates as the majority of anaerobes show a zone of inhibition, whereas aerobes grow up to the disc.

ADDITIONAL

Culture of specimen when bubonic plague is suspected

The Central Public Health Laboratory should be notified at the earliest opportunity when plague is suspected. Whenever possible, isolation of *Y. pestis* should be undertaken in this laboratory. Blood or a bubo aspirate should be sent for culturing together with a full case history and the report of the microscopical examination, i.e. whether bipolar stained organisms were seen.

Culture of specimen when infection with M. tuberculosis or M. ulcerans is suspected

The facilities of a specialist tuberculosis laboratory are required for the isolation, identification and susceptibility testing of *M. tuberculosis*, *M. ulcerans*, and other mycobacteria.

4- Examine and report the cultures

Blood agar and MacConkey agar cultures

Look especially for colonies that could be:

- ***Staphylococcus aureus***
- ***Streptococcus pyogenes***
- ***Pseudomonas aeruginosa***
- ***Proteus* species**
- ***Escherichia coli***
- ***Enterococcus* species**
- ***Klebsiella* species**

Anaerobic blood agar culture and cooked meat culture

Look for growth that could be *Clostridium perfringens*, *Bacteroides fragilis* group, or *Peptostreptococcus* species.

-*C. perfringens*: Grows rapidly in cooked meat medium with hydrogen sulphide gas production (gas bubbles in turbid medium) and reddening but no decomposition of the meat (saccharolytic reaction). On anaerobic blood agar, colonies are usually seen after 48 h incubation. Most strains produce a double zone of hemolysis (inner zone of clear hemolysis, outer zone of partial hemolysis).

-*B. fragilis*: Grows in cooked meat medium producing decomposition with blackening of the meat (foul-smelling proteolytic reaction). On anaerobic blood agar, non-hemolytic grey colonies (Gram negative pleomorphic rods) are seen, usually within 48 hours. *B. fragilis* group.

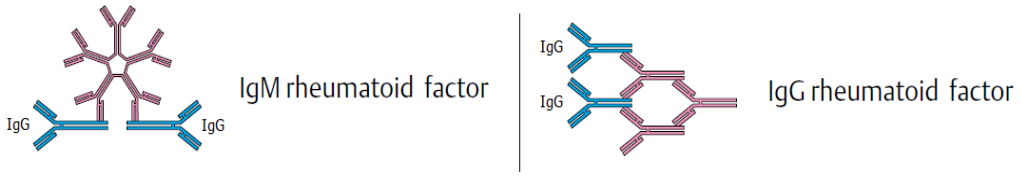
-*Peptostreptococcus*: Grows in cooked meat medium with the production of large amounts of hydrogen sulphide gas. On anaerobic blood agar, *Peptostreptococcus* produces small nonhemolytic white colonies (Gram positive cocci) after 48 h incubation. They are resistant to metronidazole (5 µg disc).

SECTION 2: MICROBIOLOGY

C- IMMUNOLOGY

SEROLOGICAL METHOD FOR TESTING RHEUMATOID FACTOR

Rheumatoid Factors (RF) are autoantibodies that react with individuals own immunoglobulin. These antibodies are usually directed against the Fc fragment of the human IgG. RF have been associated with three major immunoglobulin classes: IgM, IgG, and IgA. Of these IgM and IgG are the most common. The formation of immune complex in the joint space leads to the activation of complement and destructive inflammation, causing rheumatoid arthritis (RA).

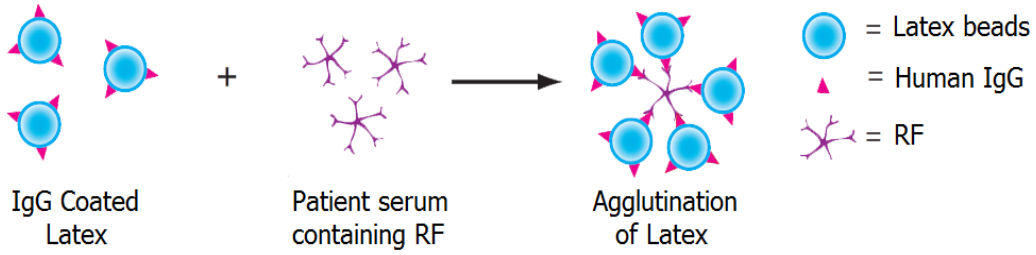


As indicated by its name, RF has particular application to diagnosis and monitoring of rheumatoid arthritis. Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting primarily the joints and periarticular tissues. Rheumatoid factor is detected in 60-80% of cases of diagnosed rheumatoid arthritis. However, it is also detectable sometimes in the serum of patients with Systemic Lupus Erythematosus (SLE) and in certain non-rheumatic conditions. Elevated values may also be observed in normal elderly population.

PRINCIPLE OF RF TEST

A number of methods are available for testing of RF. The most commonly used serological method is based on latex agglutination test. As RF is an IgM class of antibody directed against the Fc portion of the IgG molecule, it is detected by its ability to agglutinate the latex particles coated with IgG molecule.
Reagent used is a suspension of polystyrene latex particles in glycine-saline

buffer with pH: 8.6 ± 0.1, coated with human gamma globulin.



QUALITATIVE METHOD

Procedure

1. **Bring all reagents and specimens to room temperature.**
2. **Place one drop of the positive control and 40ul of the patient serum into separate circles on the slide.**
3. **Gently and add one drop of RF latex reagent on each circle of sample to be tested and control.**
4. **Use separate Applicator sticks/stir sticks to spread reaction mixture over entire area of the particular field.**
5. **Tilt the slide back and forth for 2 minutes in a rotary shaker so that the mixture rotates slowly.**
6. **Observe for agglutination after 2 minutes under bright artificial light.**

Interpretation

Agglutination of latex particles is considered a positive reaction, indicating the presence of rheumatoid factor at a significant and detectable level.
Positive result: An agglutination of the latex particles suspension will occur within two minutes, indicating a RF level of more than 18 IU/ml.
Negative result: No agglutination of the latex particles suspension within two minutes.

SEMI-QUANTITATIVE METHOD

Procedure

1- **Using isotonic saline prepare serial dilutions of the test sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16,1:32, 1:64, 1:128 and so on as follows:**

2- **For each specimen to be tested, add 100 µL of 0.9% saline into test tubes numbered 1 to 5.**

3- **Add 100 µL of specimen onto test tube 1.**

4- **Mix the mixture. Avoid formation of bubbles.**

5- **Transfer 100 µL of mixed sample from tube 1 to 2.**

6- **Repeat this serial dilution procedure in tube 3 to 4, and then 5. Dispose 100 µL from test tube 5 after mixing**

Tubes 1 to 5 now represent a dilution series as follows:

Tube Number	**1**	**2**	**3**	**4**	**5**
Dilution	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**

7- **Perform the qualitative test procedure using each dilution as test specimen.**

Interpretation

The titer is reported as the reciprocal of the highest dilution, which shows a positive test result. Read the titer in the last dilution step with visible agglutination and the approximate concentration of the rheumatoid factor can be determined as follows:

RF in IU/mL= Sensitivity of latex Gamma globulin reagent in IU/mL × Titer.

LIMITATIONS OF RF TEST

1. **RF is not detected in all patients diagnosed with RA.**
2. **RF may be detected in increased amounts in patients with infectious mononucleosis, sarcoidosis, systemic lupus erythematosus, TB or leprosy, and other conditions of acute or chronic immune response.**
3. **The significance of a positive result should be interpreted with caution. Quantitative testing should be done to confirm diagnosis of RA.**
4. **Highly hemolyzed and lipemic serum as well as plasma interfere with the test.**

C-REACTIVE PROTEIN TEST FOR DIGNOSIS OF INFLAMMATORY CYTOKINES

C-reactive protein (CRP) is a special type of protein produced by the liver in response to inflammatory cytokines such as Interleukin-6 (IL-6). CRP is classified as an acute phase reactant, which means that its levels will rise within a few hours after tissue injury, the start of an infection, or other cause of inflammation.

The most important role of CRP is its interaction with the complement system, which is one of the body's immunologic defense mechanisms.

Why CRP test is done?

C-reactive protein (CRP) test is performed to determine if a person has a problem linked to acute infection or inflammation. The CRP test is not diagnostic of any condition, but it can be used together with signs and symptoms and other tests to evaluate an individual for an acute or chronic inflammatory condition. These include:

1. **To determine if there is infection after surgery: CRP levels normally increase within two to six hours following surgery but then return to normal by the third day; if CRP levels are elevated three days after surgery it means there is an infection.**

2. **To keep track of an infection or disease that can cause inflammation: Inflammatory bowel disease (IBD), lymphoma (cancer of the lymph nodes), immune system diseases such as lupus (SLE), rheumatoid arthritis (swelling of the tissues that line the joints) and osteomyelitis (infection of the bone) are some conditions in which inflammation can be monitored with a CRP test.**

3. **To monitor treatment of a disease such as cancer or infection: Not only do CRP levels go up quickly if you have an infection but they also return to normal quickly if you are responding to the treatment.**

CRP Test Principle

CRP Test is based on the latex agglutination method introduced by Singer, et. al., in 1957. This is a slide agglutination test for the qualitative and semiquantitative detection of C-Reactive Protein (CRP) in human serum. Latex particles coated with goat IgG anti-human CRP are agglutinated when mixed with samples containing CRP. When latex particles coated with human anti-CRP are mixed with a patient's serum containing C – reactive proteins, this results in visible agglutination within 2 minutes.

CRP Test Procedure (Qualitative)

1. **Bring all reagents and serum sample to Room Temperature and mix latex reagent gently prior to use. Do not dilute the controls and serum.**

2. **Place 1 drop each of serum, positive control and negative control on separate reaction circles.**

3. **Then add CRP latex reagent 1 drop to each of the circles.**

4. **Mix with separate mixing sticks and spread the fluid over the entire area of the cell.**

5. **Tilt the slide back and forth slowly for 2 minutes observing preferably under artificial light.**

CRP Test Procedure (Semi-Quantitative)

Sera with positive results in the screening test should be retested in the semiquantitative test for obtaining the titer.

1. **Make serial two-fold dilutions of the sample in 9 g/L saline solution.**
2. **Proceed for each dilution as in the qualitative method.**

Interpretation
Agglutination of latex particles is considered a positive reaction, indicating the presence of C-reactive protein at a significant and detectable level. Specimens which do not contain human CRP will not cause agglutination.
If controls do not give expected reactions the test is invalid and must be repeated.

The titer, in semi-quantitative method, is defined as the highest dilution showing a positive result.

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

Intended Use: The Quantitative Determination of Anti *Helicobacter pylori* Specific Antibodies of the IgG, IgA or IgM type in Human Serum or Plasma by Microplate Enzyme Immunoassay, Colorimetric

SUMMARY AND EXPLANATION OF THE TEST

***Helicobacter pylori* has been shown to be the unidentified curved bacillus that was observed by Warren and Marshall in close contact with gastric epithelium in biopsy studies of patients suffering from chronic gastritis. Although the source of *H. pylori* infection is not known, the evidence is quite convincing that the bacillus can cause acute gastritis and may lead to chronic gasteritis. Sethi et al has reported that *H. pylori* was present in ninety-one (91) percent of patients with chronic superficial gastritis. Marshall[5] determined that *H. pylori was* present in ninety (90) percent of duodenal ulcer and seventy (70) percent of gastric ulcer patients.**

The use of serological testing to ascertain the immunologically produced antibody caused by *H. Pylori* infection has been suggested as the method of

choice to screen large populations. Measurements of the antibodies to *H. pylori* have been done by hemagglutination, serum complement fixation and bacterial agglutination. These tests do not have the sensitivity of enzyme immunoassay and are limited by subjective interpretation. This procedure, with the enhanced sensitivity of EIA, permits easy detectability of antibodies to *H. pylori*. In addition, the results are quantitated by a spectrophotometer, which eliminates subjective interpretation.

Microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations. In this method, serum reference, diluted patient specimen, or control is first added to a microplate well. Biotinylated *H. pylori* is added, and then the reactants are mixed. A reaction result between the autoantibodies to *H. pylori* and the biotinylated *H .pylori* to form an immune complex, which is deposited to the surface of streptavidin coated wells through the high affinity reaction of biotin and streptavidin.

After the completion of the required incubation period, aspiration or decantation separates the reactants that are not attached to the wells. An enzyme anti-human IgG, M or A conjugate is then added to permit quantitation of reaction through interacting with human IgG, M or A of the immune complex. After washing, the enzyme activity is determined by reaction with substrate to produce color. The employment of several serum references of known antibody activity permits construction of a graph of enzyme and antibody activities. From comparison to the dose response curve, an unknown specimen's enzyme activity can be correlated with autoimmune antibody level.

PRINCIPLE

A Sequential ELISA Method:

The reagents required for the sequential ELISA assay include immobilized antigen, circulating autoantibody and enzyme-linked species-specific antibody. 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 exogenous added biotinylated H. Pylori antigen.

Upon mixing biotinylated antigen, and a serum containing the antibody, reaction results between the antigen and the antibody to form an immune-complex.

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antigen. After the incubation time, the well is washed to separate the unbound components by aspiration and/or decantation. The enzyme linked species-specific antibody (anti-h-IgG, M or A) is then added to the microwells.

The anti-h-IgG, IgM or IgA enzyme conjugate that binds to the immune complex in a second incubation is separated from unreacted material by a wash step. The enzyme activity in this fraction is directly proportional to the antibody concentration in the specimen. By utilizing several different serum references of known antibody activity, a reference curve can be generated from which the antibody activity of an unknown can be ascertained

REAGENTS

Materials provided:

A-Anti-*H. pylori* Calibrators

Five (5) vials of references for anti-*H. pylori* at levels of 0(A), 10(B), 25(C), 50(D), and 100(E) U/ml* of the IgG, IgM or IgA type. Store at 2-8°C. A preservative has been added.

***Manufacturers' Reference Value**

B-*H. pylori* Biotin Reagent

One (1) vial containing biotinylated inactivated H. pylori (IgG, IgM or IgA) in a buffering matrix. A preservative has been added. Store at 2-8°C.

C-Anti-H. Pylori Enzyme Reagent

One (1) vial containing anti-human IgG, IgM or IgA- horseradish peroxides (HRP) conjugate in a buffering matrix. A preservative has been added. 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-Serum Diluent

One (1) vial of containing buffer salts and a dye. 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 A

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

H-Substrate B

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

I-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 0.010, 0.025, and 0.050ml (10, 25 & 50µl) volumes with a precision of better than 1.5%.

2-Dispenser(s) for repetitive deliveries of 0.100ml (100µ) 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 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, 0.100ml (IgM & IgA) or 0.050ml (IgG) of the diluted specimen is required.

REAGENT PREPARATION

1-Serum Diluent

Dilute the serum diluent to 200ml in a suitable container with distilled or deionized water. Store at 2-8°C.

2-Wash Buffer

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

3-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.

4-Patient Sample Dilution (1/100)

Dispense 0.010ml (10µl) of each patient specimen into 1ml of serum diluent. Cover and vortex or mix thoroughly by inversion. Store at 2-8°C for up to forty-eight (48) hours.

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, 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 diluted patient specimen into the assigned well for IgG determination. For

IgM or IgA, pipette 0.050ml (50µl) of the appropriate serum reference, control or diluted patient specimen into the assigned well.

3-Add 0.100 ml (100µl) of *H. pylori* Biotin Reagent Solution.

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, blot the plate dry with absorbent paper.

7-Add 0.350ml (350µl) of wash buffer (see Reagent Preparation Section), decant (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 *H. pylori* Enzyme Reagent to all wells.

Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION

9-Cover and incubate for thirty (30) minutes at room temperature.

10-Repeat steps (6 & 7) as explained above.

11-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

12-Incubate at room temperature for fifteen (15) minutes.

13-Add 0.050ml (50µl) of stop solution to each well and swirl the microplate gently for 15-20 seconds to mix. 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 to minimize well imperfections) in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: For re-assaying specimens with concentrations greater than 100 U/ml, dilute the sample an additional 1:5 or 1:10 using the original diluted material in the serum diluent. Multiply by the dilution factor to obtain the concentration of the specimen.

CALCULATION OF RESULTS

A reference curve is used to ascertain the concentration of anti-*H. Pylori* 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 anti-*H.Pylori* activity 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.

To determine the level of anti-*H. pylori* activity 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 1.603 intersects the dose response curve at 64.0 U/ml anti-*H. pylori* concentration

EXPECTED RANGES OF VALUES

A study of apparently healthy population (n=118) and patients suffering from gastric abnormalities (n=154) was undertaken to determine expected values for the Anti-H. Pylori ELISA test system. Based on the data, the following cut-off points were established.

Presence of H.Pylori antibodies Confirmed	
	(CONC)
IgG	> 20 U/mL
IgA	> 20 U/mL
IgM	> 40 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.

ANTI-STREPLYSIN O TEST FOR DETECTION OF STREPTOCOCCI

Anti-Streptolysin O (ASO or ASLO) is the antibody made against streptolysin O, an immunogenic, oxygen-labile hemolytic toxin produced by most strains of group A and many strains of groups C and G streptococci. In the course of streptococcal infections, the extracellular products of the bacteria act as antigens to which the body responds by producing specific antibodies. Streptolysin O (SLO) is one of two hemolysins (the other being Streptolysin S) produced by virtually all strains of *Streptococcus pyogenes*. The letter "O" indicates that this toxin is oxygen labile. The SLO toxin has direct toxic effects on heart tissue. In the course of a streptococcal infection, SLO stimulates the production of specific antistreptolysin (ASO) antibodies, which in-vitro, neutralize the hemolytic properties of the antigen.

Clinical Significance of ASO Test

An antistreptolysin titer greater than 166 Todd units (or >200 IU) is considered a positive test in adults, while the normal value for adults is less than 166 Todd units, which indicates a negative test. Measurement of ASO antibody titer is important in the investigation of post-streptococcal diseases, particularly acute poststreptococcal glomerulonephritis and rheumatic fever.

- **Over 80% of patients with acute rheumatic fever and 95% of patients with acute glomerulonephritis have elevated titers of ASO.**
- **The antibodies level starts to rise in 1-3 weeks after streptococcal infection, peaks in 3-5 weeks, and then goes back to insignificant level over 6-12 months, so a positive test can indicate current but more recent group A, C, and G streptococcal infection.**

Tests for ASO

ASO test is done by serological methods like latex agglutination or slide agglutination. ELISA may be performed to detect the exact titer value. The test principle, procedure and interpretation presented here are based on Latex Anti-Streptolysin O (ASO) test.

Principle of the Test

ASO latex agglutination is the rapid and simple test for the qualitative and semi-quantitative measurement of antibodies to Antistreptolysin-O in human serum. This method is based on an immunological reaction between streptococcal exoenzymes bound to biologically inert latex particles and streptococcal antibodies in the test sample. The reagent has been adjusted in the way that presence of an ASO titer of 200 IU/mL or higher in the serum gives a visible agglutination of the latex particles.

Qualitative Test (Screening)

Procedure:

1. **Bring all reagents and specimens to room temperature.**
2. **Place one drop (50 µl) of the positive control and 50 µl of the patient serum into separate circles on the glass slide.**
3. **Shake the ASO latex reagent gently and add one drop (45 µl) on each circle next to the sample to be tested and control.**
4. **Mix well using disposable stirrer spreading the mixture over the whole test area and tilt the slide gently. Agitate for about 2 minutes with**

rotator or by hand and observe for the presence or absence of agglutination.

Results and Interpretation:

- **Negative result:**
  No agglutination of the latex particles suspension within two minutes.
- **Positive result:**
  An agglutination of the latex particles suspension will occur within two minutes, indicating an ASO level of more than 200 IU/ml.

Semi-Quantitative Test

Procedure:

1. **Serum to be titrated is serially diluted (1:2, 1:4, 1:8 etc.) in 0.9 g/L saline solution.**
2. **Place one drop of positive control on slide. Do not attempt to dilute the ASO positive control serum for comparative or other purposes as no correlation exists between actual titer of the control and titer of unknown sera.**
3. **Place 50 ml of each serum dilution individually in successive circles on the slide and proceed as in screening methodology.**

Results and Interpretation:

The serum ASO titer can be defined as the highest dilution showing a positive result. The approximate ASO level (IU/ml) present in the sample can be obtained by the following formula:

- **ASO Titer (IU/ml) = Highest dilution with positive reaction x Reagent sensitivity (200 IU/ml)**

e.g. if the agglutination is present up to a titer 1:4, the approximate serum ASO level is 4 x 200 = 800 IU/ml

Limitations of the Test

1. **Highly hemolyzed and lipemic serum as well as plasma interfere with the test.**

2. **Non-streptococcal conditions that occasionally cause positive reactions (usually less than 500 IU/ml) in ASO antibody tests include rheumatoid arthritis, tuberculosis, pneumococcal pneumonia, gonorrhea, and hepatitis.**

3. **Since streptolysin is only one of several Streptococcus A exoenzymes, the ASO test will not detect the other antibodies to exoenzymes of Streptococcus A.**

SEROLOGICAL TESTS FOR SYPHILIS INFECTION

Syphilis is a sexually transmitted infection caused by the bacterium *Treponema pallidum* subspecies *pallidum*. ... Syphilis has been known as "the great imitator" as it may cause symptoms similar to many other diseases. Syphilis is most commonly spread through sexual activity

There are four stages of syphilis infection: primary, secondary, latent and tertiary, and a special condition of maternal–fetal transmission termed congenital syphilis. Immune responses to syphilis can be grouped into two categories: non-specific (or reaginic) and specific.

