BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

3rd Year

Prepared By

Paramedical & Allied Science Dept.

BMLT

MIDNAPORE CITY COLLEGE

3rd year BMLT PATHOLOGY
Practical (Paper-301)

ABO Forward and Reverse – Tube Method

PRINCIPLE

Testing with both Anti-A and Anti-B is necessary to determine if red blood cells possess or lack A and/or B blood group antigens. Absence of agglutination is a negative test result, which indicates the corresponding antigen is not demonstrable. Agglutination of red blood cells with a given reagent is a positive test result, which indicates the presence of the corresponding antigen on the red blood cells. (Forward Type) Direct agglutination of A1 or B reagent red cells with the patient serum/plasma indicates the presence of the appropriate ABO antibody. (Reverse type) Forward and reverse typing will be performed on samples for all patients greater than four months of age to allow for discovery of subtypes as well as to confirm typing. Individuals less than four months old may not have developed sufficient antibody to allow for detection.

SPECIMEN:

No special preparation of the patient is required prior to specimen collection. The specimen should be tested as soon as possible after collection, but EDTA and clotted specimens may be stored at 2 to 8C for up to 10 days if there is a delay in testing. (Note: Storage may result in weaker-than-normal reactions) Bacterial contamination of the specimen may cause false test results.

REAGENTS:

● Blood Grouping Reagent: Anti-A and Anti-B (forward cells)

● Reagent red blood cells: A1 and B cells (reverse cells)

● Do not use beyond expiration date. Store at 2 to 8oC. May be at room temperature (20 to 30oC) while in use.

QUALITY CONTROL

To recognize reagent deterioration the reactivity of all blood grouping reagents should be confirmed on each day of use by testing known positive and negative controls.

MATERIALS AND EQUIPMENT NOT SUPPLIED

Test tubes (12 x 75 mm), pipettes, physiologic saline, timer, centrifuge, as well as an optical aid.

Tube method procedure

(Bring all reagents to room temperature before testing)

Note: Steps 2 and 3 may be interchanged, but do it one way or the other. Be consistent.

Directions: Front Grouping

Step Action

- Prepare a 3-5% suspension of red blood cells to be tested in isotonic saline. (Washed or unwashed cells may be used)
- Place 1 drop of Anti-A and Anti-B respectively, in two small, properly labeled test tubes
- Add one drop of rbc suspension into the tube and mix.
- Centrifuge the test tube for appropriate centrifuge time
- Completely resuspend cells and examine macroscopically for agglutination.

*Note: Hemolysis may be a consequence of bacterial contamination and should not be interpreted as a positive result.

- Grade and record results

*Note: Centrifuge spin and wash time are noted on each centrifuge.

Slide method

A clean and dry glass slide is divided into two sections with a glass marking pencil. The sections are labeled as anti-A and anti-B to identify the antisera

Place one drop of anti-A serum and one drop of anti-B serum in the center of the corresponding section of the slide. Antiserum must be taken first to ensure that no reagents are missed.

Add one drop of blood sample to be tested to each drop of antiserum

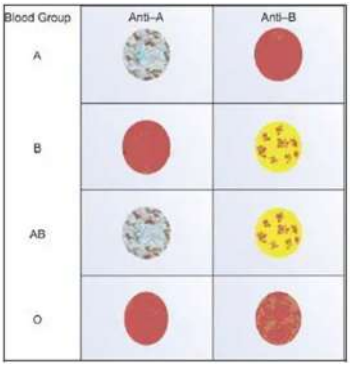
Mix antiserum and blood by using a separate stick or a separate corner of a slide for each section over an area about 1 inch in diameter

By tilting the slide backwards and forwards, examine for agglutination after exactly two minutes.

Result:

Positive (+): Little clumps of red cells are seen floating in a clear liquid.

Negative (–): Red cells are floating homogeneously in a uniform suspension.



Anti-A	Anti-B	Blood Group
+	-	A
-	+	B
+	+	AB
-	-	O

Reverse Blood Grouping /Serum Grouping

Reverse Grouping – performed on individuals greater than 4 months old

Principle

The reverse blood grouping procedure is based on the principle of direct hemagglutination. The erythrocytes of a person contain blood group antigens on the surface of the membrane. When these antigens are allowed to treat with corresponding antibodies, antigen-antibody reaction occurs and form agglutination.

Requirements

Specimen:

Serum is specimen for reverse blood grouping. No special preparation of the patient is required prior to collection. The specimen should be tested as soon as possible after collection, but specimens may be stored at 2 to 8^0C if there is a delay in testing. Storage may result in weaker-than-normal reactions.

Cell Suspension:

Although red cell reagents for serum grouping are available commercially, most laboratories prepare their own A and B test red cells from persons known to be group A and group B. Make pooled cell suspension as follows:

Label tubes as A and B.

Tube A: Place 1 drop of red cells each from 3 of A group samples.

Tube B: Place 1 drop of red cells each from 3 of B group samples.

Add Normal saline and to suspend the cells. Centrifuge the tubes for at least 1 minute at 1000 rpm. To make 5% red cell suspension, add 1 drop of RBC to 19 drops of saline. Make 20% suspension for slide method.

Test the pooled cells prepared by adding the antisera (Anti-A, B) in use.

Procedure

The reverse blood grouping can be performed in two methods: Tube and Slide method. The Tube method is preferred to slide method.

Tube Method

- Label two test tubes as A and B.
- Add two drops of serum to be tested in each tube.
- Add one drop each of A and B cells suspension to the corresponding test tubes.
- Mix well and centrifuge both tubes at 1000 rpm for 1 minute.

- Gently remove the tubes and completely resuspend cells and examine macroscopically for agglutination and if negative, microscopically.
- Record the reactions and interpret the results.

Slide Method

- Mark a clean slide into two halves, labeling the left and right side side as A and B.
- Add a drop of serum to be tested on both sides.
- Add one drop each of A and B cells suspension (20%) to the corresponding sides.
- Using a clean applicator stick, mix the serum and cell suspension on both sides separately and spread into a smooth round circle.
- Rock the slide gently for 2 minutes and look for agglutination.
- Record the reactions and interpret the results.

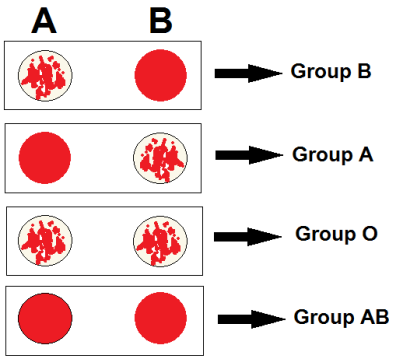
Results and Interpretation

If agglutination is observed with A cells only, then the patient's blood group is B

If agglutination is observed with B cells only, then the patient's blood group is A

If agglutination is observed with both A and B cells, then the patient's blood group is O

If agglutination is not observed with both A and B cells, then the patient's blood group is AB



Limitations

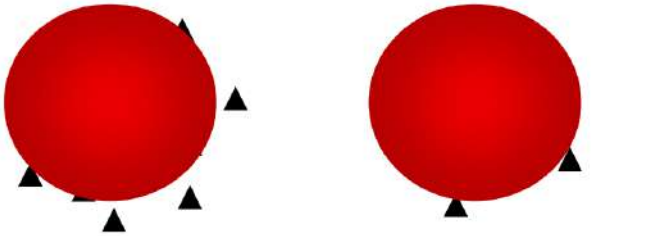
- Serum from persons with agammaglobulinemia may not contain detectable ABO antibodies.
- As naturally occuring anti-A and anti-B are only formed 3-4 months after birth, it is not suitable for newborns and infants. Antibodies at this age are commonly of maternal origin.
- If the expected reactions are not obtained an interpretation of the patient's ABO group cannot be made without further testing. ABO discrepancies should be resolved before reporting or prior to transfusion. However, Group O red blood cells is the preferred transfusion alternative for any recipient whose ABO group is in question.