The non-specific reagin is of the IgM class and reacts with an alcoholic extract of beef heart known as cardiolipin (a phospholipid). Since the reaginic antibody lacks specificity, it shows up in many other conditions and disease states unrelated to treponemal infection. In *Treponema pallidum* these cases false-positive reactions can occur. Specific antibodies to treponemes (both to *T. pallidum* and to nonpathogenic treponemes) of the normal bacterial flora of the oral or genital tract can also develop. These antibodies are of the IgG class and remain detectable throughout the life of the patient despite treatment.

Routine tests for syphilis include the rapid plasma reagin (RPR) test, the fluorescent treponemal antibody-absorbed (FTA-Abs) test and the *T. pallidum* hemagglutination (TPHA) test.

Serological procedures for syphilis include the following.:

- **Treponemal tests: detect the antibodies to Treponema pallidum. e.g. Fluorescent Treponema pallidum antibody absorption (FTA-ABS) and microhemagglutination Treponema pallidum MHA-TP).**
- **Non-treponemal tests: detect the antibodies produced in response to lipoidal material released from the damaged host cell. These antibodies are traditionally referred to as 'REAGINS'. e.g.; Venereal Disease research laboratory (VDRL) and the rapid plasma regain (RPR) tests.**

Principle

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

The FTA-Abs test is used in the confirmation of syphilis. In the first step of the test, serum is diluted in a concentrated culture filtrate of Reiter treponemes to absorb any antibodies to nonpathogenic treponemes. The serum is then layered over a glass slide on which killed *T. pallidum* organisms (Nichols strain) have been affixed. The slide is incubated, washed and overlaid with a fluorescent-labelled antihuman immunoglobulin antibody. If the test result is positive the treponemes will fluoresce.

This indirect immunofluorescence technique is highly sensitive in all stages of syphilis, especially in the very early and very late stages. Once positive this test remains positive for the life of the patient. It is not used as a screening test for syphilis because it does not detect reinfection and it is time-consuming and costly (a fluorescence microscope with a dark-field condenser is required).

The results of a test for syphilis must be interpreted according to the type(s) of test employed and the stage of the disease the patient has reached. Remember that a positive result from a screening test for syphilis may be due to other heterophile antibodies, faulty technique or to the presence of other treponemal antibodies. A negative result may mean one of the following:

_ The infection is too recent to have produced detectable levels of antibodies.

_ The test is temporarily non-reactive because of treatment the patient is receiving.

_ The test has been rendered temporarily non-reactive because the patient has consumed alcohol prior to testing.

_ The disease is latent or inactive.

_ The patient has not produced protective antibodies because of immunological tolerance.

_ The technique is faulty.

Weakly positive results may be due to:

— very early infection;

— lessening of the activity of the disease after treatment;

— nonspecific immunological reactions;

— incorrect technique.

The greatest value of the non-treponemal tests is in screening following therapy and in the detection of reinfection.

- **The *T. pallidum* hemagglutination TPHA test**

The TPHA test is also used in the confirmation of syphilis. In the first step of the test, diluted serum is mixed with absorbing diluent containing non-pathogenic Reiter treponemes. The serum is then transferred to a microtiter plate and erythrocytes sensitized with killed *T. pallidum* organisms (Nichols strain) are added. If the test result is positive the erythrocytes will form a smooth mat of agglutinated cells.

Materials and reagents

_ Test-tubes

_ Test-tube rack

_ Commercially available TPHA test kit containing microtiter plates, micropipettes (with disposable tips), absorbing diluent, erythrocytes sensitized with *T. pallidum*, unsensitized erythrocytes, positive and negative control sera

_ Distilled water.

The reagents and controls should be reconstituted before use according to the manufacturer's instructions.

Method

1. Dilute the test and control sera 1:20 with absorbing diluent.

2. Using a micropipette, dispense 25ml of the negative control serum into wells 1 and 2 of the first horizontal row of the microtiter plate.

3. Dispense 25ml of the positive control serum into wells 1 and 2 of the second horizontal row of the microtiter plate.

4. Dispense 25ml of the first test serum into wells 1 and 2 of the third horizontal row of the microtiter plate. Repeat the procedure with the remaining test sera. If necessary, use the adjacent wells.

5. Add 75ml of the control erythrocytes to the wells in the first vertical row (1) and every other row (3, 5, 7, 9 and 11), as appropriate.

6. Add 75ml of the sensitized erythrocytes to the wells in the second vertical row (2) and every other row (4, 6, 8, 10 and 12), as appropriate.

7. Rotate the plates carefully, cover and leave to stand at room temperature for the time recommended by the manufacturer. The plates should be protected from vibration, radiant heat and direct sunlight.

8. Place the plates carefully on a white background or a sintered glass plate illuminated from below or a viewing device that allows the sedimentation pattern to be seen from below through a mirror.

If the result is positive a smooth mat of agglutinated cells will be seen. The cells may be surrounded by a red circle, or may even cover the entire base of the well. If the result is negative a compact red button of non-agglutinated cells will be seen, with or without a very small hole in its center.

If the result is doubtful (borderline) a button of non-agglutinated cells with a small hole in its center will be seen.

***Note*: The results should be interpreted according to the criteria provided by the manufacturer.**

- ***Venereal Disease Research Laboratory (VDRL) Test***

The Venereal disease research laboratory (VDRL) test is a non-treponemal microflocculation test which is used for screening of syphilis. It detects the IgM and IgG antibodies to lipoidal material released from the damaged host cells, as well as to lipoprotein-like material and possibly cardiolipin released from the treponemes.

PRINCIPLE

The presence of lipoidal antibodies in patient's serum or cerebrospinal fluid (CSF) is detected by using non-specific antigen, suspended in buffered saline solution. The cardiolipin antigen is an alcoholic solution composed of 0.03% cardiolipin, 0.21% lecithin and 0.9% cholesterol.

The heat inactivated (to destroy complement) serum or CSF is mixed with VDRL antigen. If the specimen contains reagin, flocculation occurs which can be observed using microscope. Non-reactive specimens appear as homogeneous suspension.

SPECIMENS

Only serum and CSF are appropriate specimens for VDRL testing. Serum should be heated to 56-degree Celsius for 30 minutes to destroy the complement, while no heat treatment is required for CSF.

QUALITATIVE METHOD

Procedure

1. **Bring the VDRL antigen suspension, controls and samples to room temperature.**
2. **Pipette one drop (50 µl) of the test specimen, positive and negative controls onto separate reaction circles of the disposable slide.**
3. **Add one drop of well-mixed VDRL antigen next to the test specimen, positive control and negative control.**

4. **Using a mixing stick mix the test specimen and the VDRL reagent thoroughly spreading uniformly over the entire reaction circle.**
5. **Rotate the slide gently and continuously either manually or on a mechanical rotor at 180 r.p.m.**
6. **Observe for flocculation microscopically at 8 minutes.**

Interpretation

Reactive: Indicated by large or small aggregates in the center or the periphery of the test circle.
Non-reactive: Indicated by a smooth, even light gray appearance with no aggregates visible.

SEMI-QUANTITATIVE METHOD

1. **Using isotonic saline prepare serial dilutions of the test sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16,1:32, 1:64, 1:128 and so on as follows:**

1-For each specimen to be tested, add 100 µL of 0.9% saline into test tubes numbered 1 to 5.

2-Add 100 µL of specimen onto test tube 1.

3-Mix the mixture. Avoid formation of bubbles.

4-Transfer 100 µL of mixed sample from tube 1 to 2.

5-Repeat this serial dilution procedure in tube 3 to 4, and then 5. Dispose 100 µL from test tube 5 after mixing.

6-Tubes 1 to 5 now represent a dilution series as follows:

Tube Number	1	2	3	4	5
Dilution	1:2	1:4	1:8	1:16	1:32

2. **Perform the qualitative test procedure using each dilution as test specimen.**

Interpretation

The titer is reported as the reciprocal of the highest dilution, which shows a positive test result.

Limitations

1. **Biological false-positive reactions can occur with cardiolipin antigens, mainly in specimens from persons who abuse drugs; who have diseases such as lupus erythematosus, mononucleosis, malaria, leprosy or viral pneumonia; or who have recently been immunized.**
2. **Reactive specimens should be subjected to further serologic study, including quantitation, whenever feasible.**
3. **A prozone reaction may occur. In a prozone reaction, reactivity with undiluted serum is inhibited. The prozone phenomenon may be suspected when a specimen produces only a weakly reactive or a rough nonreactive result in a qualitative test.**
4. **The VDRL may be reactive in persons from areas where yaws is endemic. As a rule, residual titers from these infections will be <1:8.**
5. **There will not be a detectable immune response for 14-21 days after exposure. This test is most useful during the secondary stage of syphilis when the presence of reagin peaks, with typical results >1:32. It is less sensitive to primary syphilis, although there is a low level of <1:16 in about 80% of those who come for medical intervention in the primary stage.**

- ***Rapid Plasma Reagin (RPR) Test***

The Rapid Plasma Reagin (RPR) test is a macroscopic, non-treponemal, flocculation card test. It detects the IgM and IgG antibodies to lipoidal material released from the damaged host cells, as well as to lipoprotein-like material and possibly cardiolipin released from the treponemes.

PRINCIPLE: The antigen used in RPR is a modified VDRL (Cardiolipin) antigen, in which micro particulate charcoal particles are used to enhance the visual difference between positive and negative results. A cardiolipin lecithin-cholesterol antigen coated with carbon particle is mixed with patient's serum. If the specimen contains reagin, flocculation occurs with a coagglutination of carbon particles contained in the antigen suspension, which appears as black clumps. Non-reactive specimens appear as an even light gray homogeneous suspension.

QUALITATIVE METHOD

Procedure

1. **Bring the RPR carbon antigen suspension, controls and samples to room temperature.**
2. **Pipette one drop (50 µl) of the test specimen, positive and negative controls onto separate reaction circles of the disposable slide.**
3. **Add one drop of well-mixed RPR reagent next to the test specimen, positive control and negative control.**
4. **Using a mixing stick mix the test specimen and the RPR reagent thoroughly spreading uniformly over the entire reaction circle.**
5. **Rotate the slide gently and continuously either manually or on a mechanical rotor at 180 r.p.m.**
6. **Observe for flocculation macroscopically at 8 minutes.**

Interpretation

- **Reactive: Indicated by large or small aggregates in the center or the periphery of the test circle.**

- **Non-reactive: Indicated by a smooth, even light gray appearance with no aggregates visible.**

SEMI-QUANTITATIVE METHOD

1. **Using isotonic saline prepare serial dilutions of the test sample positive in the qualitative method 1:2, 1:4, 1:8, 1:16,1:32, 1:64, 1:128 and so on as follows:**

1-For each specimen to be tested, add 100 µL of 0.9% saline into test tubes numbered 1 to 5.

2-Add 100 µL of specimen onto test tube 1.
3-Mix the mixture. Avoid formation of bubbles.
4-Transfer 100 µL of mixed sample from tube 1 to 2.
5-Repeat this serial dilution procedure in tube 3 to 4, and then 5. Dispose 100 µL from test tube 5 after mixing.
6-Tubes 1 to 5 now represent a dilution series as follows:

Tube Number	1	2	3	4	5
Dilution	1:2	1:4	1:8	1:16	1:32

2. **Perform the qualitative test procedure using each dilution as test specimen.**

Interpretation

The titer is reported as the reciprocal of the highest dilution, which shows a positive test result.

Limitations

1. **False positive reactions occur occasionally with the RPR Carbon Antigen test. Such reactions sometimes occur in drug abuse and in such diseases as lupus erythematosus, mononucleosis, leprosy, viral pneumonia and after smallpox vaccinations.**

2. **Reactive specimens should be subjected to further serologic study, including quantitation, whenever feasible.**
3. **The RPR card test cannot be used to test spinal fluids.**
4. **There will not be a detectable immune response for 14-21 days after exposure. This test is most useful during the secondary stage of syphilis when the presence of reagin peaks, with typical results >1:32. It is less sensitive to primary syphilis, although there is a low level of <1:16 in about 80% of those who come for medical intervention in the primary stage.**

TYPHOID AND PARATYPHOID SEROLOGICAL TEST: WIDAL TEST

***Salmonella typhi* and *Salmonella paratyphi* A, B and C cause enteric fever (typhoid and paratyphoid) in human. Laboratory diagnosis of enteric fever includes Blood culture, Stool Culture and Serological test. Widal test is a common agglutination test employed in the serological diagnosis of enteric fever. This test was developed by Georges Ferdinand Widal in 1896 and helps to detect presence of salmonella antibodies in a patient's serum.**

PRINCIPLE OF WIDAL TEST

Patients infected with Salmonella produce antibodies against the antigens of the organism. Antibodies in serum, produced in response to exposure to Salmonella organisms will agglutinate bacterial suspension which carries homologous antigens. This forms the basis of Widal test.

The organisms causing enteric fever possesses two major antigens namely somatic antigen (O) and a flagellar antigen (H) along with another surface antigen, Vi. During infection with typhoid or paratyphoid bacilli, antibodies against flagellar antigen of S. typhi (H), S. paratyphi A (AH), S. paratyphi B (BH) and Somatic Antigen of S.typhi (O) usually become detectable in blood, 6 days after the onset of infection.

Those antigens specifically prepared from organism are mixed with patient's serum to detect the presence of antibodies. Positive result is indicated by the presence of agglutination Absence of agglutination indicates a negative result. The paratyphoid O antigens are not employed as they cross react with the typhoid O antigen. If agglutination occurs with O antigen then it is considered positive for Salmonella typhi. If agglutination occurs in A or B antigen then it is confirmed as positive for Salmonella paratyphi. Agglutination will occur in H antigen for all the cases of antigens like O, A, and B.

PREPARATION OF ANTIGENS

Antigen suspensions may be prepared from suitable stock cultures in the laboratory. But generally, commercially prepared suspensions are used.

- **Salmonella typhi is used to prepare S. typhi O and S. typhi H antigens.**
- **O antigens for S. paratyphi A and S. paratyphi B are not taken as they cross-react with S. typhi O antigen.**
- **H antigen suspension is prepared by treating overnight broth culture or saline suspension of Salmonella with 0.1% formalin.**
- **For preparing O antigen suspension, Salmonella are grown on phenol agar (1:800) to inhibit flagella. The growth is then emulsified in small volume of saline, mixed with 20 times its volume of alcohol, heated at 40°C to 50°C for 30 minutes and centrifuged.**
- **The antigens are treated with chloroform (preservative) and appropriate dyes are added for easy identification of antigens.**

PROCEDURE OF WIDAL TEST

The Widal test can be conducted in two ways:

- **Slide agglutination Widal test**
  1. Qualitative Slide Test
  2. Quantitative Slide Test
- **Tube agglutination Widal test**

Tube agglutination has more accuracy as compared to the slide agglutination technique. However, A slide Widal test is more popular among diagnostic laboratories as it gives rapid results.

Qualitative Slide Test

Procedure:

1. **Bring all reagents to room temperature and mix well.**
2. **Add 1 drop of test sample (25µl) into each reaction circle labeled as O, H, AH, BH according to given antigen solution.**
3. **Add 1 drop of positive control (25µl) into the circle marked as PC and 1 drop of negative control (25µl) into the reaction circle marked as NC.**
4. **Add antigen solutions of Salmonella typhi 'O', Salmonella typhi 'H', Salmonella paratyphi 'AH' and Salmonella paratyphi 'BH' to circles labeled as O, H, AH, BH respectively in which test samples has been added.**
5. **Mix it thoroughly with the aid of applicator stick and rotate the slide gently.**
6. **Observe for agglutination.**

Interpretation:

- **Positive Test: Agglutination within a minute**
- **Negative Test: No agglutination**

Quantitative Slide Test

This is performed for the samples which showed positive agglutination during qualitative test.

Procedure:

1. **Bring all reagents to room temperature and mix well.**
2. **Dispense one drop of saline into the first reaction circle and then place 5, 10, 20, 40, 80 ul of the test sample on the remaining circles.**
3. **Add a drop of the antigen, which showed agglutination with the test sample in the screening (qualitative) method, to each circle.**
4. **Mix the contents of each circle with the aid of applicator stick and rotate the slide gently.**
5. **Observe for agglutination.**

Interpretation:

The antibody titer of the test sample is its highest dilution that gives a visible agglutination. 80 µl corresponds to 1 in 20 dilution, 40 µl to 1 in 40, 20 µl to 1 in 80, 10 µl to 1 in 160 and 5 µl corresponds to 1 in 320 titers. Agglutinin titer greater than 1:80 is considered as significant infection and low titers indicate absence of infection.

Quantitative Tube Test

Procedure:

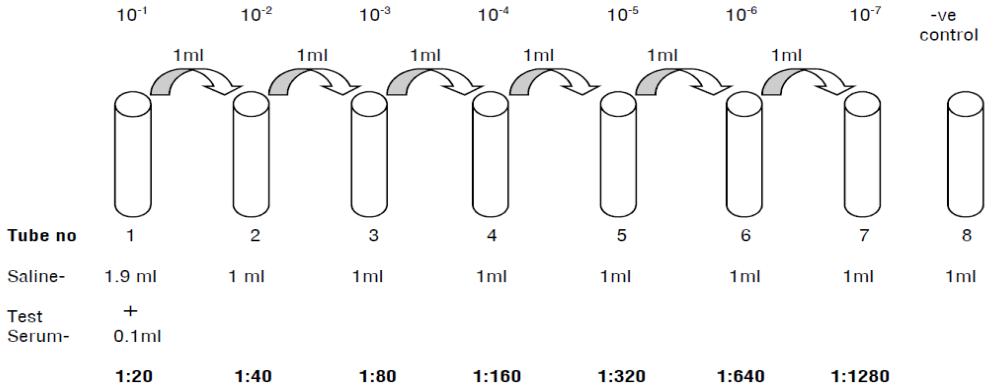
1. **Bring all reagents to room temperature and mix well.**
2. **Prepare 4 sets of test tubes for individual antigen. Each set contains 1- 8 tubes.**
3. **Add 1.9 ml of 0.85% sterile saline to tube no. 1 of each antigen set.**
4. **To tube no. 2-8 of all sets, add 1 ml of physiological saline.**
5. **To tube No. 1 of all sets, add 0.1 ml of test sample to be tested and mix well.**
6. **Transfer 1 ml of the diluted serum sample from tube No. 1 to tube No. 2 and mix well.**

7. **Transfer 1 ml of the diluted serum sample from tube No. 2 to tube No. 3 and mix well. Continue this serial dilution till tube No. 7 in each set of antigens.**

8. **Discard 1.0 ml of the diluted serum from tube No.7 of each set.**

9. **So the dilutions of the serum sample from tube No. 1 to 7 respectively in each antigen set are 1:20, 1:40,1:80, 1:160, 1: 320, 1:640, 1: 1280.**

10. **Tube no. 8 is negative control with 0.85% sterile saline.**



11. **To one set i.e. from tube no.1- 8 add 50 µl of Salmonella typhi 'O' antigen.**

12. **In second set i.e. from tube no.1- 8 add 50 µl of Salmonella typhi 'H' antigen.**

13. **Respectively for third and fourth sets, add Salmonella paratyphi 'AH' and Salmonella paratyphi 'BH' to all tubes from 1-8.**

14. **Mix well, cover and incubate these tubes overnight at 37-degree Celsius (approximately 18 hours).**

15. **After incubation dislodge the sediment and observe for agglutination.**

Interpretation:

The antibody titer of the test sample is its highest dilution that gives a visible agglutination. Agglutinin titer greater than 1:80 is considered as significant infection and low titers indicate absence of infection.

LIMITATIONS OF WIDAL TEST

1. **Tests done within 7 days of illness and after 4 weeks are usually negative.**
2. **The local titer of the place should be known for the results interpreted correctly.**
3. **This test (Quantitative) is highly time consumable.**
4. **Previous typhoid vaccination may contribute to elevated agglutinins in the non-infected population.**
5. **Other infections of non-enteric salmonella infection such as Typhus, Immunological disorders, chronic liver disease may cause false positive reaction.**
6. **Cross reaction between malaria parasites and salmonella antigens may cause false positive Widal agglutination test.**

QUALITATIVE BRUCELLA AGGLUTINATION TEST

PURPOSE: Qualitative detection and semi quantitative estimation of *Brucella* Antibodies to *Brucella* spp. by tube agglutination method. This test is useful in the laboratory diagnosis of Brucellosis. *Brucella* is a strictly aerobic, Gram-negative coccobacillus. This organism is sometimes carried by animals and only causes incidental infections in humans. The four species of this genus that can infect humans are named for the animal which they are most commonly found: *B. abortus* (cattle), *B. suis* (swine), *B. melitensis* (goats), *B. canis* (dogs).

PRINCIPLE: Antibodies in serum produced in response to exposure to *Brucella abortus/ Brucella melitensis /Brucella suis* will agglutinate the *Brucella abortus* bacterial suspension carrying homologous antigen.

The antigen is used for tube agglutination test for diagnosis of Brucellosis.

PERFORMANCE SPECIFICATIONS

• False positive results may occur due to cross reaction with cur due to cross reaction with *Yersinia, Francisella,* and *Vibrio*

• A false negative result may be obtained in the first few days after infection. A positive test result does not necessarily indicate current infection. Hence a repeat test may have to be pe a rise in titer.

PRIMARY SAMPLE

• Use only serum as specimen for the test.

• Collect 2 mL of venous blood in a plain red topped vacutainer tube or 0.1 N HCl washed tubes.

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

• Process all samples within 8 hours of collection

• If sample is to be processed on another day or after 8 hours, separate the serum and store it at 2-8 C for up to 8 days or at -20 C up to 3 months.

• Do not use hemolyzed or lipemic samples for testing

ADDITIVE OR PRESERVATIVE

No need to add any anticoagulant or preservative while sample. Collect venous blood in a plain red topped vacutainer washed (0.1 N HCl) tube.

CONSUMABLES/REAGENTS

***Brucella abortus* plain antigen: *Brucella abortus* plain antigen of is a suspension of a pure smooth culture of *Brucella abortus* strain 99 in phenol saline.**

Carbol saline (Normal saline containing 0.5% Carbolic acid)

EQUIPMENT

- **Incubator at 37°C**
- **Glass test tubes**
- **Micropipettes/ serological pipettes**
- **Test tube stand**

STEP BY STEP PROCEDURE

- **Place five Felix tubes in the test tubes rack**
- **Add 0.8 mL of carbol saline in the first tube and 0.5 mL in all the other tubes.**
- **Add 0.2 mL of serum to the first tube, mix well and transfer 0.5 ml to the second tube. Mix thoroughly and transfer 0.5 ml to the third tube**
- **Continue this process up to the fifth tube and discard 0.5 ml from last tube, after mixing**
- **Add 0.5 mL of the standardized *Brucella abortus* plain antigen to each. Mix by rolling by rolling in between palms or in a cyclomixer and incubate at 37 C for 16-18 hours (overnight).**

Perform the same test with a positive control.

ANTIGEN CONTROL TUBES

For each day's work, put a set of control tubes, as shown below for comparing the results of the test sample

Antigen Control tubes	0.5% control saline	Antigen	Degree of agglutination

Tube 1	**1.0 ml**	**1.0 ml**	**No agglutination -**
Tube 2	**1.25 ml**	**0.75 ml**	**25% agglutination +**
Tube 3	**1.50 ml**	**0.50ml**	**50% agglutination + +**
Tube 4	**1.75 ml**	**0.25 ml**	**75% agglutination +++**
Tube 5	**2 ml**	**nil**	**100% agglutination ++++**

Incubate the antigen control tubes, at 37°C for 24 hours along with the test samples

Each batch of test sample should include a positive control and antigen control tube. INTERPRETATION OF RESULTS

Positive: **Presence of granular agglutination in any of the tubes indicates a positive test**

Negative: **Absence of agglutination in all tubes indicates a negative test**

The titer of the serum is the reciprocal highest dilution of the serum which shows agglutination.

Results of agglutination should be noted after keeping the tubes for an hour or two on the bench at the room temperature.

Examine all the incubated tubes against light and compare the tubes in the test series, with the antigen control tubes. Note the degree of agglutination for each sample of serum as shown under.

The degree of agglutination is to be judged by opacity of the supernatant fluid.

++++ (Comparable with tube 5 of the antigen control series)

+++ (Comparable with tube 4 of the antigen control series)

++ (Comparable with tube 3 of the antigen control series)

+ (Comparable with tube 2 of the antigen control series)

(No agglutination) (Comparable with tube 1 of the antigen control series)

++ (50% agglutination) should be considered as end-point.

REFERENCE RANGE/SIGNIFICANT TITRE

A titer of 1: 80 or greater is considered significant.

SAFETY PRECAUTIONS

- **Handle all samples as potentially infectious**
- **Handle all reagents with care and avoid contact with eye, mouth and Skin.**
- **Do not perform mouth pipetting**
- **Discard used reagents and sample as per disposal procedure**

POTENTIAL SOURCES OF VARIABILITY

Ensure that the sample is free of fibrin/particles by centrifuging may interfere with test results. As a positive test does not differentiate between a past and current infection, a repeat test may have to be performed on a fresh sample after 10-12 days to demonstrate a rise in titer.

SEROLOGIC DIAGNOSIS OF RICKETTSIACEAE: WEIL FELIX TEST

The human pathogens in the family Rickettsiaceae are small bacteria of the genera Rickettsia, Orientia, and Erlichia. They are obligate intracellular parasites and, except for Q fever, are transmitted to human by arthropods such as fleas, lice, mites and ticks. Many rickettsia is transmitted transovarial in the arthropod, which serves as both vector and reservoir.

Rickettsial infections, except Q fever and the ehrlichiosis, typically are manifested by fever, rashes, and vasculitis. They are grouped on the basis of their clinical features, epidemiologic aspects, and immunologic characteristics. Rickettsia can be grown in the laboratory only in cultures of living cells. Diagnosis of most of the rickettsia diseases is facilitated by the development in

the blood of infected patients of specific antibodies that can be detected by serologic tests.

Serologic Diagnosis

Weil Felix (WF) test

This test is dependent on cross-reaction that exists between the antigens of certain rickettsiae and those of selected strains of Proteus vulgaris and Proteus mirabilis.

Suspensions of three proteus strains, OX – 19, OX – 2 and OX- K, are added to dilutions of patient serum. After appropriate incubation, the tubes are examined for agglutination of the proteus suspension. The end point is determined.

Result interpretation:

Either a four – fold or greater rise in titer between acute and convalescent sera or a single specimen titer of greater than or equal to 1:320 is considered to be evidence of certain rickettsial infections.

Note: False negative reactions are common in scrub typhus.

False positive reactions may occur in Proteus infections, relapsing fever, brucellosis and other acute febrile illnesses.

Table: Weil Felix Reaction

Organisms	OX-19	OX-2	OX-K
Typhus group			
- R. prowazekii	**+++**	**+/-**	**-**
- R. typhi	**+++**	**+/-**	**-**
Scrub Typhus group			
- R. trsutsugamushi	**-**	**-**	**+++/-**
spotted Fever group			
- R. conori	**+/++**	**+/++**	**-**
- R. conoripijperi	**+/++**	**+/++**	**-**
- R. siberica	**+/++**	**+/++**	**-**
- R. rickettsi	**+/++**	**+/++**	**-**

SEROLOGIC DIAGNOSIS OF TOXOPLASMOSIS

It is caused by the protozoan *Toxoplasma gondii,* a member of the sporozoan. The tachyzoite directly destroys cells and has a predilection for parenchymal cells and those of the reticuloendotelial system. Humans are relatively resistant, but a low-grade lymph node infection resembling infectious mononucleosis may occur. When a tissue cyst ruptures releasing numerous bradyzoites, a local hypersensitivity reaction may cause inflammation, blockage of blood vessels and cell death near the damaged cyst.

The organism in humans produces either congenital or postnatal toxoplasmosis. Congenital infection develops only when non immune mothers are infected during pregnancy. Postnatal toxoplasmosis is usually much less severe. Congenital infection leads to still births, intracerebral calcification and

psychomotor disturbance when the mother is infected for the first-time during pregnancy. Prenatal toxoplasmosis is a major cause of blindness and other congenital defects.

Most human infections are asymptomatic. However, fulminating fatal infections may develop in patients with AIDS presumably by alteration of a chronic infection to an acute one or changing hosts resistance. Some acquired immunity may develop in the course of infection. Antibody titers in mothers as detected in either blood or milk tend to fall within a few months. Yet, the fact that prenatal infection is limited to infants born of mothers who were first exposed during their pregnancy, strongly suggests that the presence of circulating antibody is at least partially protective.

Serological tests

The Sabin Feldman dye test depends upon the appearance in 2-3 weeks of antibodies that will render the membrane of laboratory cultured living *T. gondii* impermeable to alkaline methylene blue. Thus, organisms are unstained in the presence of positive serum. This test is being replaced by the IHA, Indirect FAT, and ELISA tests. A CFT may be positive (1:8 titer) as early as 1 month after infection, but it is valueless in many chronic infections. The Indirect FAT and IHA tests are routinely used for diagnostic purposes. Blood (Buffy coat of heparinzed sample), bone marrow, CSF and other body fluids can be tested. In addition, Frenkel's intracutaneous test is use full for epidemiological surveys.

Complement Fixation Test

The complete fixation test (CFT) is used to detect the presence of specific antibodies in the patient's serum. This test is based on the use of complement, a Biologically labile serum factor that causes the immune cytolysis i.e. lysis of antibody coated cells.

Principle of complement fixation test

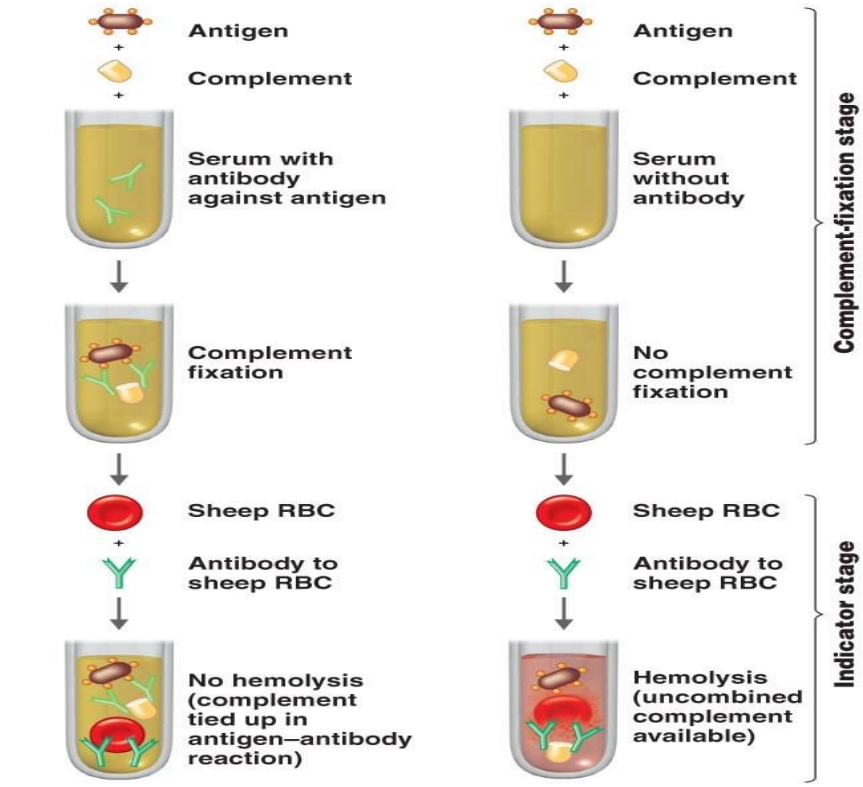
It is the nature of the complement to be activated when there is formation of antigen-antibody complex.
The first step is to heat the serum at 56°C to destroy patient's complement. A measured amount of complement and antigen are then added to the serum. If there is presence of antibody in the serum, the complement is fixed due to the

formation of Ag-Ab complex. If no antibody is present then the complement remains free. To determine whether the complement has been fixed, sheep RBCs and antibodies against sheep RBCs are added.

In the positive test: The available complement is fixed by Ag-Ab complex and no hemolysis of sheep RBCs occurs. So, the test is positive for presence of antibodies.
In the negative test: No Ag-Ab reaction occurs and the complement is free. This free complement binds to the complex of sheep RBC and its antibody to cause hemolysis, causing the development of pink color.

Procedure

The following figure shows the steps involved in the procedure of complement fixation test.



(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

Interpretation

Positive test: The available complement is fixed by Ag-Ab complex and no hemolysis of sheep RBCs occurs. So, the test is positive for presence of antibodies.

Negative test: No Ag-Ab reaction occurs and the complement is free. This free complement binds to the complex of sheep RBC and its antibody to cause hemolysis, causing the development of pink color.

SARS-CoV-2: AN OVERVIEW OF VIRUS DETECTION

INTRODUCTION: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is an enveloped, single-stranded RNA virus of the family Coronaviridae. Coronaviruses share structural similarities and are composed of 16 nonstructural proteins and 4 structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Coronaviruses cause diseases with symptoms ranging from those of a mild common cold to more severe ones such as Coronavirus Disease 2019 (COVID-19) caused by SARS-CoV-2.

SARS-CoV-2 is transmitted from person-to-person primarily via respiratory droplets, while indirect transmission through contaminated surfaces is also possible. The virus accesses host cells via the angiotensin-converting enzyme 2 (ACE2) receptor, which is most abundant in the lungs.

The incubation period for COVID-19 ranges from 2 - 14 days following exposure, with most cases showing symptoms approximately 4 - 5 days after exposure. The spectrum of symptomatic infection ranges from mild (fever, cough, fatigue, loss of smell and taste, shortness of breath) to critical. While most symptomatic cases are not severe, severe illness occurs predominantly in adults with advanced age or underlying medical comorbidities and requires intensive care. Acute respiratory distress syndrome (ARDS) is a major complication in patients with severe disease. Critical cases are characterized by e.g., respiratory failure, shock and/or multiple organ dysfunction, or failure.

Definite COVID-19 diagnosis entails direct detection of SARS-CoV-2 RNA by nucleic acid amplification technology (NAAT). Serological assays, which detect

antibodies against SARS-CoV-2, can contribute to identify individuals, which were previously infected by the virus, and to assess the extent of exposure of a population. They might thereby help to decide on application, enforcement or relaxation of containment measures.

Upon infection with SARS-CoV-2, the host mounts an immune response against the virus, including production of specific antibodies against viral antigens. Both IgM and IgG have been detected as early as day 5 after symptom onset. Median seroconversion has been observed at day 10 - 13 for IgM and day 12 - 14 for IgG, while maximum levels have been reported at week 2 - 3 for IgM, week 3 - 6 for IgG and week 2 for total antibody. Whereas IgM seems to vanish around week 6 - 7, high IgG seropositivity is seen at that time. While IgM is typically the major antibody class secreted to blood in the early stages of a primary antibody response, levels and chronological order of IgM and IgG antibody appearance seem to be highly variable for SARS-CoV-2. Anti-SARS-CoV-2 IgM and IgG often appear simultaneously, and some cases have been reported where IgG appears before IgM, limiting its diagnostic utility.

After infection or vaccination, the binding strength of antibodies to antigens increases over time - a process called affinity maturation. High-affinity antibodies can elicit neutralization by recognizing and binding specific viral epitopes. In SARS-CoV-2 infection, antibodies targeting both the spike and nucleocapsid proteins, which correlate with a strong neutralizing response, are formed as early as day 9 onwards, suggesting seroconversion may lead to protection for at least a limited time.

COVID-19 Testing: PCR, Antigen, and Antibody Tests Explained

There are three types of tests available for COVID-19: polymerase chain reaction (PCR), antigen, and antibody (serology) testing. PCR and antigen tests detect whether a person is currently infected, and serology detects whether a person had an infection in the past. This document is designed to explain the differences between PCR, antigen, and serology testing, and when one test might be used over another.

Topic	PCR Test	Antigen Test	Antibody (Serology) Test
Why is the test used?	PCR tests look for pieces of SARS-CoV-2, the virus that causes COVID-19, in the nose, throat, or other areas in the respiratory tract to determine if the person has an active infection.	Antigen tests look for pieces of proteins that make up the SARS-CoV-2 virus to determine if the person has an active infection.	Serology looks for antibodies1against SARS-CoV-2 in the blood to determine if there was a past infection.
How is the test performed?	In most cases, a nasal or throat swab is taken by a healthcare provider and tested. Sometimes the test can be run while you wait, and sometimes the swab needs to be sent to a lab for testing.	In most cases, a nasal or throat swab is taken by a healthcare provider and tested. Sometimes the test can be run while you wait, and sometimes the swab needs to be sent to a lab for testing.	In most cases, a blood sample is taken and sent to a lab for testing.
What does a positive test result mean?	A positive PCR test means that the person being tested has an active COVID-19 infection.	A positive antigen test means that the person being tested has an active COVID-19 infection.	A positive antibody test means that the person being tested was infected with COVID-19 in the past

			and that their immune system developed antibodies to try to fight it off.

-Antibodies are formed by the body to fight off infections. Immunoglobulin M (IgM) is the first antibody that is formed against a germ, so it appears on tests first, usually within 1-2 weeks. The body then forms immunoglobulin G (IgG), which appears on tests about 2 weeks after the illness starts. IgM usually disappears from the blood within a few months, but IgG can last for years. Some antibody tests test for IgM and IgG, and some only test for IgG.

1- NUCLEIC ACID AMPLIFICATION TECHNOLOGY

CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay

Important note:

- **Conducting the CDC Flu SC2 Multiplex Assay required Letter of Authorization from official healthcare agency.**
- **Users should refer to the CDC Influenza SARS-CoV-2 Multiplex Assay Instructions for Use posted on the FDA website for all IVD products used under Emergency Use Authorization,http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm.**

Intended Use

The Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay is a real-time RT-PCR multiplexed test intended for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A virus, and/or influenza B virus nucleic acid in upper or lower respiratory specimens (such as nasopharyngeal, oropharyngeal and nasal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate)

collected from individuals suspected of respiratory viral infection consistent with COVID-19 by a healthcare provider

The Flu SC2 Multiplex Assay is a molecular *in vitro* diagnostic test that aids in the detection and differentiation of RNA from SARS-CoV-2, influenza A virus, and/or influenza B virus and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®), and control material used in rRT-PCR for the *in vitro* qualitative detection and differentiation of SARS-CoV-2 virus RNA, influenza A virus RNA and/or influenza B virus RNA in upper and lower respiratory specimens.

Principles of the Procedure

The Flu SC2 Multiplex Assay is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The Flu SC2 Multiplex Assay contains three primer/probe sets (InfA, InfB, and SC2) that target the RNA of influenza A virus, influenza B virus, and SARS-CoV-2 virus, respectively. The assay also contains primers and a probe to detect the human RNase P gene (RP) in clinical specimens or control samples. The oligonucleotide primers and probe for detection of SARS-CoV-2 were selected from an evolutionarily conserved region of the 3' terminus of SARS-CoV-2 genome and include part or the carboxy-terminal portion of the nucleocapsid (N) gene. Primers and probes for the detection of influenza A viruses were selected from an evolutionarily well conserved region of the matrix (M1) gene. The primers and probe selected for detection of influenza B viruses were selected from a conserved region of the nonstructural 2 gene (NS2). The assay is a multiplex assay, run in a single well/vessel, designed for detection and differentiation of RNA from SARS-CoV-2 virus, influenza A viruses, and/or influenza B viruses.

Regions of the nucleic acids extracted from upper and lower respiratory specimens that are complementary to the oligonucleotide primers are reverse transcribed into cDNA and amplified by polymerase chain reaction using either the Applied Biosystems TaqPath™ 1-Step Multiplex Master Mix (No ROX), or Quantabio UltraPlex™ 1-Step ToughMix® (4X), using an Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4.1 software. If the target

nucleic acids are present, they are amplified, and the probe(s) anneal to specific complementary sequences located between the corresponding forward and reverse primers during the PCR process. During the extension phase of the PCR, the 5' nuclease activity of DNA polymerase degrades the probe bound to the specific target, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. Probes specific to each virus generate a fluorescent signal at different wavelengths, enabling the instrument to differentiate between the signals. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by Applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4.1 software.