- Serum reverse group may be unreliable in infants under 6 months. Antibodies detectable in the serum of infants prior to this age are most commonly of maternal origin.
- Serum from persons with agammaglobulinemia may not contain detectable ABO antibodies.
- The reactivity of Reagent Red Blood Cells may diminish over the dating period.
- Aged samples, subgroups, cold agglutinins, some diseased states, or patient age may impair test

Method for Rh Typing

Weak D

Inheritance of D genes which result in lowered densities of D Antigens on RBC membranes, gene codes for less D.



RBC with normal expression of D antigen

Decreased number of D antigens in Weak

Method for Weak D Testing

• Add 1 drop of 2-5 % suspension of D negative red cells to a

test tube and add 2 drops of Anti D (blend of IgG + IgM)

• Incubate at 37□C for 30 minutes.

• Wash three times with normal saline.

• Make dry red cell button and add polyspecific AHG reagent.

• Look for agglutination.

Results:

• If there is agglutination Du Positive.

• If there is no agglutination Du Negative.

Significance of Weak D

Donors

• Weak D testing on donors required.

• Labeled as D positive

• But as recipient D negative

Patients

• Weak D testing on patients not required.

• Standard practice to transfuse with D negative

Weak D is much less antigenic in comparison to D, however, such red cells may be destroyed if transfused to a patient already having anti-D. Hence, weak D donor units are labeled as Rh positive.

• The weak D positive recipients are classified as Rh negative and safely transfused with Rh negative blood • Du positive infant can suffer from HDN if the mother possess anti-D antibodies

• Rh immunoprophylaxis is recommended for the Rh negative mother if the newborn is D^u positive.

Coomb's test

- It was discovered by Coombs, Mourant and Race in 1945 originally for the detection of incomplete anti-Rh antibodies.
- In the test, incomplete antibodies do not agglutinate erythrocytes. Incomplete antibody antiglobulin coats the surface of erythrocytes but does not cause any agglutination.
- When such erythrocytes are treated with antiglobulin or Coombs' serum then the cells are agglutinated.
- Coombs' Serum or Coomb's reagent is a special serum from a rabbit or other animal previously immunized with purified human globulin to prepare antibodies directed against IgG and complement (eg. rabbit antiserum against human globulin).
- It is used in the direct and indirect Coomb's tests and also called antihuman globulin.

Objectives

To detect red blood cells sensitized with IgG alloantibodies, IgG autoantibodies or complement components.

Principle

Under certain conditions, complement proteins or more commonly, incomplete antibodies (IgG) attach to red cell membrane by the Fab portion of the immunoglobulin. These cells are said to be sensitized. Sensitization of red cells can occur in vivo or vitro. The IgG molecules attached to the red cells are unable to bridge the gap between sensitized red cells which are separated from each other by the negative charge on their surface and as a result of which the sensitized red cells do not agglutinate. Adding of the Coomb's reagent (antiglobulin serum) however, completes the reaction. When the serum is added to the sensitized cells, the Fab portion of the anti-human globulin molecule (anti-IgG) reacts with the Fc portions of two

adjacent IgG molecules attached to red cells thereby bridge the gap between sensitized red cells and cause agglutination. Thus, if human IgG antibody has already attached to the patient's red cells in vivo (in the bloodstream), or the patient serum contains incomplete antibodies that can attach to RBCs invitro, then the addition of anti-human IgG will cause the cells to agglutinate. This is a positive Coomb's test.

Types of Coombs test

There are two types of Coombs tests: the direct Coomb's test and the Indirect Coomb's test.

Direct Coomb's Test (Direct Antiglobulin Test)

The direct test is more common and checks for antibodies that are attached to the surface of red blood cells.

In this test, the sensitization of red blood cells (RBCs) with incomplete antibodies takes place *in vivo*.

The cell-bound antibodies can be detected by this test in which antiserum against human immunoglobulin is used to agglutinate patient's red cells.

Indirect Coomb's Test (Indirect Antiglobulin Test)

The indirect test checks for unattached antibodies that are floating in the bloodstream.

- In this test, the sensitization of RBCs with incomplete antibodies takes place *in vitro*.
- The patient's serum is mixed with normal red cells and antiserum to human immunoglobulin is added. Agglutination occurs if antibodies are present in the patient's serum

Requirements of Coombs Test

Test tubes, centrifuge, Anti-human globulin (AHG) reagent, pre-sensitized red cells (Coombs' control cells), Saline.

Procedure

The use of antihuman globulin serum to detect sensitization of red cells *in vitro* is a two-stage technique constitute indirect antiglobulin test (IAT). On the other hand, sensitization of red cells in vivo is detected by one stage technique – the direct antiglobulin test (DAT).

Direct Coomb's Test

1. Red cells suspected of being sensitized is washed 3 to 4 times in large volume of saline.

2. Two drops of anti-human globulin serum is added to the sedimented cells.

3. It is mixed well and centrifuged at 1500 rpm for one minute.

4. Agglutination is examined by holding against a lighted background and tapping the bottom of the tube.

5. If the agglutination is not seen, the tube is left at room temperature for 10 min. then recentrifuged and read. A weaker reacting antibody may will show delayed reaction and this is considered as positive.

6. If haemagglutination is not seen in step 5, one drop of presensitized red blood cells (5% suspension is saline) is added. This should result in haemagglutination of pre-sensitized cells indicating that the antihuman globulin (AHG) is reactive and the result is valid.

Indirect Coomb's Test

1. % saline suspension of the test cells is prepared.

2. Two drops of cell suspension is added to a small test tube.

3. Two drops of antiserum is added to the cell suspension.

4. It is incubated in a water bath at 37°C for 30 min.

5. The tube from the water bath is removed and washed 3 to 4 times with large volume of saline. It is completely decanted after last washing.

6. Two drops of anti human globulin (AHG) is immediately added and mixed well.

7. It is centrifuged at 1500 rpm for one minute and examined for haemagglutination.

8. In case of negative haemagglutination, pre-sensitized reagent cells are added to test the reactivity of AHG. Agglutination must be seen with the addition of Coombs' control cells.

Result Interpretation of Coomb's Test

Positive: A clumping of the red blood cells (agglutination) during the test.

Agglutination of blood cells during a direct Coomb's test suggests that antibodies may be present on red blood cells of the patient and that the condition of hemolysis may persist. Agglutination of blood cells during an indirect Coomb's test suggest the presence of antibodies circulating in bloodstream that could cause the immune system to react to any red blood cells that are considered foreign to the body — particularly those that may be present during a blood transfusion.

Negative: No clumping or agglutination of red blood cells.

Application of Coomb's Test

1. Coomb's test is one of the blood tests employed to help find out the kind of anemia an anemic patient is suffering from.

2. Indirect test is administered to determine if there was a potential bad reaction to a blood transfusion.

3. Blood banks use the indirect Coombs test to determine whether there is likely to be an adverse reaction to blood to be transfused.

4. Coombs' tests are used for detection of anti-Rh antibodies.

5. It is also used to detect incomplete antibodies in brucellosis and other diseases.

6. The indirect Coombs test is used in prenatal testing of pregnant women.

7. The test is done on the newborn's blood sample, usually in the setting of a newborn with jaundice.

8. It helps in the detection of conditions like hemolytic anemia, chronic lymphocytic leukemia, erythroblastosis fetalis, infectious mononucleosis, mycoplasmal infection, syphilis, systemic lupus erythematosus etc.