2- SEROLOGY TESTING

COVID-19 IgG/IgM ANTIBODY RAPID TEST

Antibody testing could be a vital tool for determining who has already been infected and might have immunity to the coronavirus. Serology testing for SARS-CoV-2 is at increased demand in order to better quantify the number of cases of COVID-19, including individuals that may be asymptomatic or have recovered. Many individuals may benefit from serology testing as nearly 81% of infected people are asymptomatic or have mild to moderate symptoms

For clinicians, knowing whether a patient has developed IgG antibodies in response to SARS-CoV-2 is important. With this insight, clinicians can counsel the individual on prognosis, personalized care and immunity specific to their situation.

In various studies, it has been seen that seroconversion for SARS-CoV-2 IgG and IgM may occur simultaneously or sequentially. Thus, it's important to identify both IgG and IgM individually. An IgM assay may provide information about current or recent infection; while IgG may signal a more sustained immune response.

Generally, IgM is the first antibody developed against an antigen and is detectable from 4 to 7 days—while, IgG is developed 7 to 14 days after infection

and can be detectable for months and years, depending upon the antigen and the individual.

IgM IgG SEROLOGY TESTING RESULT INTERPRETATION

- - No exposure to the virus, or could be consistent with very early active infection prior to IgM production

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+ - Acute infection; IgM develops within a few days of exposure in most individuals

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+ - There is a short window period—probably at around two weeks into infection—where both IgM and IgG are detectable

- + Pattern expected in those who have successfully cleared the virus and now may have a degree of immunity

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COVID-19 IgG/IgM Rapid Testing Kit

COVID-19 (Corona Virus Disease) is an infectious disease caused by the most recently discovered coronavirus. COVID-19 IgG/IgM Rapid Test (Serum/Plasma/Whole Blood) is a rapid chromatographic immunoassay for the qualitative of IgG and IgM antibodies to COVID-19 in human serum, plasma or whole blood.

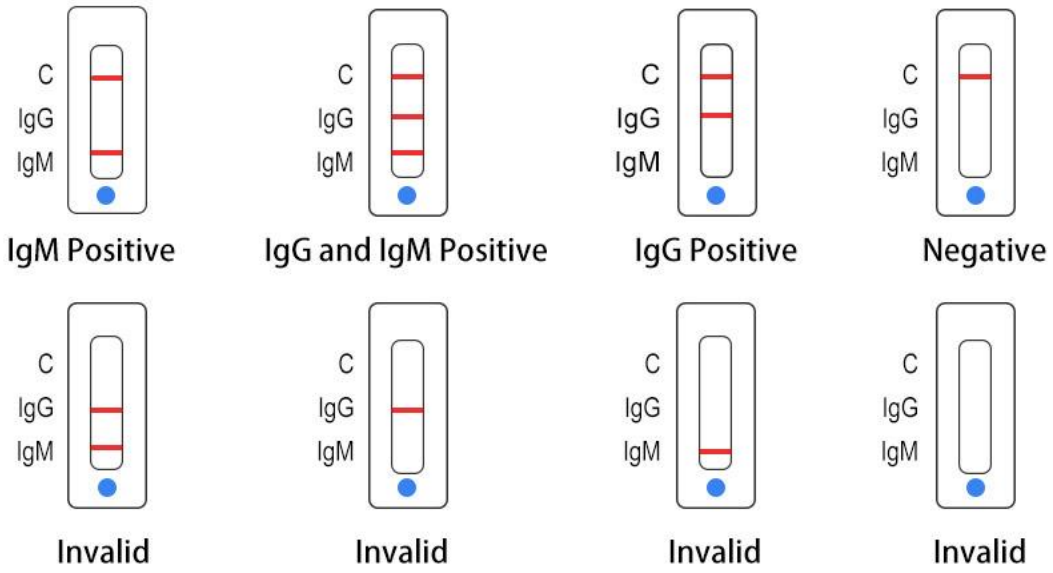
Product Features

Easier: No special equipment needed, Intuitive visual interpretation.
Rapid: Quick sampling by fingertip blood, Result in 10 minutes.
Accurate: Results with IgG and IgM respectively, validated using PCR and CT.
Application: For suspicious patients with symptoms, mild symptoms, or even without symptoms, also for testing people with close contact of infected patients and people under quarantine control.

How Does the Coronavirus Rapid Test Kit Work?

1. Sample collection and preparation. Keep the kit and sample to room temperature prior to testing.
2. Add sample (20 µL of serum/plasma/whole blood) to the sample well by using a micropipette/dropper.
3. Add buffer (1 drop for serum/plasma or 3 drops for whole blood) to the sample well immediately.
4. Wait for the colored line(s) to appear and read the result at 10 minutes. The result is valid within 20 minutes.

Testing Results



IgM POSITIVE: Two lines appear.

Colored lines should be in the control line region (C) and IgM test line region. No line appears in IgG test line region.

IgG and IgM POSITIVE: Three lines appear.

Colored lines should be in the control line region(C), IgG line test region and IgM test line region. The color intensities of the lines do not have to match.

IgG POSITIVE: Two lines appear.

Colored lines should be in the control line region(C) and IgG test line region. No line appears in IgM test line region.

NOTE: The intensity of the color in the IgG and/or IgM test line region(s) will vary depending on the concentration of COVID-19 antibodies in the sample. Therefore, any shade of color in the IgG and/or IgM test line region(s) should be considered positive.

NEGATIVE: One colored line should be in the control line region (C). No line appears in IgG and IgM test line region(s).

INVALID: Control line fails to appear.

Insufficient buffer volume or incorrect procedural techniques are the most likely reasons for control line failure. Review the procedure and repeat the procedure with a new cassette. If the problem persists, discontinue using the test kit and contact your local distributor.

Kit Components & Storage

Materials supplied:
***Test cassettes**
***Droppers**
***Package inserts**
***Buffer bottle**
Storage：
The kit can be stored at room temperature or refrigerated (2-30℃). The test cassette is stable before the expiration date printed on the sealed pouch. The test cassette must remain in the sealed pouch until use. DO NOT FREEZE. Do not use after the expiration date.

Limitations of COVID-19 IgM/IgG Rapid Test Kit

1.COVID-19 Rapid Test is for in vitro diagnostic use only. The test should be performed using serum, plasma or whole blood samples only. Neither the quantitative value nor the rate of increase in COVID-19 antibody concentration can be determined by this qualitative test.

2.In the early onset of fever, anti-COVID-19 IgM concentrations may be below detectable levels.

3.The continued presence or absence of antibodies cannot be used to determine the success or failure of therapy.

4.Results from immunosuppressed patients should be interpreted with caution.

5.As with all diagnostic tests, all results must be interpreted together with other clinical information available to the physician.

6.If the test result is negative and clinical symptoms persist additional testing using other clinical methods is recommended. A negative result does not at any time preclude the possibility of COVID-19 infection.

RAPID ANTIGEN TESTING FOR SARS-CoV 2

General Guidance

Rapid antigen tests are commonly used in the diagnosis of respiratory pathogens, including influenza viruses and respiratory syncytial virus (RSV). Antigen tests are immunoassays that detect the presence of a specific viral antigen, which implies current viral infection. Antigen tests are currently authorized to be performed on nasopharyngeal or nasal swab specimens placed directly into the assay's extraction buffer or reagent. The currently authorized antigen tests are not restricted to use on persons of a certain age. Antigen tests are relatively inexpensive and can be used at the point-of-care.

The clinical performance of rapid antigen diagnostic tests largely depends on the circumstances in which they are used. Rapid antigen tests perform best when the person is tested in the early stages of infection with SARS-CoV-2 when viral load is generally highest. They also may be informative in diagnostic testing situations in which the person has a known exposure to a confirmed case of COVID-19. Rapid antigen tests can be used for screening testing in high-risk congregate settings in which repeat testing could quickly identify persons with a SARS-CoV-2 infection to inform infection prevention and control measures, thus preventing transmission. In this case, there may be value in providing immediate results with antigen tests even though they may have

lower sensitivity than RT-PCR tests, especially in settings where a rapid turnaround time is required.

The specificity of rapid antigen tests is generally as high as RT-PCR – the first antigen tests that have received FDA EUAs have reported specificity of 100% – which means that false positive results are unlikely.

EXAMPLE

Sofia SARS Antigen Fluorescent Immunoassay

The Sofia SARS Antigen Fluorescent Immunoassay (FIA) uses advanced immunofluorescence-based lateral flow technology in a sandwich design for qualitative detection of nucleocapsid protein from SARS-CoV-2. The Sofia SARS Antigen FIA, with the Sofia 2 and Sofia Fluorescent Immunoassay analyzers, provides automated and objective results in 15 minutes, allowing for testing of patients suspected of COVID-19/2019-nCoV in near-patient testing environments.

PRINCIPLE OF THE TEST

The Sofia SARS Antigen FIA employs immunofluorescence technology in a sandwich design that is used with Sofia and Sofia 2 to detect nucleocapsid protein from SARS-CoV and SARS-CoV-2. This test allows for the detection of SARS-CoV and SARS-CoV-2. The test detects, but does not differentiate, between the two viruses. The patient sample is placed in the Reagent Tube, during which time the virus particles in the sample are disrupted, exposing internal viral nucleoproteins. After disruption, the sample is dispensed into the Test Cassette sample well. From the sample well, the sample migrates through a test strip containing various unique chemical environments. If SARS-CoV or SARS-CoV-2 viral antigen is present, they will be trapped in a specific location. NOTE: Depending upon the user's choice, the Test Cassette is placed inside Sofia or Sofia 2 for automatically timed development (WALK AWAY Mode) or placed on the counter or bench top for a manually timed development and then placed into Sofia or Sofia 2 to be scanned (READ NOW Mode). Sofia and Sofia 2 will scan the test strip and measure the fluorescent signal by processing the results using method-specific algorithms. Sofia and Sofia 2 will display the test results (Positive, Negative, or Invalid) on the screen.

REAGENTS AND MATERIALS SUPPLIED

25-Test Kit:

- **Individually Packaged Test Cassettes (25): Monoclonal anti-SARS antibodies**
- **Reagent Tubes (25): Lyophilized buffer with detergents and reducing agents**
- **Reagent Solution (25): Ampoules with salt solution ν Sterile Nasal Swabs (Kit #20374) or Nasopharyngeal Swabs (Kit #20383) (25)**
- **Small, Clear 120 µL Fixed Volume Pipettes (25)**
- **SARS Positive Control Swab (1): Swab is coated with non-infectious recombinant SARS antigens**
- **Negative Control Swab (1): Swab is coated with heat-inactivated, non-infectious Streptococcus C antigen ν Package Insert (1)**
- **Quick Reference Instructions (1)**
- **QC Card (located on kit box)**

MATERIALS NOT SUPPLIED IN KIT

- **Timer or watch ν Sofia or Sofia 2**
- **Calibration Cassette (for use with either Sofia or Sofia 2)**
- **Sofia SARS Antigen Control Swab Set for additional QC (20384)**

Description

- **Rapid results in 15 minutes to support efficient dispositioning of patients**
- **Objective, accurate results without cross-reactivity to seasonal coronaviruses**
- **Dual work modes adjust to volume fluctuations and allows for significant throughput and batching of samples in READ NOW Mode**

- **Seamless test workflow follows a similar format to CLIA-waived Sofia and Sofia 2 assays**
- **Virena provides automated tracking, data capture, government reporting, and exclusive disease mapping.**
- **Fluorescent technology with automated read eliminates the subjectivity of a visual result**
- **All necessary components included in kit, ready for use for nasal swab procedure**
- **Self-contained Test Cassette that is clean, easy to use and dispose of.**

For more information of the product see:
https://www.fda.gov/media/137885/download

BinaxNOW COVID-19 Ag Card

The BinaxNOW COVID-19 Ag Card is an assay for the qualitative detection of specific antigens to COVID-19 in the human nasal cavity. A simple nasal swab is used to collect specimens from people suspected of having an active infection. No equipment is required to process samples or read test results. In addition, minimal chemical reagents are required, which lessens exposure to biohazardous materials and improves safety for those administering the test.

The BinaxNOW™ COVID-19 Ag Card EUA has not been FDA cleared or approved. It has been authorized by the FDA under an emergency use authorization for use by authorized laboratories. The test has been authorized only for the detection of proteins from SARS-CoV-2, not for any other viruses or pathogens,

Here's How It Works

- **The healthcare worker opens the card and lays it flat on a countertop.**
- **Extraction reagent is added to the test card, which is about the size of a credit card.**
- **A nasal swab is taken from the patient.**

- **A technician inserts the swab into the test card, folds over the cover and in 15 minutes, reads the result.**

For more information of the product see: https://www.abbott.com/BinaxNOW-Test-NAVICA-App.html

RAPID QUALITATIVE DETERMINATION OF HIV 1/2 ANTIBODY

PURPOSE: Qualitative determination of HIV 1/2 antibody in human serum by rapid screening technique. This test is indicated for the screening of blood and blood products for HIV 1/2 antibodies.

PRINCIPLE: HIV antigens are immobilized on a porous immunofiltration membrane. Sample and reagents pass through the membrane and absorbed by the underlying absorbent. As the patient's sample passes through the membrane, HIV antibodies, if present, bind to the immobilized antigens, Conjugate binds to the Fc portion of the HIV antibodies to give the distinct pinkish purple color DOTs against a white background.

PRIMARY SAMPLE

• Use only serum as specimen for the test

• If plasma is used, use only heparinized plasma.

• Do not use pooled specimens.

• Collect 2 mL of venous blood in a plain tube/plain 0.1 N HCl washed tube.

• Process the sample on the same day within 2 hours of collection

• If the specimen contains precipitates, it must be centrifuged or filtered.

• If the analysis is not done within 2 hours of collection, separate the serum and store it at 2-8°C for up to 3 days or at -20°C if longer storage is required.

REAGENTS/CONSUMABLES

• Disposable HIV 1/2 tri dot test device.

• Dropper

• Buffers

• Protein A conjugate

• Sodium hypochlorite solution (1%) or other suitable disinfectant for treating the specimens before disposal.

PROCEDURE

• Bring the specimen, buffer and pouch containing the Tri-dot device to room temperature before use.

• Add 3 drops of buffer solution to the center of the device.

• Hold the dropper vertically and add 1 drop of patient's serum. Use separate dropper for each specimen to be tested.

• Add 5 drops of buffer solution.

• Add 2 drops of liquid conjugate directly from the conjugate vial.

• Add 5 drops of buffer solution and read the results.

RESULT

Reactive: **If two dots, one for the control and the other for HIV-1 appear, the specimen is reactive for antibodies to HIV-1.**

If two dots, one for the control and the other for HIV-2 appear, the specimen is reactive for antibodies to HIV-2.

Nonreactive: **Presence of a single dot, only at the control spot.**

Wrong result: **If two dots are present one in the HIV-1 and other with HIV-2 with no dot in control then test has to be repeated. If no dot appears in either control or HIV-1/HIV-test needs to be repeated.**

LIMITATIONS

• The kit works well with fresh samples. Frozen and thawed samples may block the membrane.

• HIV 1 and HIV-2 viruses share many morphological and biological characteristics. It is likely that due to this, their antibodies have a cross reactivity of 30-70%.

SAFETY PRECAUTIONS

• Handle all samples as potentially infectious

• Handle all reagents with care and avoid contact with eye mouth and skin

• Discard used reagents and sample as per disposal procedure.

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

PURPOSE: To detect the presence of antibodies to HIV-1 and HIV-2 in serum by ELISA method using commercially available bioelisa HIV 1+2 (rec).

PRINCIPLE: Bioelisa HIV-1+2 (rec) is a third-generation solid phase enzyme immunoassay in which highly purified recombinant antigens are used for the combined detection of antibodies to HIV A, HIV 2 and HIV-1 subtype O. The immunodominant N- terminal parts of the transmembrane proteins gp41 and the highly reactive core antigen p24 of HIV-1 and gp36 of the produced by means of recombinant technology. The microplate coated with a mixture of these highly purified recombinant antigen. Serum or plasma samples are added to microplate wells. If HIV- specific antibodies are present in the sample, they will form complexes with the antigens on the well. Unspecific antibodies are removed by subsequent washing step. The antigen antibody complexes formed

during the first incubation step are detected with highly purified peroxidase labeled HIV-1 and HIV-2 recombinant antigens. The enzyme activity of bound conjugate is then determined by addition of a substrate solution ntaining a chromogen. This solution will develop a blue color if the sample is positive. The blue color changes to yellow after blocking the reaction with sulphuric acid. The intensity of color is proportional to the oncentration of HIV antibodies in the sample. Wells containing negative samples remain colorless.

PERFORMANCE SPECIFICATION

Patients with recent exposure to EB virus may show false positive result. Performance of this test with other body fluids as specimen is not well established.

A sample reactive may be negative nonreactive retesting due to presence of fibrin clots or cellular materials or particulate matter.

PRIMARY SAMPLE

• Use only serum free from fibrin as specimen for the test

• Plasma collected in Lithium Heparin Sodium Citrate, ACD, CPDA-1, and EDTA tubes may also be used • Collect 2 mL of venous blood from a peripheral vein in a plain redtopped vacutainer tube

• Do not use hemolyzed/contaminated samples/serum containing fibrin and particulate matter for testing

• Separate serum within 30 minutes of collection by centrifugation

• Process the sample on the same day within 3 hours of collection, separate the serum and store it at 2-8°C for up to 14 days OR at -10°C for up to 12 weeks.

REAGENTS/CONSUMABLES

As per Product Insert.

COMPONENTS

- **Microplate: 12 x 8 wells coated with HIV-1 and HIV-2 recombinant antigens. Round bottom.**

- **Concentrate conjugate: HIV-1 and HIV-2 recombinant antigens conjugated with peroxidase. Contains stabilizers protein and preservatives. Dilut 1/51 with the conjugate diluent before use.**
- **Conjugate diluent: Phosphate buffer containing stabilizers protein and preservatives.**
- **Sample diluent: Phosphate buffer with stabilizers protein and preservatives Ready to use.**
- **Washing solution: Concentrate saline buffer (20x) with additives. Dilute 1/20 with distilled or deionized water before use.**
- **Substrate buffer: Citrate-acetate buffer saline containing hydrogen peroxide.**
- **Chromogen: 3,3'5,5'-Tetramethylbenzidine (TMB) in solution. Dilute 1/100 with the substrate buffer before use.**
- **HIV-1 positive control: Diluted and inactivated human serum positive for antibodies to HIV-1. Contains preservatives. Ready to use.**
- **HIV-2 positive control: Diluted and inactivated human serum positive for antibodies to HIV-2. Contains preservatives. Ready to use.**
- **Negative control: Normal human serum, negative for antibodies to HIV-1 and HIV-2. Contains preservatives. Ready to use.**
- **Stopping solution: 1N sulfuric acid. Ready to use.**
- **Adhesive seals: To cover plate during incubations.**
- **Resealable bag: For storage of unused strips.**

PROCEDURE

• Previous operations

• Allow all the reagents to reach room temperature (20-25°C) before running the assay

• Gently mix all liquid reagents before use

• Dilute the concentrate washing solution 1/20 with distilled or deionized water. For example: 50 mL of concentrate washing solution in 950 ml of water. If the kit is stored for long time crystals may appear in the washing solution. To dissolve them put the bottle in a water bath a 37°C. Homogenize the solution before use.

• Dilute the concentrate conjugate with the conjugate diluent according to Table given below. Mix well. It is recommended to dilute the conjugate 10 minutes before use.

Preparation of working dilution of conjugate (1:5 dilution)

Strips required	**1**	**2**	**4**	**6**	**8**	**10**	**12**
Conjugate diluent ml	**1.0**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**	**12**
Concentrate conjugate ul	**20**	**40**	**80**	**120**	**160**	**200**	**240**

ASSAY PROCEDURE

- **Determine the total number of wells needed for the assay. In addition to test samples, it is necessary to include 1 well for the substrate blank, 2 wells for HIV-1 positive control and 1 well for HIV-2 positive control.**
- **Dispense 100 uL of sample diluent in each well except blank well. Dispense 50 uL of each sample in the designated well. Dispense 50 uL of negative control, 50 uL of HIV-1 positive control and 50 uL of HIV-2 positive control in the designated wells.**
- **Cover the plate with the adhesive seal, mix gently and incubate at 37°C for 60 minutes.**
- **Remove and discard the adhesive seal. Aspirate the content of the wells and fill them completely (approximately 300 uL) with the diluted washing solution. Repeat the process of aspiration and washing 4 more times.**

Ensure that each column of well soaks for at least 15 seconds before the next aspiration cycle.

- **Transfer 100 uL of diluted conjugate into each well of the microplate except the blank. Prepare the diluted conjugate during the last 10 minutes of step 3.**
- **Cover the plate with the adhesive seal and incubate at 37°C for 30 minutes.**
- **During the last 15-20 minutes of this incubation prepare the substrate chromogen solution.**

The substrate buffer and the chromogen must be at room temperature, before preparing the working solution. Prepare the required volume according with Table given below. Mix well. The final solution should be colorless; discard if it becomes blue.

Preparation of substrate (1:101 dilution)

Strips required	**1**	**2**	**4**	**6**	**8**	**10**	**12**
Substrate buffer mL	**1.0**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**	**12.0**
Chromogen (TMB) Ul	**10**	**20**	**40**	**60**	**80**	**100**	**120**

- **Remove and discard the adhesive plate cover. Aspirate and wash the plate as in step 4.**
- **Add 100 uL of substrate- TMB solution to each well including the blank. Incubate for 30 minutes in the dark at room temperature (20-25C).**
- **Stop the reaction by adding 100 uL of stopping solution in the same sequence and time intervals as for the substrate-TMD.**

- **Blank the reader at 450 nm with the blank well and read the absorbance of each well, within 30 minutes. It is recommended to read in bichromatic mode using a 620-630 nm reference filter.**

POTENTIAL SOURCE OF VARIABILITY

This test does not detect all potentially infectious individuals/ blood units A negative test result does not rule out exposure to or infection with HIV 1 and/or HIV 2.

This test does not differentiate between HIV 1 and 2 antibodies.

INTERPRETATION OF RESULTS

Calculation

The system calculates the cut off rate from the mean rate of two-index calibrator. It replicates and stores the result. The cut off rate is determined by multiplying the mean index calibrator rate by 2.5. Cut off Rate (CO) = INDEX CALIBRATOR MEAN RATE x 2.5

The HIV - /HIV-2 protocol calculates a result based on the ratio of the samples rate to the cut off ratio (CO) for each specimen and control

S/CO = SAMPLE RATE/CUT OFF RATE

INTERPRETATION

• Specimens with S/CO VALUES <1.00 are considered NEGATIVE

• Specimens with S/CO VALUES > or equal are considered POSITIVE

• Repeat all positive results obtained with MEIA Technique by SANDWICH ELISA technique through freshly collected samples and also confirmed by Western Blot Technique by sending a fresh sample to an approved External Laboratory.The Assay will be completed within 40 minutes

Dipstick test for HIV antibody

Principle: Dipstick tests were developed for the rapid detection of antigens and antibodies in human serum. These tests are generally employed in situations

where quick decisions may need to be taken and often require no equipment other than that provided in the kits. In the dipstick test for HIV antibody, which is commercially available, a polystyrene strip is coated with HIV antigen and allowed to react with serum. Any HIV antibody present will then bind to the HIV antigen. After subsequent incubation with a substrate solution, a colored spot that indicates the presence of HIV antibody will develop.

Materials and reagents

_ Timer

_ Absorbent towels or filter-paper

_ Commercially available test kit containing dipsticks, reagents and positive

and negative controls

_ Weak-positive in-house control serum.

Method

Follow the instructions provided in the kit. A positive result is indicated by any color development on the antibody coated spot. A spot should be visible on the positive control and no color should be seen on the negative control. Weak-positive in-house controls should be included to help in reading results. The test run is invalid if the results obtained using the controls are not as described above.

WESTERN BLOTTING TECHNIQUE CONFIRMATION TEST FOR DETECTION OF

HIV-1/2 ANTIBODIES

PURPOSE: Qualitative determination of HIV 1/2 antibody concentration in human serum by western blot technique. This test is indicated for the confirmation of blood and blood products for HIV 1/2 antibodies.

PRINCIPLE: To perform the assay the strip is incubated with the patient serum/plasma diluted in a buffer. Antibodies to HIV 1 and HIV 2 if present in the serum, binds to the antigens located on the strip. Unbound materials are

washed away. Then the strip is incubated with antihuman IgG conjugated to alkaline phosphatase. After washing the unbound conjugate, substrate is added which results in the initiation of the color bands.

PRIMARY SAMPLE

- **Use only serum as specimen for the test**
- **If plasma is used use only heparinized plasma**
- **Do not use pooled specimens**
- **Collect 2 mL of venous blood in a plain tube/plain 0.1 N HCl washed tube**
- **Process the sample on the same day within 2 hours of collection**
- **If the specimen contains precipitates, it must be centrifuged or filtered**
- **If the analysis is not done within 2 hours of collection, separate the serum and store it at 2-8°C for up to 48 hours or at -20°C if longer storage is required.**

REAGENTS/ CONSUMABLES

- **Disposable HIV 1/2 Membranes/Test strips**
- **Dropper**
- **Forceps**
- **Buffers**
- **Preparation of reagents for one strip**

Working each buffer:

Wash buffer concentrate	**- 1 mL**
Distilled water	**- 19 mL**

Working diluent buffer:

Diluent buffer concentrate	**- 0.5 mL**
Distilled water	**- 4.5 mL**

No. of spoons of blotting powder - 2 spoons

Working conjugate:

Working diluent buffer - 2 mL

100X conjugate - 20 µL

Substrate: Ready to use

Centrifuge

Sodium hypochlorite solution (2%) or other suitable disinfectant for treating the specimens before disposal.

PROCEDURE-RAPID METHOD

• Bring the specimen, buffer and pouch containing the to room temperature before use.

• Positive and negative controls should be included with each run of the test

• Using forceps remove required number of strips from the pouch place it on clean flat surface. Ensure that the numbered sides of strips are facing up. Note down the strip number with respect to samples and control on the worksheet for correct identification.

• Proper and gentle shaking of the tray at 50-60 rpm is extremely important. Improper shaking may affect the sensitivity of the test

• Reagents and samples should be added only to the ends of the trays and not to the strips directly.

• Use separate tips for all reagents including serum.

• Add 2 mL of working wash buffer per strip to each tray and incubate for 5 minutes on rotary shaker and aspirate wash buffer.

• Prepare working diluent buffer according to the number of tests and controls to be run.

• Add 2 mL of the working buffer and 20 uL serum and controls to appropriate wells.

• Incubate for one hour at room temperature (25-30°C) on rotary shaker. The covers of the wells should also be marked to prevent interchange of covers which may lead to cross-contamination.

• Carefully remove covers, aspirate solution completely from tray and discard into sodium hypochlorite solution.

• Wash 3 times with 2 mL working wash buffer per strip for 5 minutes each on W.blot/rotary shaker. Prepare working conjugate solution according to the number of strips to be run.

• Add 2 mL of working conjugate per strip.

• Incubate for 1 hour on W. blot/rotary shaker.

•Aspirate conjugate. Wash 4 times with 2 mL working wash buffer per strip for 5 minutes each on W. blot/rotary shaker.

• Add 2 mL ready to use substrate per strip. Incubate 0.5-15 min away from the light preferably in dark till bands develop

• Continue to observe the reaction till gp160/gp120/gp41 appeal stop the reaction after their appearances. However, in case the ands do not appear, then continue the reaction up to the point.

• Before strong background is formed on the strip.

• Up to 15 minutes, whichever is earlier.

• Aspirate substrate and add distilled water and wash strips reaction

• Remove strips on paper towels and mount on work sheet keeping number side up.

INTERPRETATION OF RESULTS

Negative control: **No HIV-1 and HIV-2 specific bands should be observed on the negative control strips only the band for serum control should be visible.**

Positive control: **Almost all the virus specific bands at positions gp 160. gp 120, p66, p55/51, p31, p24, p17 and HIV-2 specific band should be visible along with the serum control band.**

The results should be interpreted as positive, indeterminate, negative or invalid based on the interpretation table in the instructions manual of the kit.

INTERPRETATION TABLE

INTERPRETATION	PATTERN		
POSITIVE, HIV-1 POSITIVE	a) 2 ENV (either of 2 ENV gp 160, gp41, gp120)	+ +/-	1GAG (p 24) 1 POL (p31, p51, p66)
HIV-1 POSITIVE With HIV-2 INDICATED	b) 2 ENV (either of 2 ENV; gp 160, gp41, gp120)		1 GAG (p24) +/- 1 POL (p31, p51,p66) + HIV-2 $BAND_Z$
HIV-1 NEGATIVE HIV-2 Indicated	Only control band + HIV-2 BAND		
INDETERMINATE Viral specific bands present but pattern does not meet the criteria for POSITIVE	a) 1 ENV (either of 1 ENV; gp 160, gp 41, gp120) b) GAG (p17, p24, p55)	+ GAG (p17, p24, p 55) +	+ POL (p31,p51 p66) POL (p31, p51, p66)

c) Only GAG	**(p17, p24, p55)**
d) Only POL	**(p31, p51, p66)**

INDETERMINATE	**Viral specific bands present but pattern does not meet the**
with HIV-2 Indicate	**criteria for POSITIVE + HIV-2 BAND**

NEGATIVE	**Only control band or control band with p51/55** band**

INVALID	**No control band**

LIMITATIONS

• The test has to be repeated when the test sample shows indeterminate results with the rapid procedure, with overnight incubation

• Dark color background or greenish tinge on the bands p24, p55/51 and p66 will occur due to overexposure of strips to substrate buffer.

•In the indeterminate cases the test has to be retested using fresh specimen after 4-6 months. HIV-1 and HIV-2 viruses share many morphological and biological characteristics. It is likely that due to this, their antibodies have a cross-reactivity of 30-70%.

SAFETY PRECAUTIONS

• Handle all samples as potentially infectious

• Handle all reagents with care and avoid contact with eye, mouth and skin

• Discard used reagents and sample as per disposal procedure.

RAPID DETECTION OF HEPATITIS B SURFACE ANTIGEN BY IMMUNOFILTRATION METHOD

PURPOSE: Qualitative determination of antibodies to Hepatitis B Surface antigen (HBsAg) concentration in human serum/plasma by Immunochromatography. This test is indicated for the screening of blood and blood products to be used for transfusion and an aid for the diagnosis of existing or previous hepatitis B infection.

PRINCIPLE: The test is a one-step Immunochromatography, which employs monoclonal antibodies conjugated to colloidal gold conjugate and polyclonal antibodies immobilized on a nitrocellulose strip in a thin line. The strip is coated with monoclonal antibodies specific for HBsAg. The sample flows laterally from the well through an absorbent pad and mixes with the signal reagent. If the sample contains HBsAg, the colloidal gold antibody (mouse) conjugate binds to the antigen forming an antigen antibody complex. The complex then migrates through the nitrocellulose strip by capillary action. Which are stopped by immobilized Ab zone forming a purple band.

PERFORMANCE SPECIFICATION

Sensitivity: This test can detect hepatitis B antigen in serum or plasma in a concentration as low as 1 ng/mL.

PRIMARY SAMPLE

- **Use only serum/plasma as specimen for the test.**
- **Do not use pooled specimens.**
- **Collect 2 mL of venous blood in a plain tube/plain 0.1 N HCl washed tube.**
- **Process the sample on the same day within 2 hours of collection**
- **If the specimen contains precipitates, it must be centrifuged or filtered**
- **If the analysis is not done within 2 hours of collection, separate the serum and store it at 2-8°C for up to 3 days or at -20°C C if longer storage is required.**

REAGENTS/ CONSUMABLES

• Disposable HEPA cards

• Dropper

• Sodium hypochlorite solution (1%) or other suitable table disinfectant for treating the specimens before disposal.

PROCEDURE

• Bring the specimen and pouch containing the HEPA card to room temperature before use.

• Remove one test card from the pouch and place it on clean flat surface Using the dropper provided add 2-3 (70 µL-100 µL) drops of serum sample into the sample well. Avoid overflowing.

• Let the reaction to proceed up to 20 minutes.

• Read the results after 20 minutes.

• Strong positive reactions may be visible in 5 minutes.

• If negative or questionable results are obtained, and HBV infection is suspected, the test should be repeated on a fresh serum specimen.

RESULT

Positive: **If a distinct purple line is formed at the test zone marked 'T' (Test line) and the control zone marked 'C' (control line)-indicating the sample contains Hepatitis B antigen.**

Negative: If a distinct purple line is formed only at the control zone marked 'C' (control line)-indicating the sample is negative.

LIMITATIONS

This test will indicate only the presence or absence of the Hepatitis B surface antigen in the specimen.

SAFETY PRECAUTIONS

• Handle all samples as potentially infectious

• Handle all reagents with care and avoid contact with eve, mouth and skin

• Discard used reagents and sample as per disposal procedure

• Potential sources of variability

• HBsAg card is used for the detection of HBsAg in human serum or plasma. Based on a single reactive test result, a sample should not be considered HBsAg positive. Further testing, including confirmatory testing, should be performed before a specimen is considered positive for HBsAg

• A non-reactive test result does not exclude the possibility of exposure to hepatitis B virus, Levels of HBsAg may be undetected both in early infection and on specimens containing precipitate give inconsistent test results.

ELISA TECHNIQUE FOR HEPATITIS B SURFACE ANTIGEN

Hepatitis refers to an inflammatory condition of the liver. It's commonly caused by a viral infection, but there are other possible causes of hepatitis. These include autoimmune hepatitis and hepatitis that occurs as a secondary result of medications, drugs, toxins, and alcohol.

Routine tests for hepatitis include the use of markers for hepatitis A, B and C viruses. Hepatitis A is most common in children, especially in nurseries; however, it is not routinely tested for, except in cases of epidemics. Hepatitis B and C viruses are transmitted through blood products, body fluids, contaminated needles and other contaminated materials.

Hepatitis B virus has several markers which include:

— surface antigen (HBsAg)

— antibody to surface antigen (anti-HBs)

— envelope antigen (HBeAg)

— antibody to envelope antigen (anti-HBe)

— antibody to core antigen (anti-HBc).

The concentrations of these markers vary during the course of an infection. The antigen markers appear first or earlier on after exposure to the virus. Seroconversion (antibody production) often occurs several weeks or months after exposure. Hepatitis testing is routinely done by solid-phase ELISA and radioimmunoassay methods. Commercial kits for detection of hepatitis markers are available and specific criteria and instructions are provided with each kit. The general principles of the ELISA technique for one of the markers for hepatitis B virus are outlined below.

ELISA for hepatitis B surface antigen

Materials and reagents

_ Micropipettes

_ Incubator or water-bath

_ Washer or vacuum pump

_ Spectrophotometer (reader)

_ Commercially available test kit containing solid-phase support system, reagents and controls

_ Distilled or deionized water.

Method

1. Add the test (serum) samples and controls to the anti-HBs precoated solid phase support system and incubate according to the manufacturer's instructions.

2. Using a vacuum pump or automated washer, carefully aspirate the liquid from the solid phase and wash the system.

3. Add the specified amount of conjugate (enzyme-linked anti-HBs) and incubate according to the manufacturer's instructions.

4. Aspirate the liquid and wash to remove any unbound conjugate.

5. Add the specified amount of substrate (usually *o*-phenylenediamine) and incubate in the dark. (This is the color development stage and should be protected from light.)

6. Add the stopping solution as specified. The stopping solution (usually an acid) inhibits any further reaction between the enzyme and the substrate.

7. Read the results in a spectrophotometer at the specified wavelength.

8. Calculate the cut-off value for the test run as instructed by the manufacturer.

The test run is invalid if the positive control values are less than the cut-off value. In such cases the assay must be repeated.

Precautions

The ELISA method is fairly easy to perform, but pay attention to the following:

_ Make sure that the reagents and samples are brought to room temperature.

_ Make appropriate dilutions of reagents or specimens if required.

_ Make sure that the pre-coated antigen or antibody (solid phase) is not disturbed during the addition of the sample or of beads.

_ Prepare only enough chromogen solution for a single test run. Store the solution in a closed container, in the dark. If color develops prior to application, a new solution should be prepared.

_ Avoid cross-contamination of samples.

_ Adhere strictly to the incubation times, temperatures and other conditions specified in the manufacturer's instructions.

Dipstick test for hepatitis B surface antigen

Principle: The dipstick test for the detection of hepatitis B surface antigen (HBsAg) takes advantage of the formation of a visible spot by precipitating immunocomplexes. Conjugates of monoclonal antibodies against HBsAg coupled to colloidal gold particles are adsorbed to one area of a nitrocellulose strip.

Polyclonal antibodies against HBsAg are chemically fixed to another area of the strip. A drop of human serum is applied to zone A. The HBsAg antigen in the serum binds to the antibody conjugate and the gold–HBsAg immunocomplex migrates along the strip until it reaches the fixed polyclonal antibodies in zone B. The polyclonal antibodies precipitate the gold–HBsAg immunocomplex, and form a visible red band in zone B. No red band is formed if the serum does not contain HBsAg.

Materials and reagents

_ Commercially available test kit containing dipsticks, reagents and controls.

Positive reaction

Method

1. Label the test strip with the patient's name and/or number.

2. Add a drop of serum to zone A as recommended by the manufacturer.

3. Allow the serum fluid to migrate to zone B on the test strip.

4. Inspect zone B after 10–20 minutes for the appearance of a spot indicating a positive reaction.

DETECTION OF HEPATITIS C VIRUS ANTIBODIES BY TRIDOT TECHNIQUE

PURPOSE: Qualitative determination of anti HCV antibodies in human serum by TRIDOT technique. This test is indicated for the screening of blood and blood products for HCV antibodies.

PRINCIPLE: HCV antigens are immobilized on a porous immunofiltration membrane. Sample and reagents pass through the membrane and absorbed by the underlying absorbent. As the patient's sample passes through the membrane, HCV antibodies, if present, bind to the immobilized antigens. Conjugate binds to the Fc portion of the HCV antibodies to give the distinct pinkish purple color dots against a white background.

PRIMARY SAMPLE

• Use only serum/plasma as specimen for the test

• Do not use pooled specimens

• Collect 2 mL of venous blood in a plain tube/plain 0.1 N HCl washed tube.

• Process the sample on the same day within 2 hours of collection.

• If the analysis is not done within 2 hours of collection, separate the serum and store it at 2–8°C for up to 3 days or at -20°C if longer storage is required

• If the specimen contains precipitates, it must be centrifuged and the clear supernatant to be used for test.

REAGENTS/ CONSUMABLES

• Disposable HCV Tri dot test device

• Dropper

• Buffers

• Protein A conjugate, positive and negative control.

• Sodium hypochlorite solution (1%) or other suitable disinfectant for treating the specimens before disposal.

PROCEDURE

• Bring the specimen, buffer and pouch containing the tri-dot device room temperature before use.

• Add 3 drops of buffer solution to the center of the device.

• Hold the dropper vertically and add 1 drop (50 µL) of patient sample. Use separate dropper for each specimen to be tested.

• Add 5 drop of buffer solution.

• Add 2 drops of protein A conjugate directly from the conjugate vial.

• Add 5 drops of buffer solution and read the results.

RESULT

It can be found by comparing the test strip with the given standard strip.

Reactive

• Appearance of two dots, one at the control region "C" and other at the test region "T_1", indicates that the sample is REACTIVE for antibodies to HCV.

• Appearance of two dots, one at the control region "C" and other test region "T_2 "indicates that the sample is REACTIVE for antibodies to HCV.

• Appearance of all the three dots one each at "C", T1" and T2. indicates that the specimen is reactive for antibodies to HCV.

• Invalid result: If no dot appears after the completion of test, either with clear background or with complete pinkish/purplish background the test indicates ERROR.

Non-reactive: Appearance of dot only at "C" and no dot at 'T1' or "T2".

LIMITATIONS

The kit works well with fresh samples. Frozen and thawed samples may block the membrane.

SAFETY PRECAUTIONS

• Handle all samples as potentially infectious

• Handle all reagents with care and avoid contact with eye, mouth and skin

• Do not perform mouth pipetting

• Discard used reagents and samples as per disposal procedure.

SEROLOGICAL TEST FOR INFECTIOUS MONONUCLEOSIS

Infectious mononucleosis is also called a glandular fever, an acute infectious disease that primarily affects the lymphoid tissue, caused by Epstein Barr Virus (EBV Belong to the gammaherpesvirus subfamily of herpes viruses). The virus enters the body via the respiratory tract and replicates with in the epithelial cells of the nasopharynx and salivary glands. Lysis of cells of the salivary glands' releases EBV into saliva. Exchange of saliva is important in the transmission of EBV leading to IMN. During the course of the disease, B lymphocytes become infected and a state of latency is established in which the viral genome persists within the B cells. Cytotoxic T lymphocytes recognize and the virally infected B cell & epithelial cells. The T lymphocytes develop cellular abnormalities that are seen as atypical lymphocytes that characterize IMN (despite the name of this disease, the abnormal cells are lymphocytes and not monocytes). The symptoms and signs of IMN include a sore throat, low grade fever, enlarged and tender lymph nodes. The virus has been associated with subsequent development of two forms of cancer; Burkett's lymphoma and naso pharyngeal carcinoma in different population group.