Limitations of Coomb's Test

- Sometimes, especially in older adults, a Coomb's test will have an abnormal result even without any other disease or risk factors.
- The test can only be rarely used to diagnose a medical condition.

CROSS MATCHING IN BLOOD

Introduction:

• Cross Matching is a procedure performed prior to a blood transfusion to determine whether donor blood is compatible (or incompatible) with recipient blood.

• Compatibility is determined through matching of different blood group systems, the most important of which are the ABO and Rh system, and/or by directly testing for the presence of antibodies against a sample of donor tissues or blood.

Purpose of Cross Matching

• The crossmatch is routinely used as the final step of pretransfusion compatibility testing. The purposes of compatibility testing are to detect: irregular antibodies; errors in ABO grouping, and clerical errors in patient identification and result recording.

• The crossmatch will detect the following:

1. Most recipient antibodies directed against antigens on the donor red blood cells.

2. Major errors in ABO grouping, labeling, and identification of donors and recipients.

Principle

Cross-matching will detect incompatibilities between the donor and recipient that will not be evident on blood typing. There are two types of cross-matches: Major cross-match and Minor cross-match. The major crossmatch involves testing the patient's serum with donor cells to determine whether the patient has an antibody which may cause a hemolytic transfusion reaction or decreased cell survival of donor cells. This is the most important cross-match. The minor crossmatch involves testing the patient's cells with donor plasma to determine whether there is an antibody in the donor's plasma directed against an antigen on the patient's cells.

Procedure

• Prepare donor and recipient blood samples:

For Major crossmatch : Donor's red cell and recipient serum or plasma

For Minor crossmatch : Recipient red cells and donor's serum or plasma

• Prepare 3 – 5% cell suspensions of red cells.

Major Crossmatch:

Label a test tube. Add two drops of the patient serum and one drop of the appropriate donor cell suspension.

Minor Crossmatch:

Label a test tube. Add two drops of the appropriate donor serum and one drop of the patient cell suspension.

• Mix the tubes and incubate at 37°C for about 45 minutes.

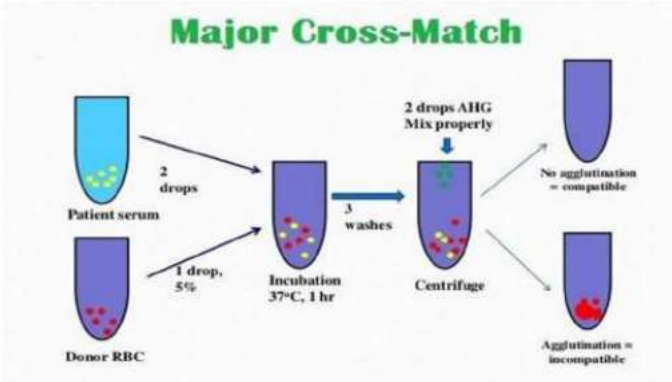
• Add two drops of AHG (Antihuman globulin) and mix well.

• Centrifuge for 1 minute at 1500 rpm

• Read macroscopically and microscopically and record the results

Interpretation:

The mixture of erythrocytes and serum are observed for hemolysis or microscopically for agglutination. Any evidence of hemolysis/agglutination indicates an incompatible cross-match. Negative results are taken to indicate compatibility.



Determination of clotting time

Background

Whenever a great blood vessel ruptures bleeding continues. In a few minutes blood loses its fluidity and sets into a semisolid mass. The mass is referred as clot and the phenomenon as coagulation. Clotting time is defined as the time interval in between onset of bleeding and appearance of semisolid mass i.e. clot. Normal value of clotting time is 3-4 minutes. Clotting time is determined using two methods viz. Capillary glass method and Wrights Coagulometer. The aim of the experiment is to determine the clotting time of the subject.

REQUIREMENTS:

Spirit, cotton, needle, capillary tube, stop watch.

Procedure:

Capillary glass method:

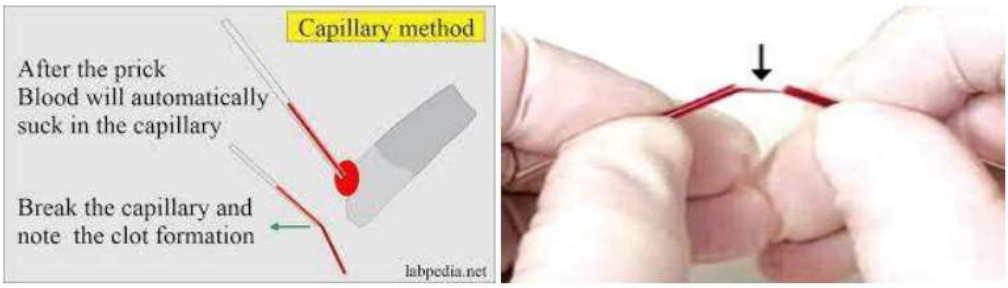
1. The finger tip of the subject Is sterilized and a bold prick Is made in the fingertip with a sterilized needle to have free flow of blood.

2. The blood coming out of the puncture Is sucked into a capillary glass tube of 15 cm long.

3. Then the tube is kept undisturbed horizontally for about 1-2 minutes.

4. A small bit of the glass tube is broken off every 30 seconds until a fine thread of clotted blood appears while the capillary tube is broken.

5. When the thread appears the stop watch is stoped. This gave us the clotting time. The period in between appearance of blood in finger and formation of clot was taken as clotting time.



Determination of bleeding time

Definition:

The bleeding time is the time required for a small cut to stop bleeding. When a blood vessel is injured, blood comes out for some time and then it stops because of the formation of platelet plug. The duration of bleeding is the bleeding time. Normal value for bleeding time is 1-3 minutes.

Significance:

The bleeding time is mainly used in the diagnosis and treatment of the haemorrhagic diseases. The bleeding time is also useful just before operations such as tonsillectomy. In such cases it may point out an abnormal bleeding process. This will aware the physician to take proper precaution. The bleeding time may be performed by following methods: Duke Method, Ivy Method, Macfarlane Method.

The aim of the experiment is to determine the bleeding time of the subject.

Requirements:

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

Procedure:

Duke method for bleeding time:

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

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

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

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

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

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

CEPHALOPLASTIN REAGENT FOR PARTIAL THROMBOPLASTIN TIME (APTT) DETERMINATION USING ELLAGIC ACID, AS AN ACTIVATOR

WARRANTY

The product is designed to perform as described on the label and the package insert. The manufacturer disclaims any implied warranty of use and sale for any other purpose.

BIBLIOGRAPHY

1. Biggs, R., ed: Human Blood Coagulation, Haemostasis and Thrombosis, Blackwell Scientific Publications Oxford, England, 1972.
2. Hoffmann, J.J.M.L. and Neulerdijk P.N.: Thrombos. Haemosta. (Stuttgart) 39, 640 (1978).
3. CRC, Handbook Series in Clinical Laboratory, Science, Section 1: Haematology, Volume III, 1980. CRC Press, Inc. Boca Raton, Florida.
4. NCCLS guideline H21-A3, Vol.18, No. 29.
5. Data on file: Tulip Diagnostics (P) Ltd.

SYMBOL KEYS

	Temperature limitation		Manufacturer	LOT	Batch Number/ Lot Number		This side up	PS	Production Site
	Use by		Consult Instructions for Use	IVD	In vitro Diagnostic Medical Device	REAGENT	Description of reagent		
	Date of Manufacture	REF	Catalogue Number	APTT	Partial thromboplastin time test	EC REP	Authorised Representative in the European Community		

TULIP DIAGNOSTICS (P) LTD.

FS

GITANJALI, TULIP BLOCK, DR. ANTONIO DO REGO BAGH, ALTO SANTACRUZ, BAMBOLIM COMPLEX P.O., GOA-403202, INDIA. Website: www.tulipgroup.com

PLOT NOS. 92/96, PHASE II C, VERNA IND. EST., VERNA, GOA-403722, INDIA.

EC REP

CMC Medical Devices & Drugs S.L., Cl Horacio Lengo No. 18, CP 29006, Malaga, Spain

1117-MDR-01

CE

LIQUICELIN-E®

CEPHALOPLASTIN REAGENT FOR PARTIAL THROMBOPLASTIN TIME (APTT) DETERMINATION USING ELLAGIC ACID, AS AN ACTIVATOR

SUMMARY

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of a series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues.

Activated Partial Thromboplastin Time is prolonged by a deficiency of coagulation factors of the intrinsic pathway of the human coagulation mechanism such as factor XII, XI, IX, VIII, X, V, II and Fibrinogen.

Determination of APTT helps in estimating abnormality in most of the clotting factors of the intrinsic pathway including congenital deficiency of factor VIII, IX, XI and XII and is also a sensitive procedure for generating heparin response curves for monitoring heparin therapy.

PRESENTATION

REF	10630003	10630123	106300004	106300124	10631025
LIQUICELIN-E®	3 ml	12 X 3 ml	4 ml	12 X 4 ml	2.5 ml
Pack insert	1	1	1	1	1

REAGENT

LIQUICELIN-E® is a liquid ready to use activated cephaloplastin reagent for the determination of Activated Partial Thromboplastin Time. It is a phospholipid preparation derived from rabbit brain with ellagic acid as an activator.

Each batch of the reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

REAGENT STORAGE AND STABILITY

a) Store the reagent at 2-8°C. **DO NOT FREEZE.** b) The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. Once opened the reagent is stable for **3 months at 2-8°C, 1 week at 18-25°C, 2 days at 37°C provided it is not contaminated and capped tightly when not in use.**

PRINCIPLE

Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions.

APTT is prolonged by a deficiency of one or more of these clotting factors of the intrinsic pathway and in the presence of coagulation inhibitors like heparin.

NOTE

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. LIQUICELIN-E® reagent is not from human source hence contamination due to HBsAg and HIV is practically excluded.
3. Reagent contains 0.01% Thimerosal as preservative.
4. It is **very important** that clean and dry micropipette tips be used to dispense the reagent.
5. **Avoid exposure** of the reagent to elevated temperature and contamination. **Immediately** replace cap after use and store at recommended temperatures **only**.
6. Do not use damaged or leaking reagents.

SAMPLE COLLECTION AND PREPARATION

No special preparation of the patient is required prior to sample collection by approved techniques. Withdraw blood without undue venous stasis and without frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into tubes, after detaching the needle from the syringe.

Mix exactly nine parts of freshly collected blood with one part of tri-sodium citrate (0.11 mol/l, 3.2%). Centrifuge immediately for 15 minutes at 1500 g and transfer the plasma into a clean test tube. **Plasma must be tested within three hours of blood collection.** For heparin determination, platelet deficient plasma should be used, hence higher centrifugation time is required.

FNP COLLECTION

Prepare a plasma pool (FNP) of freshly collected blood from at least five normal healthy donors and process as above. Plasma must be tested within three hours of blood collection.

ADDITIONAL MATERIAL REQUIRED

(a) 12 x 75 mm glass test tubes. (b) precision pipettes. (c) Stop watch. (d) Water bath or heating block at 37°C. (e) FRESH NORMAL POOLED PLASMA. (f) $CaCl_2$ (0.025mol/l)*.

*AVAILABLE FROM TULIP DIAGNOSTICS; CAT. NO: 10633010,10633100

TEST PROCEDURE

Manual Method

1. Before use, the reagent should be mixed well by gentle swirling. Do not shake.
2. Aspirate from the reagent vial enough reagent for the immediate testing requirement in a thoroughly clean and dry test tube. Bring this reagent to room temperature before prewarming at 37°C for testing purposes.
3. Separate test tubes containing LIQUICELIN-E® and TULIP Calcium Chloride Solution should be brought to 37°C. (Depending on volume, approximately 5 to 10 minutes required). Do not incubate the test plasma.
4. To a 12 x 75 mm test tube, add 100µl test plasma and 100µl LIQUICELIN-E®. Shake tube briefly to mix the reagent and plasma; place tube at 37°C for 3 to 5 minutes.
5. Following incubation period, add forcibly 100µl of prewarmed calcium chloride into the plasma and LIQUICELIN-E® mixture; simultaneously start a stopwatch. Shake tube briefly to mix contents, keep at 37°C for 15 seconds.
6. Following 15 seconds incubation, remove the tube; gently tilt back and forth until a gel clot forms; stop the watch; record time.
7. Repeat steps 4-6 for a duplicate test using the same test plasma.
8. Find the average from the duplicate test values. This is the Activated Partial Thromboplastin Time (APTT of patient plasma).
9. Similarly repeat steps 4-6 twice, and record duplicate values using FNP in place of test plasma (APTT of FNP).

If a coagulation instrument is being used to perform the tests, the instrument manufacturers instructions must be strictly adhered to.

Calibration Curve Method (For determination of heparin concentration):

1. Dilute heparin (as used for treatment) with physiological saline to a concentration of 10 U/ml.
2. Mix 200µl of 10 U/ml diluted heparin with 1.8 ml of FNP to give a heparin standard of 1 U/ml concentration.
3. Dilute the heparin standard as prepared above (1U/ml) with FNP as follows:

Test tube no.	1	2	3	4	5	6	7
Heparin standard (1 U/ml) in µl	500	400	300	200	100	100	-
FNP in µl	-	100	200	300	400	900	500
Heparin Concentration (U/ml)	1.0	0.8	0.6	0.4	0.2	0.1	0.0

4. Pipette 100µl each of the seven heparin dilutions into clean test tubes.
5. Add 100µl LIQUICELIN-E® reagent to each test tube.
6. Mix well and incubate each test tube at 37°C for exactly 3 minutes before testing.
7. **Forcibly** add 100µl calcium chloride (prewarmed at 37°C) to each test tube, one by one and simultaneously start the stop watch.
8. Gently tilt the tube back and forth and stop the stopwatch as the first fibrin strand is visible and the gel / clot formation begins. Record the time in seconds.
9. Repeat steps 4-8 for each dilution for duplicate test, and find the average of the duplicate test values.
10. Plot the mean of the double determination in 'seconds' against each heparin concentration using LIQUICELIN-E® graph paper.
11. Clotting times (APTT) of test specimens can be interpolated against the heparin concentration to determine the heparin concentration of the sample in U/ml.

CALCULATION AND REPORTING OF RESULTS

Manual Method

a) The results may be reported directly in terms of the mean of the double determination of the APTT of the test plasma clotting time. It is suggested that the results be reported to the clinicians in conjunction with the normal range.

OR

b) As a ratio as follows:

$$R = \frac{\text{APTT of patient plasma (in seconds)}}{\text{APTT of FNP (in seconds)}}$$

c) Calibration Curve Method

Heparin concentration in the test sample can be directly obtained from the LIQUICELIN-E® calibration curve by interpolating the test plasma clotting time against heparin concentration in U/ml.

EXPECTED VALUES

Reference values for healthy individuals may vary from laboratory to laboratory depending on techniques and instrumentation used.

In a study of 96 apparently healthy individuals using LIQUICELIN-E® reagent on an Opto-mechanical instrument, a reference range of 22-35 seconds was obtained. Each laboratory must establish the reference range for a reagent with instrument, specimen collection and testing techniques used in that laboratory.