Three distinct groups of antibodies are found in infectious mononucleosis: -

o Heterophil antibodies

o Epstein Barr virus (EBV) antibodies

o Hetero antibodies

Heterophil antibodies

Are antibodies that react with an antigen entirely different from and phylogenetically unrelated to the antigen responsible for their production. Are agglutinins that react particularly to sheep and horse red cells and are mainly class IgG. Are detected by Paul – Bunnell test. Antibodies to EBV are produced early in the disease and can be detected by complement fixation tests and Immunofluorescence techniques. Heterophil antibodies are present in low titer in the serum of normal persons and are known as forssman antibodies. They resemble the antibodies found in IMN in that they agglutinate sheep red blood cell, but differ from them in that they are absorbed by an emulsion of guinea pig

kidney which is rich in forssman antigen and are not absorbed by beef cell which are poor in forssman antigen.

In cases of serum sickness or sensitization to animal (usually horse) serum, another type of sheep red cell agglutinating antibody is found and may be present in high titer. However, this is again distinguished from the antibody of IMN by being absorbed by guinea pig kidney and froms forssman antibody by being absorbed by beef red cells. This comparison is used as the basic for presumptive and differential tests. The sheep cell agglutinins of IMN can be distinguished from those of serum sickness and other conditions by means of a differential test using absorption with guinea pig kidney and beef red cell Antigens. The antibody that can be removed by absorption with guinea pig kidney is known as the forssman antibody and the guinea pig kidney as the forssman antigen.

- The classical sheep red cell agglutination test is carried out in two steps.

1. The presumptive test of Paul - Bunnell

2. The differential test of Paul Bunnell and Davidsohn

- Modifications of these classical procedures utilize horse red cells instead of sheep red cells.

- Under normal circumstances, rapid screening tests for IMN are done for the presence of heterophil antibody. Horse red cells are usually used rather than sheep red cells, as they are more sensitive to heterophil antibodies.

- Persons suffering from IMN begin developing heterophil antibody shortly after the appearance of the symptoms, usually during the first 2 weeks.

- Highest titers are found during the second and third weeks of the illness. The titer, however, bears no relationship with the severity of the illness.

Note Heterophil sheep cell agglutinins appear in only 50 -80% of cases of IMN

- **Negative result can be obtained when the disease is present. Negative tests therefore, don not rule out the possibility of the disease.**

- **The test for heterophil antibodies is of confirmatory diagnostic importance in case of IMN with typical clinical and hematologic findings. Recently, faster and easier screening tests have been introduced and have replaced the laborious presumptive and differential tests.**

- **These tests are done on a slide. The serum from the patient is mixed thoroughly with guinea pig kidney on one spot of the slide and with beef red cell stromata on another spot.**

- **The unwashed preserved horse red cells are added immediately to both spots. Agglutination is observed on both spots of the slide one minute after the final mixing.**

Interpretation of slide agglutination

If agglutination is stronger on the spot where the guinea pig kidney suspension was mixed with the patient's serum, the test is positive. If it is stronger on the spot where the beef red cells were mixed with the patients' serum, the test is considered negative. If agglutination is equal on both spots, the test is negative. If no agglutination appears on either spot, the tests is negative.

N.B - The glass slide used for these rapid screening tests must be carefully cleaned under running water. Use of detergent could cause errors in the result. Most of the widely used immunologic assays for IMN are highly sensitive.

SEROLOGIC METHODS FOR DETACTION OF CYTOMEGALOVIRUS

Cytomegalovirus is a ubiquitous human viral pathogen. Human CMV is classified as a member of the herpes viruses, are relatively large, enveloped DNA viruses that undergo a replicative cycle involving DNA expressions and nucleocapsid assembly within the nucleus.

Although the herpes family produces diverse clinical diseases, the viruses shares the basic characteristics of being cell associated. These characteristics may play a role in the ability of the virus to produce sub clinical infections that can be reactivated under appropriate stimuli. Dissemination of the virus may occur by oral, respiratory, or venereal route. It may also be transmitted preferably by organ transplantation or by transfusion of fresh blood.

Persistent infections characterized by periods of reactivation of CMV are frequently termed latent infections, although this condition has not been clearly defined for CMV. Acquired CMV infection is usually asymptomatic and can persist in the host as a chronic or latent infection. In most patients, CMV infection is asymptomatic, occasionally a self-limited, heterophil negative, mononucleosis like syndrome results. CMV infections is known to alter the immune system and produce overt manifestations of infection. Infection interferes with immune responsiveness in both normal and immunocompromised individuals. This diminished responsiveness results in a decreased proliferation response to the CMV antigen, which persists for several months. In patents with CMV monucleosis like syndrome, alterations of T. lymphocytes subsets result producing an increase in the absolute number of suppressor (CD) lymphocytes and a decrease in helper (CD) lymphocytes.

In cells infected by CMV, several antigens appear at varying times after infection. Before replication if viral DNA takes place, immediate early antigen and early antigens are present in the nuclei of infected cells.

Diagnostic Evaluation

Serologic methods to defect the presence of IgM antibodies can aid in the diagnosis of primary infection. Detection of CMV-specific IgM can represent primary infection or rare reactivation of infection.

Detection of significant increases in CMV- specific IgG antibody by methods such as complement fixation (CF), anti-complement immunofluorescense (ACIF) and Enzyme Immunoassay (EIA) suggest, but do not prove recent infection or reactivation of latent infection. The EIA method for IgM and IgG, antibodies to CMV has replaced CF, ACIF, and IFA. Latex particle agglutination and indirect hemogglutination are useful screening methods to obtain sero negative blood donors.

Newer CMV detection methods are being explored. CMV antigen detection in urine by EIA and cDNA is being developed. RNA transcript of CMV DNA is detectable in peripheral blood mononuclear cells of sero positive individual by in situ hybridization (ISH) with DNA of CMV.

SECTION 3: HISTOPATHOLOGY

FIXATION OF SPECIMENS

"Fixation is the preparation of a histologic or pathologic specimen in a physical and also partly in a chemical state for the purpose of maintaining the existing form and structure of all of its constituents". It is one of the important requirements in preparing good tissue sections.

Purpose

The purposes of fixation are as follows

1. **To prevent post-mortem changes such as putrefaction and autolysis, by inhibiting autolytic enzymes and killing the organisms that cause decomposition.**
2. **To preserve the various tissue constituents nearly as possible to their original form.**
3. **To harden the naturally soft tissue permitting easier and safer manipulation during subsequent processing.**
4. **To render the various tissue constituents receptive to subsequent staining.**

The essentials for good fixation are:

1. Fresh tissue: Surgical specimens should be immersed in the fixing solution as soon as possible. Specimens obtained post-mortem would have undergone les autolysis if it is placed under refrigeration as soon as possible

2. Proper penetration of the tissue by the fixative: Inadequate penetration produces poor nation and subsequent bad staining. The mass of tissue excised may be so large that the fixative will not penetrate the tissue within a reasonably short time. In such situation specimen is fixed for longer time. ie. 48 to 72 hours depending on the size of the specimen

CHOICE OF FIXATIVE SOLUTION

Choice of fixative is made with several factors in mind, e.g. structures and entities to be demonstrated and the effects of short-term and long-term storage. Some fixatives are restrictive others are multi-purpose. Most commonly used fixatives are listed below:

1.10% Neutral Buffered Formalin

Preparation

37 to 40% Formaldehyde ……..100 ml

Disodium hydrogen phosphate…….6.5 gm

Sodium dihydrogen phosphate ……4.029 gm

Distilled water……..900 ml

Except in special circumstances it is used routinely for fixation of all biopsy specimens.

Advantages

1. **Sections prepared from tissue fixed in formalin can be stained with almost any kind of special stains,**
2. **Formalin fixed tissue lends itself well to preparation of frozen sections and staining for fat.**
3. **Formalin does not cause excessive hardness or brittleness of the tissue.**

2. Glutaraldehyde

• Glutaraldehyde penetrates more slowly than formaldehyde and is useful in electron microscopy and in enzyme histochemistry.

• There are many variations in the preparation of this fixative, including the percentage of glutaraldehyde, other additives, and buffers. Small blocks of tissues 1-2 mm in size, fix well at cold temperature 1°- 4°C, and fixed tissue specimens can be stored in buffered solution for many months. • The slow penetration, cold temperature, and the need for a storage medium prevent the use of this fixative in routine diagnostic histotechnology. Electron microscopists, however, are able to use it with continued success.

3.Alcohol (Propanol/ Methanol/ Ethanol)

It is used for cytology specimens.

Following fixation, tissue is transferred to a tissue cassette. These come in various sizes and hold and protect the tissue whilst it undergoes processing. Once the embedding stage is reached, the cassette lid is snapped off and the main part of the cassette forms a base for the paraffin wax block. The cassettes can be labelled by hand (with pencil!)

DECALCIFICATION

Cutting of thin sections by ordinary method is impossible when the issue has become partly calcified. Such tissues must be treated to remove the calcium and phosphate salts, which are deposited in them. Methods of decalcification involve the use or acids, in which the bone salts are dissolved. All such acid solutions are in ingenious to the organic ground substances of the tissues, which must therefore be protected by adequate fixation before decalcification.

Fixation

Fix all tissue samples in 10% neutral buffered formalin. All fixed specimens from biopsies are washed in slowly running tap water for a minimum of 30 minutes. Larger specimens are washed up to a maximum of one hour. Avoid rinsing in rapidly running tap water. To avoid losing small biopsy specimen carefully decan the fixative. The volume of decalcification used should be at least one oz/gm of tissue and should be changed once or twice a day until decalcification is completed.

DECALCIFYING FLUID

A. Formic Acid-Sodium citrate mixtures

1. Stock citrate solution

Sodium citrate……..100 gm

Water……..500 ml

2. Stock formic acid

Concentrated formic acid……..250 ml

Distilled water……250 ml

Well-fixed calcified tissues are placed in a mixture of equal parts of stock citrate and formic acid solutions. Change daily until decalcification is complete. Then wash in running water for 4 to 8 hours. Then dehydrate, clear and embed.

ADVANTAGES OF FORMIC ACID DECALCIFICATION

• This has proved to be one of the most popular methods since formic acid is gentler on tissues than nitric acid.

• Tissues are not ruined if they remain in formic acid beyond the completion of decalcification.

• It is safer to handle

• Addition of citrate accelerates decalcification by chelating the calcium as it is liberated from the bone.

B. Von Ebner's hydrochloric acid-Sodium chloride mixture

Conc, hydrochloric acid (Specific gravity 1.19) ..... 15 ml

Sodium chloride ..... 175 gm

Distilled water ..... 1000 ml

Procedure: Add 1 ml of conc. Hydrochloric acid to each 200 ml of the above mixture, place the fixed calcified tissues in the solution. Change daily until decalcification is complete.

END-POINT OF DECALCIFICATION

End-point of decalcification can be checked by the following tests:

Physical Test: The physical test includes bending the specimen or inserting a pin, scalpel directly into the tissue. The disadvantage of inserting a pin or scalpel is the introduction of tears and pin hole artifacts slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

Chemical Test:

1.Stock 5% Ammonium hydroxide 28%.....5 mL

Distilled water .....95 mL

2. Stock 5% Ammonium oxalate..... 5 gm

Distilled water .....100 mL

Working ammonium hydroxide/ammonium oxalate solution

Add equal parts of 5% ammonium hydroxide and 5% ammonium oxalate solution

Procedure: To 5 mL of decalcification solution withdrawn from underneath the specimen, add working 10 mL of ammonium hydroxide/ammonium oxalate solution, mix well and allow to stand for 15-30 minutes. When no precipitate is observed, it indicates the completion of decalcification. Wash the tissue thoroughly in running water, prior to processing.

TISSUE PROCESSING

The term tissue processing refers to treatment of tissue necessary to impregnate them with solid medium to facilitate the production of sections for microscopy. The aim of tissue processing is to embed the tissues in a solid medium firm enough to support the tissues and give it sufficient rigidity to enable thin sections to be cut, and yet soft enough to enable the knife to cut the sections with little damage to the knife or tissue.

Automatic tissue processor: Is a compact and sturdy instrument designed with latest technology for complete automatic dehydration and filtration of human, animal and plant's tissues, up to final fixing in wax

Introduction

The more satisfactory embedding material for routine histology is paraffin wax. It is essential that the embedding medium thoroughly permeates the tissue in fluid form and that it solidifies with little damage to the tissue. when the tissue is received it is usually partly or completely fixed in a suitable fixative, nearly always an aqueous fixative. Before the tissue can be embedded in paraffin wax, the tissue must be subjected to the following:

1. Completion of fixation.

2. Gentle but completes dehydration to remove aqueous fixative and any tissue water

3. Clearing with a substance which is totally miscible with both the dehydrating agent, which precedes it and the embedding agent which follows it.

4. Embedding: These four processes depend on complete impregnation of the tissue by the fluid being used.

(i) Dehydration

First stage in the processing of fixed tissue involves the removal of aqueous and some of the lipid tissue fluids by a variety of compounds, many of which are alcohols of varying types. Several are hydrophilic and attract water from the tissues, whereas others affect dehydration by repeated dilution of the aqueous tissue fluids. Numerous dehydrants are readily available and are used in a series of increasing strength, beginning by immersing the tissue in, for example, 70% alcohol, progressing through 95% and finally several changes of absolute alcohol. Commons dehydrating fluids used are isopropyl alcohol, ethyl alcohol, methanol. Whatever agent is used its amount in each stage should not be less than ten time the volume of tissue to be dehydrated. Dehydration is also essential before stained sections are mounted.

(ii) Clearing or Dealcoholizing

The use of a clearing agent becomes necessary as the dehydrating agent i.e., alcohol is not miscible with the impregnating medium (paraffin wax). The essential requirements of a clearing agent are that it is not both dehydrating

agent and embedding agent. The word "Clearing agent" is used because in addition to removing alcohol many of these substances have the property of making the tissue transparent. This is possible because the refractive index of the clearing agent is approximately equal to that of the tissues. Clearing agents suitable for routine use are: Xylene, Chloroform.

(ii) Paraffin Wax Impregnation

This process involves the impregnation of the tissues with a medium that will fill all natural cavities, spaces and interstices of tissue the spaces within the constituting cells and that will set to sufficiently a firm consistency to allow the cutting of thin sections without undue distortion and without alteration of the spatial relationships of the tissue and cellular elements.

Vacuum Impregnation

This technique is the transferring of cleared issues in a heated sealed container of molten paraffin wax and applying suction to the container. Vacuum impregnation or embedding under reduced is of use, for certain purposes:

a. Lung or other tissues that contain much air

b. Dense pieces of tissues, eg skin and embryo which tend to become excessively hard in routine processing

c. To rapidly eliminate the clearing agents

MANUAL PROCESSING SCHEDULE

When tissues are to be processed regularly or manual means, an arrangement of containers allows fewer possibility of error and greater speed.

Table: Processing Schedule for smaller biopsies measuring less than 5 mm thickness

Container	*Fluid*	*Time*
1.	**100% alcohol**	**30 min**
2.	**100% alcohol**	**30 min**
3.	**100% alcohol**	**30 min**
4.	**Xylene**	**30 min**
5.	**Xylene**	**30 min**
6.	**Paraffin**	**30 min**
7.	**Paraffin**	**1 hr**
8.	**Paraffin under Vacuum**	**30 min**
9.	**Embed**	

Table: Manual processing schedule for thick specimen

Container	*Fluid*	*Time*
1.	60% alcohol	Leave overnight
2.	80% alcohol	8 hours
3.	95% alcohol	Overnight
4.	100% alcohol	24 hours
5.	Xylene	8 hours
6.	Xylene	Leave overnight
7.	Wax	2 hours
8.	Wax	4 hours
9.	Embed	

AUTOMATIC TISSUE PROCESSING

Automatic tissue processor enhances the processing of tissue specimens by using heat, vacuum, pressure and agitation. Processor also allows the three stages to be carried out and without the presence of personnel. Routine use of graded alcohols from a lower to higher concentration is standard. The sequential steps given in the processing schedules those follow are for small biopsies and routine specimens.

SHORT SCHEDULE (BIOPSIES AND SMALL FRAGMENTS OF TISSUE MEASURING LESS THAN 5 MM THICKNESS)

Total processing time--3 to 4 hours (approximately)

- **Hold if necessary, in 80% alcohol**
- **95% alcohol 3 changes, 15 to 20 mins each**

- **Absolute alcohol, 3 changes, 15 mins each**
- **Equal parts of absolute alcohol and xylene--15 mins**
- **Xylene, two changes 15 mins each**
- **Paraffin, 3 changes-15 mins each**
- **Paraffin under vacuum - 15 to 20 mins and then embed**

OVERNIGHT SCHEDULE (FOR ROUTINE SPECIMENS)

Total processing time- 14 to 16 hours (approximately)

- **80% alcohol 1 hour**
- **95% alcohol, 3 changes 1 hour each**
- **Absolute alcohol, 3 changes-1 hour each**
- **Xylene, 3 changes. 1 hour each**
- **Paraffin, 3 changes--1 hour each**
- **Paraffin, under vacuum 1 hour and then embed.**

OVERNIGHT PROCEDURE FOR EXENTERATED SPECIMENS AND LARGER BIOPSIES MEASURING MORE THAN 20 MM)

Total processing time 17 hours (approximately)

- **60% alcohol 4 hours**
- **95% alcohol 2 changes-1 1/2 hours each**
- **Absolute alcohol - 4 changes-45 mins each**
- **Xylene - 3 changes--1 hour each**
- **Paraffin - 2 changes-2 hours each**
- **Embed**

Delay in operation of tissue processor:

During the overnight processing (14-16 hrs schedule), the processing schedule can be delayed by 4-5 hrs with the help of the timer provided in the instrument, so that the tissues can be prevented from over exposure in hot paraffin which might cause shrinkage. In such conditions, the tissues would remain in 80% alcohol during the delayed period. In case the tissue processor needs to be operated during holidays, the processing schedule can be delayed by 24 hrs or more depending on number of holidays. During this period of delay, the first jar should be filled with 10% neutral buffered formalin so that the tissues remain in the fixative itself and avoid over exposure of alcohol.

EMBEDDING

Tissues that have been completely dehydrated and cleared are impregnated with paraffin wax by immersion in a succession of molten wax baths. A physical advantage is provided in the handling of small specimens by surrounding the specimen with a mask or block of embedding material, thus allowing the specimens to be handled and fixed to the microtome block without damage to the actual tissue.

PARAFFIN WAX EMBEDDING

Paraffin embedding is the technique used in virtually all-general path laboratories

Advantages of Paraffin Embedding

1. Sections embedded in paraffin can be cut thinner.

2. A wide variety of special histochemical stains can be used

3. Serial sections can be readily prepared. Remaining uncut tissue residing in the paraffin block can be kept permanently

EMBEDDING EQUIPMENT

There are several specialized materials and equipment that facilitate paraffin embedding.

There are several methods involved:

1 . Paper boat method

2 . Ice tray methods

3. L-mold method

4. Plastic molds

5 . Plastic embedding ring

6. Disposable molds

7. Steel molds

Embedding molds are used for casting shaping liquid paraffin into blocks. These are:

The most useable mold right now is:

i) Stainless steel molds are perhaps the most widely used and are considered ideal for embedding purpose. It is manufactured in various Sizes to accommodate different sizes of tissue specimens. They are re-usable but periodic cleaning is required.

ii) L pieces consist of two L-shaped pieces of metal resting on a flat metal base. The L pieces can be moved to adjust the size of the mold so that it will match the size of the tissues

PARAFFIN EMBEDDING PROCEDURE

Fresh molten wax (melting point of 56°C to 60°C) is poured into the mold, which is to be used for embedding. The wax touching the mold will quickly form a thin solid layer. The tissues are then lifted from the final wax with previously warmed forceps and placed in the bottom of the mold. The side of the tissue from which it is desired to take sections is placed face down and all other tissue must be carefully oriented, so that the plane of the sectioning will be correct. It is necessary to press down the tissue specimen in the mold for few seconds until it is held by the cooling wax. It is also necessary to flame the forceps periodically to prevent wax and tissue from adhering to its points. A label bearing the pathology number of the tissue in the mold next to the tissue. When the wax

becomes partly solid the mold should be placed in a basin of cold water (10° to 18° C) or refrigerator to cool. This method of cooling lessens the tendency of some waxes to crystallize when allowed to set at room temperature give blocks of uniform, smooth and solid consistency.

TRIMMING

When blocks are hardened it is removed. Excess wax is cut leaving 2- 3 mm of wax between edge and tissue, so that the block forms a four- sided prism. A small paper tag bearing the tissue number is affixed to block with the help of hot knife or spatula.

To attach the block to the wooden block holder, simultaneously heat both and press together while molten and seal the edges by means of a hot knife.