REMARKS

1. Due to inter and intra laboratory variations users must establish their own normal population range as well as normal and abnormal range.
2. It is recommended that controls with known factor activity should be run simultaneously with each test series routinely.
3. Incorrect mixture of blood and tri-sodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware etc. are potential source of errors.
4. Incorrect dilution of heparin is also a potential source of error.
5. Oxalated plasma may induce prolonged clotting times.
6. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
7. Abnormalities of coagulation factor VII, factor XIII and platelets are not detected by this test procedure.
8. For automated equipment it is strongly recommended that the equipment manufacturers methodology be strictly adhered to.
9. In heparin monitoring time of collection of blood sample is important since the *in-vivo* half-life of heparin is approximately 1.5 hours. When it is administered intravenously it has an immediate anti-coagulant effect but its efficacy decreases rapidly with time.
10. Platelet factor IV, a heparin-neutralising factor can be released due to platelet aggregation or damage. In order to prevent this phenomenon *in-vitro* the specimen should be collected with a minimum of trauma.
11. Decrease in APTT time is observed in males under estrogen therapy and oral contraceptive administration in females.

PERFORMANCE CHARACTERISTICS

Heparin sensitivity

The sensitivity of LIQUICELIN-E® to heparin was determined by adding known amounts of heparin to pooled normal plasma and performing the APTT. The following results were obtained on Hemostar-XF a coagulometer based on turbodensitometric method of clot detection with one Lot of LIQUICELIN-E®:

Heparin concentration (U/ml)	APTT (seconds)
0.0	25.3
0.1	31.4
0.2	40.2
0.4	52.0
0.6	80.3
0.8	120.8
1.0	158.3

Each laboratory must establish its own heparin calibration curve using the same source of heparin used for therapy in that institution. Variations can result from different brands of heparin, tissue origin and salt forms.

Factor sensitivity

An APTT reagent with good sensitivity must demonstrate a prolonged clotting time in samples having ≤ 30-40% factor VIII and factor IX activity. LIQUICELIN-E® was evaluated by diluting a normal pooled plasma with factor deficient plasma and measuring the clotting time.

Sensitivity to Factor VIII		Sensitivity to Factor IX	
Activity of Factor (%)	APTT (seconds)	Activity of Factor (%)	APTT (seconds)
100	33.7	100	43.8
50	42.0	50	47.5
25	46.8	25	52.0
12.5	50.0	12.5	56.8
6.25	62.4	6.25	60.0
3.12	67.0	3.12	68.0
1.5	73.6	1.5	70.0

The above values should only be used as guidelines. Each laboratory should establish sensitivity to individual factors using techniques, reagents and instruments used in their laboratory.

SENSITIVE THROMBOPLASTIN REAGENT FOR PROTHROMBIN TIME (PT) DETERMINATION

SENSITIVE THROMBOPLASTIN REAGENT FOR PROTHROMBIN TIME (PT) DETERMINATION

SUMMARY

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues.
Tissue Thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, Prothrombin and Fibrinogen.
During oral anticoagulant therapy most of the Vitamin K dependent factors such as II, VII, IX, X, Protein C and Protein S are depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired.
Prothrombin Time determination is the preferred method for presurgical screening, as a liver function test, determination of congenital deficiency of factors II, V, VII and X and for monitoring of patients on oral anticoagulant therapy.

PRESENTATION

REF	*10621025	10620005	10620125
UNIPLASTIN®	5 ml	5 ml	12 X 5 ml
3.2% Tri-Sodium Citrate	12. 5 ml	-	-
INR conversion table	1	1	1
Pack insert	1	1	1

* REF 10621025 represents Precision combi pack which contains Uniplastin reagent along with 3.2% Tri-Sodium Citrate solution used for blood collection.

REAGENT

UNIPLASTIN® is a novel, highly sensitive, low opacity, **ready to use liquid Calcified Thromboplastin Reagent,** which is derived from rabbit brain.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

REAGENT STORAGE AND STABILITY

(a) Store the reagent at 2-8°C. **DO NOT FREEZE**. (b) The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. The uncontaminated reagent is stable as per the **labeled shelf life at 2-8°C, 1 week at 18-25°C, 2 days at 37°C.**

PRINCIPLE

Tissue Thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When UNIPLASTIN® reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time. The time required for clot formation would be prolonged if there is acquired or congenital deficiency of factors / factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of Vitamin K dependent clotting factors during oral anticoagulant therapy.

NOTE

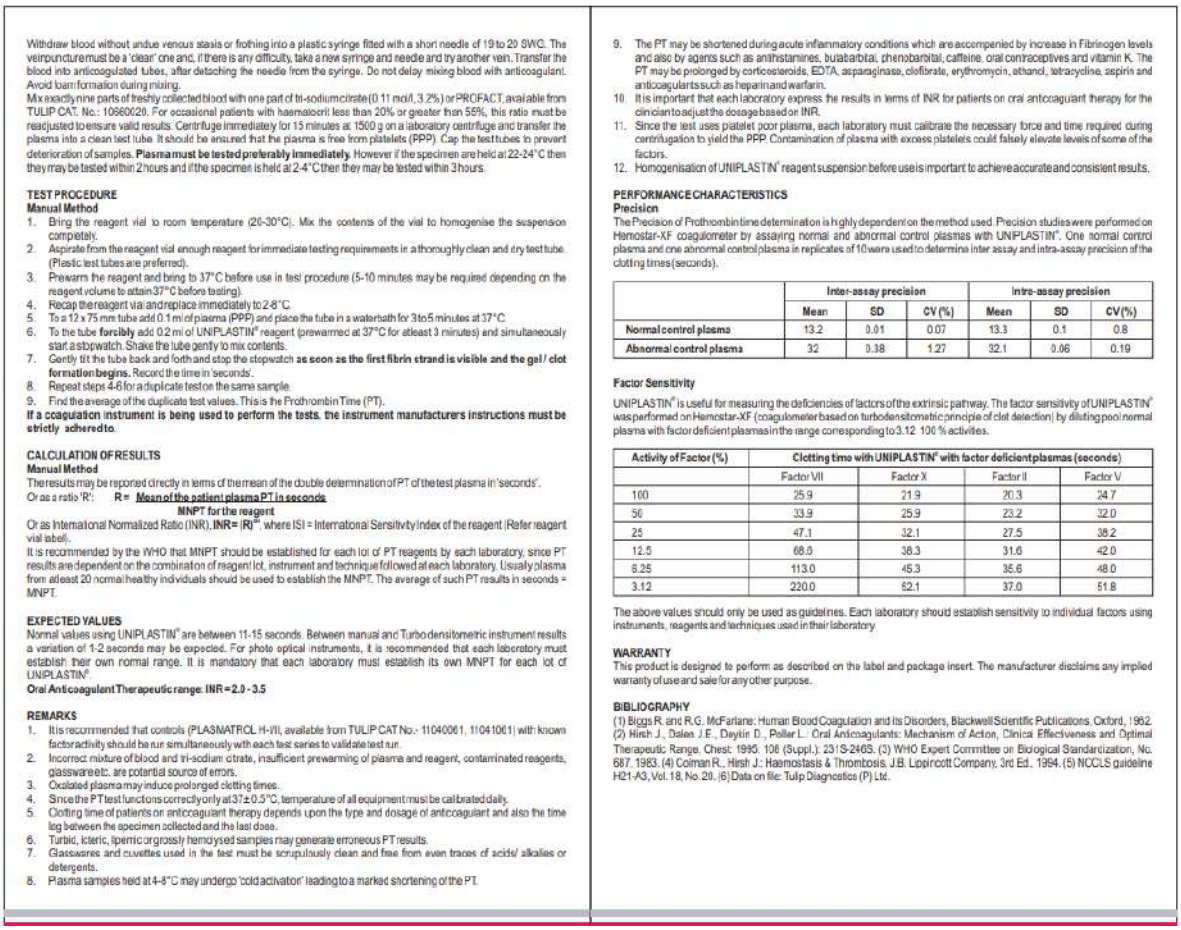
(1) In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use. (2) UNIPLASTIN® reagent is not from human source hence contamination due to HBsAg and HIV is practically excluded. (3) Reagent contains 0.01% Thimerosal as preservative. (4) It is **very important** that scrupulously clean and dry micropipette tips be used to aspirate / dispense the reagent. (5) **Avoid exposure** of the UNIPLASTIN® reagent to elevated temperatures, contamination and undue stress due to high and low temperature exposure cycles. **Immediately** replace reagent cap after use and store at recommended temperatures **only.** (6) On prolonged storage at 2-8°C the thromboplastin suspension has a tendency to settle down. Homogenise the reagent by resuspending before use. (7) Do not use damaged or leaking reagents.

ADDITIONAL MATERIAL REQUIRED

12 x 75 mm test tubes (plastic tubes are preferred), 0.1 ml and 0.2 ml pipettes, Stop watch, Water bath or heating block at 37°C, Fresh normal plasmas for establishing MNPT.

SAMPLE COLLECTION AND PREPARATION OF PPP

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light non-fatty meals prior to blood collection provide samples with a desirable lower opacity.



Withdraw blood without undue venous stasis or frothing into a plastic syringe fitted with a short needle of 19 to 20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into anticoagulated tubes, after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing.
Mix exactly nine parts of freshly collected blood with one part of tri-sodium citrate (0.11 mol/l, 3.2%) or PROFACT, available from TULIP CAT. No.: 10660020. For occasional patients with haematocrit less than 20% or greater than 55%, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500 g on a laboratory centrifuge and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. **Plasma must be tested preferably immediately.** However if the specimen are held at 22-24°C then they may be tested within 2 hours and if the specimen is held at 2-4°C then they may be tested within 3 hours.

TEST PROCEDURE
Manual Method
1. Bring the reagent vial to room temperature (20-30°C). Mix the contents of the vial to homogenise the suspension completely.
2. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube. (Plastic test tubes are preferred).
3. Prewarm the reagent and bring to 37°C before use in test procedure (5-10 minutes may be required depending on the reagent volume to attain 37°C before testing).
4. Recap the reagent vial and replace immediately to 2-8°C.
5. To a 12 x 75 mm tube add 0.1 ml of plasma (PPP) and place the tube in a waterbath for 3 to 5 minutes at 37°C.
6. To the tube **forcibly** add 0.2 ml of UNIPLASTIN® reagent (prewarmed at 37°C for atleast 3 minutes) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
7. Gently tilt the tube back and forth and stop the stopwatch **as soon as the first fibrin strand is visible and the gel / clot formation begins.** Record the time in 'seconds'.
8. Repeat steps 4-6 for a duplicate test on the same sample.
9. Find the average of the duplicate test values. This is the Prothrombin Time (PT).

If a coagulation instrument is being used to perform the tests, the instrument manufacturers instructions must be strictly adhered to.

CALCULATION OF RESULTS
Manual Method
The results may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds'.
Or as a ratio 'R': $R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent}}$
Or as International Normalized Ratio (INR), $\mathbf{INR = (R)^{ISI}}$, where ISI = International Sensitivity Index of the reagent (Refer reagent vial label).
It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from atleast 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

EXPECTED VALUES
Normal values using UNIPLASTIN® are between 11-15 seconds. Between manual and Turbodensitometric instrument results a variation of 1-2 seconds may be expected. For photo optical instruments, it is recommended that each laboratory must establish their own normal range. It is mandatory that each laboratory must establish its own MNPT for each lot of UNIPLASTIN®.
Oral Anticoagulant Therapeutic range: INR = 2.0 - 3.5

REMARKS
1. It is recommended that controls (PLASNATROL H-I/II, available from TULIP CAT No.- 11040061, 11041061) with known factor activity should be run simultaneously with each test series to validate test run.
2. Incorrect mixture of blood and tri-sodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glasswareetc. are potential source of errors.
3. Oxalated plasma may induce prolonged clotting times.
4. Since the PT test functions correctly only at 37 ± 0.5°C, temperature of all equipment must be calibrated daily.
5. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
6. Turbid, icteric, lipemic or grossly hemolysed samples may generate erroneous PT results.
7. Glasswares and cuvettes used in the test must be scrupulously clean and free from even traces of acids/ alkalies or detergents.
8. Plasma samples held at 4-8°C may undergo 'cold activation' leading to a marked shortening of the PT.
9. The PT may be shortened during acute inflammatory conditions which are accompanied by increase in Fibrinogen levels and also by agents such as antihistamines, buatabrbital, phenobarbital, caffeine, oral contraceptives and vitamin K. The PT may be prolonged by corticosteroids, EDTA, asparaginase, clofibrate, erythromycin, ethanol, tetracycline, aspirin and anticoagulants such as heparin and warfarin.
10. It is important that each laboratory express the results in terms of INR for patients on oral anticoagulant therapy for the clinician to adjust the dosage based on INR.
11. Since the test uses platelet poor plasma, each laboratory must calibrate the necessary force and time required during centrifugation to yield the PPP. Contamination of plasma with excess platelets could falsely elevate levels of some of the factors.
12. Homogenisation of UNIPLASTIN® reagent suspension before use is important to achieve accurate and consistent results.

PERFORMANCE CHARACTERISTICS
Precision
The Precision of Prothrombin time determination is highly dependent on the method used. Precision studies were performed on Hemostar-XF coagulometer by assaying normal and abnormal control plasmas with UNIPLASTIN®. One normal control plasma and one abnormal control plasma in replicates of 10 were used to determine inter assay and intra-assay precision of the clotting times (seconds).

	Inter-assay precision			Intra-assay precision		
	Mean	SD	CV (%)	Mean	SD	CV (%)
Normal control plasma	13.2	0.01	0.07	13.3	0.1	0.8
Abnormal control plasma	32	0.38	1.27	32.1	0.06	0.19

Factor Sensitivity
UNIPLASTIN® is useful for measuring the deficiencies of factors of the extrinsic pathway. The factor sensitivity of UNIPLASTIN® was performed on Hemostar-XF (coagulometer based on turbodensitometric principle of clot detection) by diluting pool normal plasma with factor deficient plasmas in the range corresponding to 3.12 100 % activities.

Activity of Factor (%)	Clotting time with UNIPLASTIN® with factor deficient plasmas (seconds)			
	Factor VII	Factor X	Factor II	Factor V
100	25.9	21.9	20.3	24.7
50	33.9	25.9	23.2	32.0
25	47.1	32.1	27.5	38.2
12.5	68.6	38.3	31.6	42.0
6.25	113.0	45.3	35.6	48.0
3.12	220.0	52.1	37.0	51.8

The above values should only be used as guidelines. Each laboratory should establish sensitivity to individual factors using instruments, reagents and techniques used in their laboratory.

WARRANTY
This product is designed to perform as described on the label and package insert. The manufacturer disclaims any implied warranty of use and sale for any other purpose.

BIBLIOGRAPHY
(1) Biggs R. and R.G. McFarlane: Human Blood Coagulation and its Disorders, Blackwell Scientific Publications, Oxford, 1962. (2) Hirsh J., Dalen J.E., Deykin D., Poller L.: Oral Anticoagulants: Mechanism of Action, Clinical Effectiveness and Optimal Therapeutic Range. Chest. 1995. 108 (Suppl.): 231S-246S. (3) WHO Expert Committee on Biological Standardization, No. 687. 1983. (4) Colman R., Hirsh J.: Haemostasis & Thrombosis. J.B. Lippincott Company, 3rd Ed., 1994. (5) NCCLS guideline H21-A3, Vol. 18, No. 20. (6) Data on file: Tulip Diagnostics (P) Ltd.