PARAFFIN SECTION CUTTING

Equipment required

1. Microtome

2. Water bath, thermostatically controlled

3. Drying oven

4. Forceps and small squirrel hair brush

5. Coated slides

6. Slide rack

7. Tissue paper

8. Ice cubes.

1. Microtome: It is an instrument designed for the accurate cutting of thin sections of tissues. Two types of microtomes used for light microscopy are rotary microtome where the block moves, is the most widely used. Sliding microtome where the knife moves, it is particularly useful when sectioning large blocks.

2. Water bath: With temperature of about 54°C to 58°C as required.

3. Drying ovens: The fixing of sections to slides can be done in an oven at 54 to 58 C.

4. Forceps and brush: It is necessary for the manipulation of sections during cutting and for removal of folds and creases in sections after floating out.

5. Section adhesive: Certain adhesive mixtures are used for coating the slides so that the section remains adherent to the slide during subsequent staining. They are all protein solution and since proteins retain many stains, the amount and concentration of the adhesive used for the slides must be kept to a minimum. These adhesives mainly act by reducing the surface tension and thus produce closer capillary adhesion of the section to the slide.

Coating of Slides

1.Chrome alum gelatin solution

Gelatin .....3 gm

Chromium potassium sulfate……0.5 gm

Distilled water…….... 1000 mL

Method:

Heat the water to 60°C and completely dissolve the gelatin with the aid of a magnetic stirrer. Stir in the chromium potassium sulfate (the solution should turn pale blue) add few crystals of thymol as a preservative. Dip clean slides in the warm solution, blot the edges and then stand the slides on air dry. Once dried, store in dust-free container until ready for use.

2. 3-Aminopropyltriethoxy silane (APES)

Method:

1. Place slides in a rack and immerse in acetone for 5 min.

2. Immerse in a solution of 2% silane in acetone v/v for 5 min.

3. Rinse in two consecutive baths of acetone for 5 min. each.

Allow to dry in room temperature or 60° in oven for 45 minutes and store at room temperature indefinitely.

PRACTICAL SECTION CUTTING

Section cutting may be carried out with the operator standing or sitting.

Setting of Microtome (Rotary)

The floatation bath should be filled with distilled water at the appropriate temperature. The block is removed from the ice, wiped dry and clamped firmly in the clamp of the microtome. The object holder is moved until the surface of the wax just touches the knife edge, cutting then being commenced with regular even strokes (there must be no jerking motion).

It is important to tighten knife clamp screws securely, similarly block amp screws must also be firm.

Trimming of tissue block: In order to trim away any surplus wax and to expose a suitable area of the tissue for sectioning, the section thickness is adjusted over 5 to 10 microns. A series of glass slides is numbered with a diamond marker pencil. When the block has been trimmed a new blade is used for cutting sections. The section thickness is now set to appropriate level, for routine purpose, 4-6 microns.

Cutting Sections

Paraffin blocks are cooled with ice cubes before cutting, such cooling can cause the production of flat sections and also make the wax block harder and firmer, thus facilitating the cutting of thin sections.

It is important that the upper and lower surfaces of the block to be cut are parallel in order that straight ribbons of sections are obtained. The first section is raised carefully with the index finger or camel's hairbrush. As the knife strikes the block to start cutting the next section, locally generated heat and pressure weld the edge of section number 2 to the back edge of section number one. This continues with succeeding sections. When the ribbon is several cms long, handling is greatly eased, the first section being held with the finger or forceps

and the last section being detached from the knife by means of a small brush. The ribbon of sections may be immediately floated onto the water bath. To obtain flat sections, it is necessary to spend time in cutting and gentle stretching of the ribbon, before floating on the water bath.

Floating out sections: The action in floating out must be smooth, with the trailing end of the ribbon making contact with the water first. The slight drag produced when the ribbon touches the water surface is sufficient to produce tension in the ribbon and remove some folds from the sections. When the ribbon has come to rest on the water, any remaining wrinkles and folds are removed by tearing apart, using forceps. Complete flattering expansion of sections is achieved after minutes on the surface, this may be hastened by transferring the sections on a slide to second water bath at higher temperature. Prolonged floating out of section on the water bath may be avoided as tissues may expand beyond their original size and become distorted

Picking up section: The ribbons of sections floating water may be split into individual or groups of section by the use of forceps. Picking up of a section on slide is achieved by immersing the slide, lightly smeared with adhesion, in the water bath to the three-fourth of its length and maneuvering the section into contact with the slide. On lifting the slide vertically from water, the section will flatten onto the side. The sections are correctly positioned on the side and blotted lightly with moistened blotting paper to remove excess water and to increase contact between section and the slide.

Drying sections: The sections are dried in an oven at 54 C to 58 C temperature, minimum of 45 minutes. The advantage of oven drying is that dust is less likely to settle on sections. After removal of sections the oven, the cool sections are stored in dust free container.

FROZEN SECTION

Purpose: To prepare the slides for histopathologic diagnosis we sunt duration to rule out malignancy. Various special stain techniques on diagnosis are performed on fresh or fixed or frozen tissues Inspire for all tissues required except for aspirates or scrapped sample

Principle: When the tissue is frozen, the water within the tissue turns to ice and in this stage, the tissue is firm with the ice acting as the embedding sample.

A cryostat consists of a rotary type microtome enclosed in mechanically refrigerated cabinet; virtually, it is a microtome in a deep freeze. It has a platform or chamber to freeze tissue rapidly and mostly have self-defrosting capability. In the cryostat's-controlled environment, the microtome knife, cabinet interior and instruments are all maintained at the same operating temperature, so that the sectioning operation is rarely affected by room temperature. The freezing platforms or chambers eliminate the need to use liquid nitrogen or other freezing agents for the vast

Cryostat Knife/Disposable

Blades In a disposable knife system the blade must be cleaned before it is mounted in the knife holder. Most disposable blades are coated with silicone or oil, which can be removed with xylene followed by absolute alcohol. Knife angle is important but seems to be a little less critical than in the paraffin technique.

Temperature

Each type of tissue has an optimum cutting temperature, but it is impossible especially during sectioning for rapid diagnosis, to adjust the cryostat temperature for every tissue. Machines are set at (-19°C to - 21°C) and performed adequately for most tissues. The cabinet, microtome, microtome knife and any tools (forceps, paintbrushes, etc.) must all be at the same temperature. It is a good idea to keep a spare sharp microtome knife stored in the cabinet so that if the regular knife becomes dull or nicked one can instantly change knifes without regard for cool-down time.

Sections Adhesion

Adhesives are infrequently necessary when performing an H and E stain on fresh tissue. The proteins in the tissue and the tissue fluids coagulate in the first alcohol or fixative and help the section adhered to the slide. The sections of fixed tissue have a greater tendency to float off the slide, and many special staining techniques will loosen both fresh and fixed tissues. Thorough washing of fixed tissues before sectioning can also help prevent section loss. If the

section just will not stay, several adhesives may be used, provided that the adhesives not interfere with the stain to be performed. The common adhesive is chrome alum gelatin solution (as in case of paraffin sections).

Cutting Technique

• A small amount of commercially available liquid embedding medium is spread evenly on the appropriate object holder. The tissue is placed on the surface of the object holder and completely surrounded with embedding medium. The embedding medium should provide adequate support for sectioning and have at least 2 mm margins. Care must be taken to orient the tissue properly.

• The object holder is placed on the freezing platform in the cryostat to start the freezing process. If necessary, more embedding medium is added as the tissue freezes. The freezing process can be accelerated if the object holder is at -20°C, the same temperature as the cryostat chamber

• When the tissue is completely frozen, the object holder is securely mounted to the microtome head. • Preliminary facing of the tissue:

- Adjust the knife holder so that the tissue just clears the knife

- Release the automatic advance pawl from the toothed ratchet wheel

- Manually advance the ratchet wheel until the tissue extends over the knife edge approximately 0.1-0.5 mm

- Release the lock on the drive wheel

- Rotate the drive wheel counter clock-wise 180° to cut the rough tissue section. Immediately return the drive wheel to the upright position.

- Repeat the above steps until the desired cutting plan is reached. Lock the drive wheel.

• Engage the automatic advance pawl and the toothed ratchet wheel. Carefully clean the knife edge with dry gauze.

• Unlock the drive wheel. Slowly and smoothly rotate the wheel clock Wise. As the section begins to form on the knife edge, a cold paint brush may be used to gently guide the section down the face of the knife to help keep the section flat.

Some machines have plastic anti-roll devices Attached to guide the sections over the knife.

• Mount the section on the slide by bringing the surface of room temperature slide very close to the section. The section will seem to jump on the slide and the mounting medium will melt immediately. • Slide is ready to be placed in fixative or stained.

• Remember to lock the drive wheel and clean the tissue debris from the cabinet mechanism and knife.

STAINING TECHNIQUES

ROUTINE STAINING: HEMOATOXYLIN AND EOSIN

If unstained sections of tissue are examined under the microscope with transmitted light, little details other than nuclear and cellular boundaries can be identified. Staining the sections with one or more dyes permits the evaluation of the physical characteristics and relationships of the tissues and their constituent cells.

This is facilitated if two contrasting stains are used such as hematoxylin (which stains the nuclear detail) and eosin (which stains the cytoplasmic details of the cell and extracellular tissues).

Stains for Nuclei

The most important of these is hematoxylin. This is a basic dye that causes staining of the acidic nucleoproteins. The resulting color is a bluish purplish-black. Substances that stain with this basic dye are described as basophilic.

Counter Stain

Sections that have been stained with hematoxylin alone are unsatisfactory for general examination unless a counterstain is used to demonstrate cytoplasmic details, and details of cellular tissues. Eosin is by far themes commonly used of this background or contrast stains. It gives a bright red color to erythrocytes and muscles. It imparts a pale pink color to the cytoplasm and to proteins in edema

fund. It is an acidic dye. Substances that stain with eosin are described as acidophilic.

1. Harris Hematoxylin

This stains the nuclei only. Enhancement of the blue color is accomplished by washing the slide in running tap water.

Preparation

Hematoxylin 100%.....5 gm

100% ethyl alcohol……15 ml

Potassium or ammonium alum……100 gm

Distilled water……1000 ml

Mercuric oxide……2.5 gm

Use a 2000 ml flask for the alum and a small flask for alcohol and hematoxylin, completely dissolve the alum in the distilled water with the aid of heat and a magnetic stirrer.

Vigorously shake to dissolve the hematoxylin in the alcohol at mum temperature. Remove the alum and distilled water from the heat. Slowly combine the solutions. Return the combine solution to heat. Bring to boil as rapidly as possible, approximately 1 minute or less. Remove from heat and slowly add the mercuric oxide. Return the solution to the heat until it becomes dark purple, remove it from the heat and plunge it into a basin or sink of cold water to cool. The solution is ready for use. Add 20 ml of glacial acetic acid to intensify the nuclear stain. Always filter before each use.

2. Eosin stock solution

Eosin Y. water soluble……..1 gm

Distilled water……100 ml

3. Phloxine stock solution

Phloxine B……1 gm

Distilled water……100 ml

4. Eosin-Phloxine working solution

Eosin stock solution……100 ml

Phloxine stock solution……..10 ml

95% Alcohol ……780 ml

Glacial acetic acid……..4 ml

The solution is used for approximately one week.

Routine staining of virtually all specimens are done using hematoxylin and eosin stain

Staining Procedure

a. Deparaffinize the slides

- **Xylene I ……..10 min**
- **Xylene II……..10 min**
- **Xylene III……..10 min**
- **100% Alcohol……4 min**
- **100% Alcohol II……4 mins**
- **95% Alcohol I ……4 mins**
- **95% Alcohol II.....4 mins**
- **80% Alcohol……4 mins**
- **70% alcohol ……..4 mins**
- **60% alcohol.....4 mins**
- **Water……4 mins**

b. Stain in Harris hematoxylin ……..8 mins

c. Wash in tap water……3 mins

d. Differentiate in 1% acid alcohol (99 mL of 70% alcohol + 1 mL of HCl) 1 to 2 dips

e. Wash in briefly in tap water

f. Place in weak ammonia water (0.4% of ammonia in D. water) or saturated lithium carbonate solution until the sections are bright blue

g. Wash thoroughly in running water

h. Counter stain in eosin-phloxine solution ..... 1-2 minutes

i. Dehydrate and clear through two changes

each of 95% alcohol, absolute alcohol and xylene .....2 minutes each

j. Dry and mount the slides in DPX mountant

RESULTS

Nuclei …… Blue

Cytoplasm…… Pink to red

Most other tissue structures……. Pink to red

SPECIAL STAINS: PERIODIC ACID AND SCHIFF STAIN

Stains glycogen, basement membrane, eg, descemet's membrane, Bruch's membrane, lens capsule, vascular basement membrane and fungi. Paraffin sections with thickness of 6 to 10 microns are required.

Principle: The reaction is based on oxidation of certain tissue elements to aldehydes by periodic acid. Schiff reagent is prepared by treating basic fuchsin (pararosaniline) with sulphurous acid. Reduction causes the loss of quinoid structure and masking of chromosphores. A colorless compound referred to as eucofuchsin is formed. Following the Schiff reaction, washing in running water causes the loss of the bound sulphurous acid group attached at the central carbon atom, the restoration of quinoid structure in the dye bound by the aldehyde, and the visualization of the typical schiff color.

Preparation

1. 0.5% Periodic acid solution:

Periodic acid……0.5 gm

Distilled water…… 100 mL

2. 1N Hydrochloric acid solution

Hydrochloric acid, specific gravity (1.19) .....83.5 mL

Distilled water……916.5 mL

3. Coleman's Schiff reagent

Basic fuchsin ..... 1 gm

Distilled water heat to 60°C……200 mL

and bring just to boil point.

Cool and then add

Potassium metabisulfite.....2 gm

1 N hydrochloric acid .....10 mL

Let bleach for 24 hours then

add activated carbon ..... 1 gm

***Shake for one minute, then filter through coarse filter paper. Repeat filtration until the solution is colorless. Store in refrigerator*.**

Staining Procedure 1:

a. Deparaffinize and hydrate to water

b. Oxidize in periodic acid solution .....5 minutes

c. Rinse in distilled water

d. Schiff reagent .....15 minutes

e. Wash in lukewarm tap water .....10 minutes

f. Harris hematoxylin .....6 minutes

g. Wash in tap water .....15 minutes

h. Dehydrate and clear through 2 changes of

95% alcohol, 100% alcohol and xylene ..... 2 minutes each

i. Dry and mount the slides using resinous medium

Staining Procedure 2:

a. Deparaffinize and hydrate to water

b. Oxidize in periodic acid solution ....4 minutes

c. Running tap water ..... 1 minute

d. Distilled water .....10 dips

e. Schiff reagent .....4 minutes

f. Running tap water .....5 minutes

g. Harris hematoxylin .....1 minute

h. Running tap water .....1 minute

i. Ammonia water .....5 dips

j. Running tap water……1 minute

k. 95% Alcohol……10 dips (2 changes)

l. 100% Alcohol ..... 10 dips (2 changes)

m. Xylene ..... 10 dips (2 changes)

n. Dry and mount using resinous medium

RESULTS

Glycogen, mucin, and some basement membranes.....red to purple

Fungi.....red to purple

Nuclei .....blue

SPECIAL STAINS: ALICIAN BLUE STAINING FOR ACID MUCOPOLYSACCHARIDES

Principle: Alcian blue is a copper phthalocyanine basic dye that is water soluble and is colored blue because of its copper content. When used in a 3% acetic acid solution (pH 2.5) Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucine (glycoproteins)

Reagents Required

1. 3% acetic acid

Glacial acetic .....3 ml

Distilled water .....97 ml

2. Alcian blue solution (pH 2.5)

Alcian blue 8G X..... 1 gm

3% acetic acid ..... 100 mL

3. Nuclear fast red (Kernechtrot) solution

0.1 gm nuclear fast red in 100 mL of 5% aluminum sulfate solution. Heat to boiling slowly, cool, filter and add a grain of thymol as a preservative.

Staining Procedure

a. Place in 3% acetic acid solution for 3 minutes

b. Stain in Alcian blue solution for 30 minutes

c. Wash in running water for 10 minutes

d. Rinse in distilled water

e. Counterstain in filtered nuclear fast red solution for 5 minutes

f. Wash in running water for 1 minute

g. Dehydrate and clear through 95% ethyl alcohol, absolute alcohol and xylene, 2 changes each, 2 minutes each

h. Mount with resinous media.

Result

Weakly acidic sulfated mucosubstances

Hyaluronic acid and sialomucins .....dark blue

Nuclei……..red to pink

Cytoplasm .....pale pink

SPECIAL STAINS: ALKALINE CONGO RED

Amyloid refers to the abnormal, fibrous, extracellular proteinaceous deposits found in organs such as liver, kidneys, spleen etc. This condition of deposition of amyloid in tissues is known as Amyloidosis. Congo red histological staining technique is the gold standard technique for the diagnosis of amyloidosis.

Principle

Congo red dye forms nonpolar hydrogen bonds with amyloid and red to apple green birefringence occurs when viewed by polarized light due to alignment of dye molecules on the linearly arranged amyloid fibrils. The high pH enhances the non-polar hydrogen bonding of Congo red and amyloid.

**** Paraffin sections with thickness of 8 to 12 microns required for this stain***

Reagents Required

1. 1% Congo Red solution

Congo red……..1 gm

Distilled water……..100 Ml

2. 1% Sodium Hydroxide solution

Sodium hydroxide .....1 gms

Distilled water .....100 mL

3. Alkaline-Alcohol solution

1% Sodium Hydroxide solution..... 1 mL

50% Ethyl Alcohol solution .....99 mL

Mayer's/Harri's Hematoxylin Solution

Staining procedure:

a. Stain in filtered Congo red solution for 1 hr

b. Rinse brief in distilled water

c. Differentiate rapidly in alkaline-alcohol solution

d. Wash in running tap water for 5 mins

e. Counterstain in Mayer's/Harri's hematoxylin for 5 minutes

f. Wash in running tap water for 15 minutes

g. Dry and mount using resinous media

Result:

Amyloid ..... Pink to red and "apple green" birefringence with polarized light

Nuclei .....Blue

SPECIAL STAINS: GOMORI'S ONE STEP TRICHROME STAIN FOR MUSCLE FIBERS

Principle: A plasma stain (Chromatrope 2R) and a connective tissue fiber stain (light green or aniline blue) are combined in a solution of phosphotungstic acid to which glacial acetic acid has been added Phosphotungstic acid favors the red staining of muscle and cytoplasm. The tungstate ion is specifically taken by the collagen and the connective tissue fiber stain is subsequently bound to this complex coloring the collagen green or blue.

Reagents Required

1. Bouin's fixative solution:

To 75 ml saturated aqueous picric acid solution add 250 ml formalin (37-40%) and 50 ml glacial acetic acid (saturated picric acid: 2 gm in 100 ml distilled water)

2. Weigert's iron hematoxylin:

Stock solution A:

Hematoxylin 1gm

95% ethyl alcohol……100 ml

Stock solution B:

29% Ferric chloride .....4 mL

Distilled water.....95 mL

Concentrated HCl .....1 mL

Working solution:

Equal parts of stock solutions A and B, as 100 ml A and 100 ml B

(It can be stored for about 2 weeks)

3. Gomori's trichrome stain:

Chromotope 2R .....0.6 gm

Light green (or) Aniline blue .....0.3 gm

Glacial acetic acid .....1 ml

Phosphotungstic acid .....0.8 gm

Distilled water.....100 mL

Staining Procedure

a. Place in Bouin's fixative in oven at 56°C for 1 hr or at room temperature overnight

b. Wash well in running water till sections are clear

c. Stain in Weigerts iron hematoxylin working solution for 10 minutes

d. Rinse in tap water

e. Stain in Gomori's trichrome stain for 15-20 minutes

f. Rinse in 1% acetic acid

g. Rinse in distilled water; dry and mount in resinous medium

Result

Muscle fibers .....red

Collagen .....green or blue

Nuclei…….black

SPECIAL STAINS: VERHOEFFS ELASTIC STAIN

Principle: The ferric chloride and iodine serve as mordants and they also have an oxidizing function that assist in converting hematoxylin to hematin. Differentiation is accomplished by using excess mordant of ferric chloride, to break the tissue mordant-day complex. The day will be attracted to large amount of mordant in the differentiated solution and will be removed from the

tissue. The elastic case tissue has strongest affinity for the iron hematoxylin complex and will retain the day longer than other tissue elements. This allow other tissue elements to be decolorized and the elastic fibers remain stained. Sodium thiosulfate is used to remove excess iodine, Van Gieson solution is used as counterstain.

Reagents

1. 10% Alcoholic Hematoxylin:

Hematoxylin……..10 gm

Ethanol……..100 ml

2. 10% Ferric chloride:

Ferric chloride……..10 gm

Distilled water……..100 ml

3. Verhoeff's Iodine solution:

Iodine……..2 gm

Potassium iodide……..4 gm

Distilled water……..100 ml

Mix the crystals of iodine and potassium iodide in flask, shake vigorously, then add DW 20 ml at a time.