Identification of abnormal cells in Peripheral blood smear (PBS)

Peripheral blood smear examination

- Making a quality blood smear Although there are several techniques described for making blood smears, most people use the wedge or push technique.
- Always start with room temperature, well-mixed, clot free, EDTA anticoagulated blood (heparin is used for some exotic species). If the blood has been refrigerated, allow it to return to room temperature.
- The best slides to use for making blood smears are the premium, pre-cleaned kind; those with a frosted end also facilitate easy labeling. If you are not using premium pre-cleaned slides, you will need to wipe off each slide you plan to use for making the blood smear, including spreader slides, in order to eliminate glass grit and dust which can ruin your smear.
- Always mix the specimen immediately before making the smear by gently rolling the tube several times to ensure good cellular distribution, don't shake or invert.
- To transfer blood from the tube to the slide, fill a plain microhematocrit tube with blood by capillary action and place a finger over the end to prevent the blood from running out. Do NOT try to dab blood from the cap onto the slide or use a 1 ml plastic pipette; the microhematocrit tube will give you better control and, if you have a microhematocrit

centrifuge, you can simply plug the clean end after making your slide and spin the tube for your hematocrit and total protein.

- Place an approximately 4mm diameter drop of blood on one end of the slide. If there isn't enough blood in the microhematocrit tube to produce the required droplet of blood, DO NOT repeatedly tap the tube onto the glass to try and get enough blood out; go back and get some more from the EDTA tube.
- Holding the spreader slide at an approximately 30-45º angle, back into the drop of blood and, as soon as you see the drop spreading along the edge of the spreader slide, push the spreader slide forward in a smooth, moderately fast motion; apply only enough
- pressure to keep the spreader slide on the glass, excessive pressure will push too much blood forward without allowing development of a good body and monolayer; you should be pulling the blood along the slide, not pushing it.
- If the blood is very thin (severe anemia) you may need to increase the angle of the spreader slide to avoid going off the end of the slide.

Staining smears

⇒ Prepare a thin blood smear on a clean and dry microscopic glass slide and air dry it.

⇒ Now, cover the well dried, thin blood smear with undiluted Leishman Stain solution by counting the drops of Leishman stain.

⇒ Let it stand for 2 minutes, the methanol present in the stain fixes the smear onto the glass slide.

⇒ After 2 minutes, add twice the amount of distilled water or Phosphate buffer solution and mix the content by swirling or by blowing gently. Incubate the slides for at least 10 min at 37 °C. This will stain the blood cells.

⇒ Rinse the slides thoroughly with Phosphate buffer solution up to 2 minutes or until it acquires a purple-pinkish tinge.

⇒ Air dry the slides in a tilted position so that the water easily remove out of the slides.

⇒ Now you can mount the smears with mounting media, e.g. Gurri's neutral mounting media or any other mounting medium which do not decolorizes the smear. Do not use Canada balsam as it may decolorize the smear.

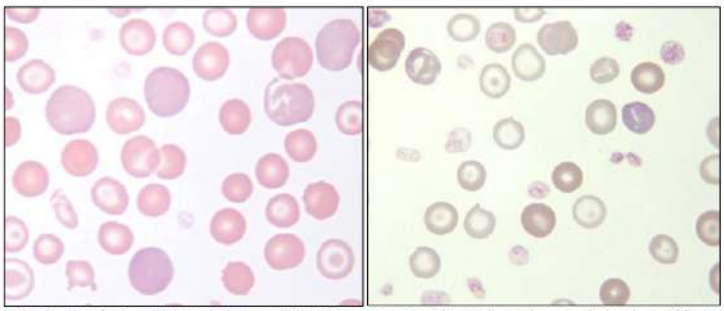
⇒ Let it dry in air for few hours and then observe the slides under oil immersion objective lens of the microscope.

Smear evaluation

Red cells

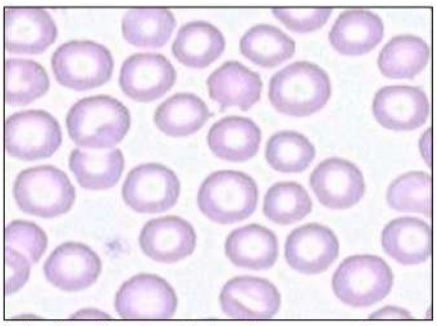
Color changes.

• Polychromasia is recognized as larger, slightly bluer-staining red cells and is an excellent indicator of a regenerative response. Note: you will have a hard time identifying polychromasia if your smear is over-stained. Polychromasia is typically semi-quantified as rare, slight, mild, moderate or marked



Red cell color changes. Right; polychromatophilic RBCs are present. These cells are larger and more basophilic than normal RBCs. This image show moderate polychromasia. Left; hypochromatic RBCs. The area of central pallor is increased and the cells, overall, are paler staining. Note a couple of polychromatophilic RBCs are also present. Images courtesy of Ms. Tillie Laws.

- Hypochromasia indicates iron deficiency and is recognized by increased central pallor AND pale color. This is recognized in dogs more often than cats. This is different from 'punched out' RBCs in which there is a distinct, round, pale central area that is sharply delineated from the rim of hemoglobin. Punched out cells have a wider rim of hemoglobin than do hypochromic cells, and the hemoglobin color is normal, not pale. Note that in iron deficiency, there often is a lot of poikylocytosis present as well.

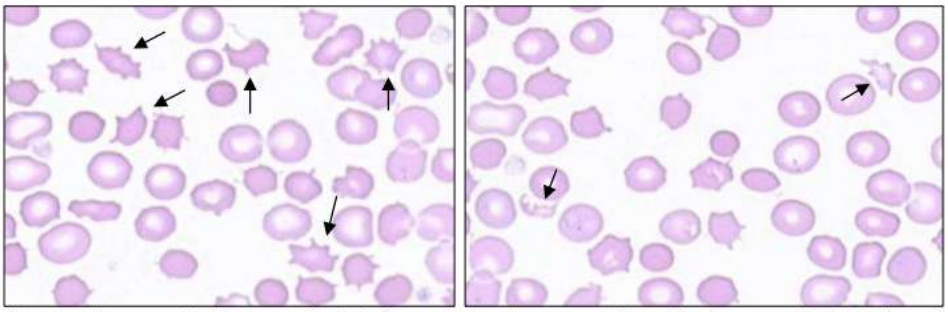


Red cells with a 'punched out' appearance; the area of central pallor appears enlarged and is sharply delineated from the cytoplasm. This has no significance and should not be confused with hypochromasia; the rim of cytoplasm is still larger than is seen in hypochromic RBCs and the color of the cytoplasm is robust.

Morphology changes

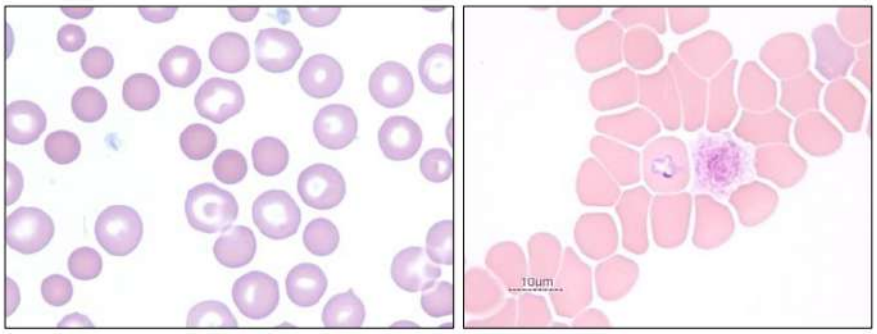
- Changes can be quantified as rare (<1/100xfield), occasional (1-3/ 100x field), few/mild (4-10/100x field), moderate (10-25/100x field) or marked/many (>25/100x field). In practice, these are estimated rather than actually counted.
- Acanthocytes. These are cells with unevenly distributed cytoplasmic projections, and are most commonly seen in feline hepatic lipidosis or in dogs with hemangiosarcoma, especially when it involves the liver.