4. Verhoeff's elastic stain working solution:

Mix equal parts of above solutions and ethanol

5. 2% Ferric chloride (Differentiation solution):

10% Ferric chloride .....20 ml

Distilled water……..80 ml

6. Van Gieson solution:

1% Acid Fuchsin……5 ml

Saturated Picric acid…… 95%

7. 5% Sodium Thiosulfate solution:

Staining Procedure

a. Stain the deparaffinized slide in Verhoeff elastic solution…15 minutes

b. Wash in lukewarm running water….20 minutes

c. Place in D water

d. Differentiate in 2% ferric chloride, check microscopically, elastic fibers are black and sharply fine and the background is gray

e. Place in 5% sodium thiosulphate solution for 1 minute

f. Wash in tap water for 5 minutes

g. Counterstain with Van Gieson solution for exactly 1 minute

h. Dehydrate through 2 changes of 95% alcohol, 100% alcohol and xylene for 2 minutes each. Dry and mount.

Results

Elastic fibers……Black

Nuclei……..Black

Other tissue structures…Yellow

SPECIAL STAINS: OIL O RED METOD FOR LIPIDS STAIN

Principle: Staining with oil soluble dyes is based on the greater solubility of the dye in the lipoid substances than in usual hydroalcoholic dye solvent.

FOR FROZEN SECTION

Reagents Required

1. Oil O Red Stain (Stock solution)

Oil O Red ……0.5 g

99% Iso-propanol ..... .100 mL

Working Solution

Oil O Red stock ..... .6 mL.

Distilled water ..... ……..4mL

2. Mayer's/Harri's Hematoxylin

Procedure

Prepare the working solution of Oil O Red and allow to stand for 5 minutes and then filter. The filtrate can be used for several hours.

a. Stain thin frozen sections for 10-15 minutes

b. Wash in water.

c. Stain nuclei briefly (10–30 seconds) in Mayer's/Harri's Hematoxylin

d. Blue in water.

e. Drain slides and mount the section with glycerol/water soluble mount

Result

Lipid ……Red

Nuclei ..... ……Blue

For Permanent Section

Reagents Required:

1. 100% propylene glycol

2. 0.5% Oil O Red solution

Oil O Red……..0.5 g

Propylene glycol .....100 mL

Add a small amount to propylene glycol to the Oil O Red and mix well. Crush larger pieces. Gradually add the remainder of the propylene glycol stirring periodically. Heat gently until the solution reaches 96°C. Do not allow to go over 100° C. Stir while heating. Pass through coarse filter paper while still warm. Allow to stand overnight at room temperature. Filter through medium glass filter with aid of vacuum. If the solution becomes turbid refilter.

3. 85% propylene glycol solution

100% propylene glycol.....85 mL

Distilled water .....15 mL

4. Mayer's/Harri's hematoxylin solution

Staining Procedure

a. Rinse the slides containing the permanent sections in distilled water

b. Place in absolute propylene glycol for 3-5 minutes

c. Stain in Oil O Red solution for 48-72 hrs

d. Differentiate in 85 % propylene glycol solution for 1-2 minutes.

e. Rinse in 2 changes of distilled water

f. Stain in hematoxylin solution for 5 minutes

g. Rinse thoroughly in distilled water

h. Mount in warmed glycerin jelly solution.

Result

Lipids..... red color

Nuclei .....blue

SPECIAL STAINS: BLEACHING OF MELANIN PIGMENTS

Principle: Melanin pigments are bleached by using oxidizing agents such as potassium permanganate followed by oxalic acid to clear the sections of color.

Reagents Required

1. 0.25% Potassium permanganate

0.25 gm Potassium permanganate .....0.25 gm

Distilled water ..... 100 mL

2. 5% Oxalic acid

Oxalic acid.....5 gm

Distilled water.....100 mL

Bleaching Technique

0.25% potassium permanganate…….5 minutes (if bleaching is not completed, time can be extended)

b. Wash in water ……..5 minutes

c 5% oxalic acid…….. 3 minutes

d. Wash in water ..... 2 minutes

Continue with routine staining

SPECIAL STAINS: PERL'S IRON STAIN FOR HEMOSIDERIN PIGMENTS

Principle: The sections are treated with an acidic solution of potassium ferrocyanide and any ferric iron present reacts to form an insoluble bright blue pigment called Prussian blue.

Reagents Required

1. 20% Hydrochloric acid

HCl. ……20 mL

Distilled water……..80 mL

2. 10% Potassium ferrocyanide stock solution

Potassium ferrocyanide …….. 10 gm

Distilled water .....100 mL

3. HCl-Potassium ferrocyanide working solution

Equal parts of 20% HCl and 10% potassium ferrocyanide solution

4. Nuclear fast red solution (Refer Alcian blue stain)

Staining Procedure

a. Place slides in freshly mixed HCl-potassium ferrocyanide working solution for 30 minutes

b. Rinse slides in distilled water

c. Counter stain with nuclear fast red solution for 5 minutes

d. Wash thoroughly in running tap water for 2 minutes

e. Mount slides with resinous medium.

Result

Hemosiderin and some oxides and salts of iron …… blue

Nuclei and cytoplasm .....pink to red

SPECIAL STAINS: ALIZARIN RED FOR CALCIUM

Principle: Alizarin red reacts with cations (calcium), forms alizarin red-calcium complex in a chelation process.

Reagents

1. Alizarin red solution:

Alizarin red……2 gm

Distilled water……100 mL

Adjust the pH to 4.0 to 4.3 by adding drop by drop of ammonium hydroxide

2. Acetone

3. Aceto-xylene solution

4. Xylene

Staining Procedure

a. Place the deparaffinzed slide in alizarin red solution 30 seconds-5 minutes examine microscopically, when orange red appears, shake off the excess stain

b. Dehydrate and clear through acetone, aceto-xylene and xylene

c. Dry and mount in resinous medium.

Results

Most calcium salts..... Birefringent red precipitate

Calcium oxalate……No reaction

SPECIAL STAINS: TOLUIDINE BLUE STAIN FOR MAST CELLS

Principle: Mast cells will stain metachromatically with toluidine blue that will stain in different color from the dye solution from rest of the tissue. The color shift called metachromasia.

Reagents

1. Toluidine blue solution:

Toluidine blue-……..0.1 gm

Distilled water……..100 mL

Staining Procedure

a. Stain the deparaffinized sections in toluidine blue solution.....10 minutes

b. Rinse in distilled water

c. Dehydrate and clear through 95% alcohol, 100% alcohol and xylene

d. Dry and mount in DPX.

Results

Mast cells ……. Deep violet

Background……..Blue

SPECIAL STAINS: PAPANICOLAOYU STAINIG FOR CELL DIFFERENTIATION

Papanicolaou stain (also Papanicolaou's stain or PAP stain) is the most important stain utilized in the practice of Cytopathology. It is a polychromatic stain containing multiple dyes to differentially stain various components of the cells. This technique was developed by George Papanicolaou, the father of Cytopathology. This method is used to differentiate cells in the smear

preparation of various gynecological specimens (pap smears), materials containing exfoliative cells and material from fine needle aspiration.

OBJECTIVES OF PAPANICOLAOU STAIN

Papanicolaou described three chief objectives for staining of cytological smears:

- **Definition of nuclear details: Because of the widespread muclear abnormalities of cancer cells and their diagnostic significance, good staining of the nucleus is of primary importance.**
- **Transparency of cytoplasm: This is of particular importance because of the varying thickness and the frequent overlapping of cells.**
- **Differentiation of cell: Differences in the staining reaction such as that between acidophilic and basophilic cells help greatly in the identification of certain cell types found in smears.**

PRINCIPLE OF PAPANICOLAOU STAIN

Papanicolaou stain includes both acidic and basic dyes. Acidic dye stains the basic components of the cell and basic dye stain the acidic components of the cell. The polychromatic PAP stain involves five dyes in three solutions.

1. **Hematoxylin: Natural dye hematoxylin is the nuclear stain which stains cell nuclei blue. It has affinity for chromatin, attaching to sulphate groups on the D.N.A. molecule. Harris' hematoxylin is the commonest cytologically although Gills' hematoxylin and Hematoxylin S can be used.**
2. **Orange Green 6: This is the first acidic counterstain (cytoplasmic stain) which stains matured and keratinized cells. The target structures are staine d orange in different intensities.**
3. **Eosin Azure: This is the second counterstain which is a polychrome mixture of eosin Y, light green SF and Bismarck brown.**
   Eosin Y gives a pink colour to cytoplasm of mature squamous cells, nucleoli, cilia and red blood cells. Staining solutions commonly used in cytology are EA 31 and EA 50, while EA 65
   Light green SF stains blue to cytoplasm of metabolically active cells like parabasal squamous cells, intermediate squamous cells and columnar cells.
   Bismarck brown Y stains nothing and sometimes it is often omitted.

COMPOSITION AND PREPARATION OF REAGENTS

1. **Harris' hematoxylin :**
   Hematoxylin = 5g
   Ethanol = 50ml
   Potassium alum = 100g
   Distilled water (50°C) = 1000ml
   Mercuric oxide = 2-5g
   Glacial acetic acid = 40ml

2. **Orange G 6 :**
   Orange G (10% aqueous) = 50ml
   Alcohol = 950ml
   Phosphotungstic acid = 0-15g

3. **EA 50 :**
   0.04 M light green SF = 10ml
   0.3M eosin Y = 20ml
   Phosphotungstic acid = 2g
   Alcohol = 750ml
   Methanol = 250ml
   Glacial acetic acid = 20ml

Filter all stains before use.

PROCEDURE OF PAPANICOLAOU STAINING

Both progressive and regressive nuclear staining techniques can be used in Papanicolaou stain. Before staining, Wet fixation immediately with Cytology spray fixative 96% ethanol for minimum 30 min is required.

Procedure of Progressive Papanicolaou Staining Method

In the progressive method, the nucleus is stained with hematoxylin to a intensity desired. The intensity of the nuclear staining is controlled by the immersion of the slide into a blueing agent. Most commonly used blueing agent is Sott's tap water (pH 8.02).

Step	Reagent	Time
1.	95% Alcohol (Fixation)	15-30 minutes
2.	80% Alcohol	2 minutes
3.	60% Alcohol	2 minutes
4.	Distilled Water	5 dips
5.	Distilled Water	5 dips
6.	Hematoxylin stain	3 minutes
7.	Distilled Water	3 minutes
8.	60% Alcohol	2 minutes
9.	80% Alcohol	2 minutes
10.	95% Alcohol	2 minutes
11.	Orange G Stain	3 minutes

12.	**95% ALcohol**	**2 minutes**
13.	**95% Alcohol**	**2 minutes**
14.	**Eosin Azure Stain**	**3 minutes**
15.	**95% Alcohol**	**2 minutes**
16.	**95% Alcohol**	**2 minutes**
17.	**95% Alcohol**	**2 minutes**
18.	**95% Alcohol**	**2 minutes**
19.	**Absolute Alcohol**	**2 minutes**
20.	**Absolute Alcohol**	**2 minutes**
21.	**Absolute Alcohol**	**2 minutes**
22.	**Absolute Alcohol+Xylene (1:1)**	**2 minutes**

23.	**Xylene**	**2 minutes**
24.	**Xylene**	**2 minutes**
25.	**Xylene**	**Till clear**
26.	**Mount in D.P.X**	

Procedure of Regressive Papanicolaou Staining Method

When using the regressive staining method, the nucleus is deliberately over-stained with a non-acidified haematoxylin. The excess stain is removed with dilute hydrochloric acid solution (acid water). The decolourising process is then stopped by immersing the slide in running tap water. Timing is crucial in the regressive method as de-staining may lead to a hyperchromatic nucleus becoming hypochromatic.

Step	Reagent	Time
1.	**90% Alcohol (Fixation)**	**15-30 minutes**
2.	**80% Alcohol**	**2 minutes**
3.	**60% Alcohol**	**2 minutes**
4.	**Distilled Water**	**5 dips**

5.	**Distilled Water**	**5 dips**
6.	**Hematoxylin stain**	**3 minutes**
7.	**Distilled Water**	**10 seconds**
8.	**1% Acid Alcohol**	**10 seconds (1 dip)**
9.	**Distilled Water**	**10 seconds**
10.	**Scott's Tap Water**	**2-3 minutes**
11.	**Running Tap Water**	**2 minutes**
12.	**60% Alcohol**	**2 minutes**
13.	**80% Alcohol**	**2 minutes**
14.	**95% Alcohol**	**2 minutes**
15.	**Orange G Stain**	**3 minutes**
16.	**95% ALcohol**	**2 minutes**

17.	**95% Alcohol**	**2 minutes**
18.	**Eosin Azure Stain**	**3 minutes**
19.	**95% Alcohol**	**2 minutes**
20.	**95% Alcohol**	**2 minutes**
21.	**95% Alcohol**	**2 minutes**
22.	**95% Alcohol**	**2 minutes**
23.	**Absolute Alcohol**	**2 minutes**
24.	**Absolute Alcohol**	**2 minutes**
25.	**Absolute Alcohol**	**2 minutes**
26.	**Absolute Alcohol+Xylene (1:1)**	**2 minutes**
27.	**Xylene**	**2 minutes**

28.	**Xylene**	**2 minutes**
29.	**Xylene**	**Till clear**
30.	**Mount in D.P.X**	

RESULTS AND INTERPRETATION OF PAPANICOLAOU STAINING

- **Nuclei: Blue**
- **Acidophilic cells: Red**
- **Basophilic cell : Blue Green**
- **Erythrocytes: Orange-red**
- **Keratin: Orange-red**
- **Superficial cells: Pink**
- **Intermediate and Parabasal Cells: Blue Green**
- **Eosinophil: Orange Red**
- **Candida: Red**
- **Trichomonas: Grey green**

IMMUNOHISTOCHEMISTRY

Examination of tissue sections with the conventional H and E stain and histochemical characterization with special stains may not be sufficient to arrive at precise diagnosis. Immunohistochemical methods will help in some of these instances by providing additional information. For example, poorly differentiated or undifferentiated tumors can be identified for the origin of tumor cell type by their specific cytoplasmic or surface antigens, utilizing monoclonal or polyclonal antibodies directed to these antigens, oxidase

antiperoxidase (PAP) method or the avidin-biotin complex (ABC) method, both of which have higher sensitivity than the Immunoperoxidase method.

FIXATION

Fresh 10% neutral buffered formalin (pH 7-7.5) is most likely the optimum for immunohistochemistry. Tissue should be exposed to the fixative just long enough to achieve good preservation and morphology. Over-fixation will cause the formation of excess aldehyde linkages that can block or mask antigen binding sites and prevent the primary antibody from linking to the antigen. Tissues that have been exposed to formalin can be digested with proteolytic enzymes such as trypsin or pepsin, can also be treated with citrate buffer (pH 6.0).

PROCESSING

• Routine paraffin procedures are adequate for immunohistochemistry.

• Do not allow processing temperature to exceed 60°C, since excess heat will destroy antigen and cellular morphology.

• It is important to use clean paraffin compounds and to be certain that all traces of paraffin and plastic additives are removed from the tissue during the deparaffinizing and hydration phase. Residual embedding media can cause nonspecific staining, incomplete staining or suppress staining entirely.

SECTIONING

Paraffin sections are cut at 5 microns and the selected sections are picked up with pre-coated slides and dried horizontally. Optionally slides may be dried at 60°C for 30 mins but great care must be taken to ensure that the slides are completely dry and not overheated.

CONCENTRATIONS

Valid staining of the target antigen can only be achieved if the antibody solution is used at ideal concentration (as indicated by the manufacturer). Correct antibody dilution is affected by every solution and procedure performed on the specimen: fixation, processing, buffer selection, staining temperature, humidity

and staining, just to mention a few. Key to uniform, valid and significant result is consistency during all phases of tissue handling and staining.

Methods

Avidin Biotin Complex (ABC) Technique

This technique uses three reagents a primary antibody, a secondary antibody. That is chemicals bound to vitamin biotin and a complex of the glycoprotein avidin that is bound to biotin and peroxidase. Avidin has the ability to bind nonimmunologically for molecules of biotin.

Labeled Avidin-Biotin Technique

The localization of tissue antigens.

Principle

This method utilizes three reagents: Primary antibody, biotinylated secondary antibody, and avidin conjugated with a marker enzyme. The primary antibody is specific for the antigen. The secondary or "link" antibody is capable of binding to the primary antibody and then binding the avidin conjugated with the marker enzyme, usually horseradish peroxidase or alkaline phosphatase. Avidin, either an egg-white protein or streptavidin, has the ability to nonimmunologically bind four molecules of the vitamin biotin. The chromogen is applied to develop an observable color.

GENERAL LABORATORY TECHNIQUES

The solutions used in these techniques can be very expensive. To minimize solution wastage slides can be stained horizontally on elevated rods in a humidity chamber. Use of humidity chamber is important as the slides must not be allowed to dry during any step of the procedure. Staining protocol is to be followed from the kit insert as provided by the manufacturer.

FLUORESCENT ANTINUCLEAR ANTIBODY TEST

PURPOSE: Indirect immunofluorescence staining for detection of antinuclear antibodies present in human serum.

PRINCIPLE: The antibodies in the patient's serum combines with the tissue antigen to form a complex. This complex binds with the fluorescent anti-human globulin conjugate and thus fluoresce in ultra-violet illumination.

PERFORMANCE SPECIFICATIONS

Always perform the test with freshly coated slides. Slides should be thoroughly washed after each step to get rid of non-specific fluorescence which might interfere with reading of the result. Reading of the result should be done by experienced personnel.

PRIMARY SAMPLE

- **Use only serum as specimen for the test.**
- **Collect 2 mL of venous blood in a plain red topped vacutainer tube or 0.1 N HCl washed tubes.**
- **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 or contaminated serum.**
- **Process the specimen on the same day.**

REAGENTS/ CONSUMABLES

- **Phosphate buffered saline at pH 7.2.**
- **Acetone**
- **Fluorescein conjugated anti-human immunoglobulin (polyvalent)**
- **Mounting medium - 50% glycerol in 0.05 M sodium barbital pH 8.6**

Preparation of Glass Slides

On the glass slides five circles are marked using diamond marker and the slides are dropped in distilled water containing 0,025% sodium meta silicate and it is kept in room temperature for half an hour. Then the slides are cleared well with tissue paper, then with methanol dipped tissue paper and again with clean tissue paper. Finally, slides are wrapped with aluminum foil and it is kept in hot air oven for sterilization at 160°C for 1 hour.

Coating of Slides

• Inoculate into the wells of the specially made slides with HEP - 2 cells on the previous day which is incubated in 10% Carbon dioxide atmosphere overnight.

• Next day observe the growth of the cells.

• Then wash the slides in three changes of phosphate buffered saline (pH - 7.2) and fix the slides in cold acetone for 10 minutes.

STEP BY STEP PROCEDURE

• Inactivate the patient's serum before performing the test.

• Dilute the serum in saline from 1 in 4 up to 1 in 32 (four-fold dilution).

• Include positive control

• Include negative sera control at the same dilution as test. Mark the slide with diamond marker.

• Then add 10 uL of the 1 in 8, 1 in 16 and 1 in 32 diluted samples to three wells of the slide coated with cell lines.

• Keep in a moist chamber for 30 minutes. Wash thrice with PBS in slide chamber.

• Then add 15µl of 1 in 20 diluted (diluted in PBS pH -7.2) rabbit antihuman Fluorescent Isothiocyanate (FITC) conjugate polyvalent serum to each of the wells.

• Keep for 30 minutes in moist chamber. Wash thrice in PBS and mount with glycerol mounting fluid and observe under 20X of fluorescent microscope with blue filter.

RESULTS

Staining character	*Antigenic determinant*	*Antibodies to*
Rim and homogeneous	**dS DNA**	**dS DNA**
	DNA - histone complex	**DN protein**
	Classes of histones	**LE - Cell ab**
Variable large speckles	**H3**	**Histone**
Speckled	**Small nucleolar RNP**	**Smith Ag**
	RNA protein	**Nuclear RNP**
	Complex protein	**NSP I, NSPII**
Nucleolar & Cytoplasm	**Ribosomal fraction**	**Cytoplasmic RNP**
Variable speckled in some cells	**33 KD a protein**	**PCNA**

Discrete speckled Centromere	**Protein**

SAFETY PRECAUTIONS

- **Handle all samples as potentially infectious**
- **Handle all reagents with care and avoid contact with eye, mouth and skin**
- **Do not perform mouth pipetting**
- **Discard used reagents and sample as per disposal procedure.**

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