- Schistocytes. These are small, irregular fragments of cells and are most commonly seen in hemangiosarcoma, microangiopathies, DIC and iron deficiency.



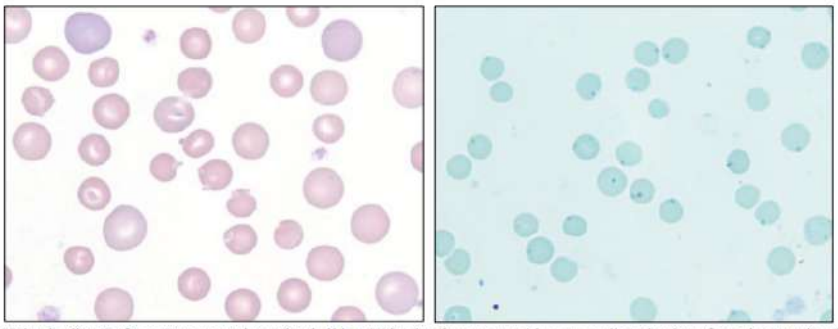
Many acanthocytes and schistocytes. On the left, several acanthocytes are indicated by the arrows. On the right , two schistocytes are indicated. Acanthocytes have irregularly spaced cytoplasmic projections that are typically blunted and may have a knob-like end. Schistocytes are irregular, torn pieces of RBC cytoplasm formed by sheering of cells.

- Spherocytes. These are red cells that are round instead of flat and most commonly indicate IMHA, but can also form after removal of Heinz bodies by the spleen and low numbers are noted in fragmentation anemia. Because they are rounded, they appear smaller and darker than normal red cells and there is a lack of central pallor. Spherocytes are best appreciated in dogs due to the prominent central pallor in the normal canine RBC, and are more difficult to detect in other species. Be careful not to interpret RBCs along the feathered edge as spherocytes; cells in these areas (where there are large open spaces) tend to round up and lose their central pallor normally.

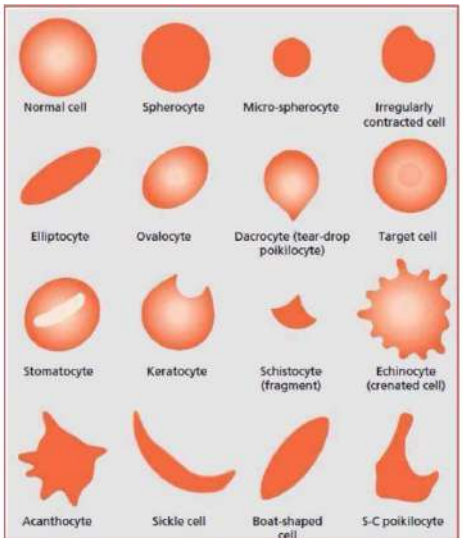


Right; an example of spherocytes in a dog. Spherocytes classically are small, round cells that lack central pallor and appear darker red than surrounding blood cells. Sometimes, when they have all the features of spherocytes but do not appear darker than surrounding cells, they are referred to as 'pre-spherocytes' or 'imperfect' spherocytes and may also indicate immune mediated targeting of RBCs. Left; RBCs from the feathered edge of a canine blood smear. Note that the cells appear to have lost their central pallor – don't mistake RBCs without central pallor on the feathered edge for spherocytes, this is normal. This dog also has a large platelet and, in the RBC in the center, a *Babesia canis* piroplasm.

- Heinz bodies. These indicate oxidative damage to hemoglobin, and large numbers are seen with oxidative hemolytic anemia. They appear as small, roundish structures can protrude from the margin of the cell as a pale structure, or appear as a small pale dot near the edge of the cell. Cats often develop large Heinz bodies when it's the result of an oxidative drug or plant; Heinz bodies seen in sick cats (renal disease, lymphoma, hyperthyroidism) tend to be smaller and present in lesser numbers.



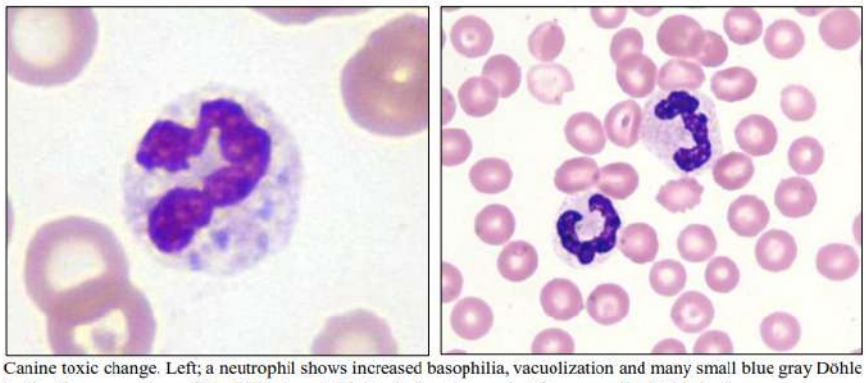
Heinz bodies. Left; on Romanowsky stained slides, Heinz bodies appear either as small projections from the margin of the cell, or pale stained regions on the interior of the cell. Right; with new methylene blue staining, Heinz bodies are basophilic blue dots.



White cell changes

Toxic change

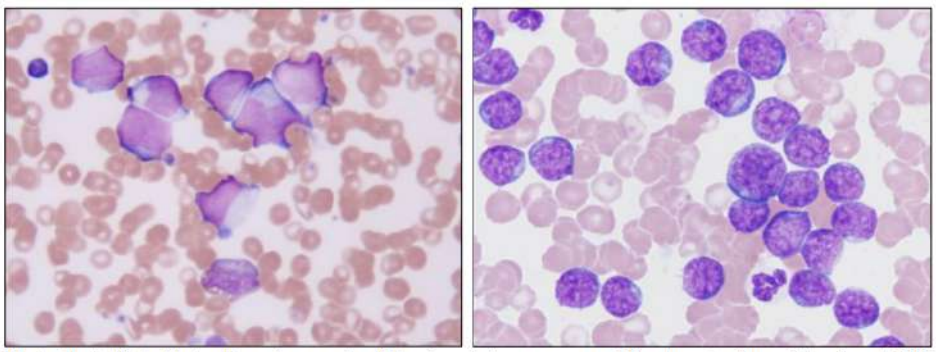
- Toxic change indicates acute, systemic inflammation and, when moderate or marked, is most closely associated with toxemia from bacterial infections. Slight toxic change is common in cats and slight to mild toxic change can be seen in a variety of disease processes that are not necessarily infectious (immune mediated disease, neoplasia, drugs) or bacterial (fungal disease, protozoal infection, viruses). Toxic change consists of 4 potential components;
- Cytoplasmic basophilia due to retained ribosomes. This is often the last change to resolve.
- Döhle bodies. These are small, cytoplasmic, angular, grey-blue aggregates of retained rough ER. Cats develop these commonly and a rare Döhle body can be seen in healthy animals.
- Cytoplasmic vacuolation due to granule dissolution, it's identified as cytoplasm with a frothy appearance. This is often seen in conjunction with increased basophilia but is a more serious manifestation of toxic change.
- Toxic granulation from retained primary granules. This is a severe change and is rarely seen in small animals (I've yet to see a convincing case in a dog or cat), is seen occasionally in large animals, and is most commonly seen in avian and reptile species. Toxic granules are pinkpurple in color.



Canine toxic change. Left; a neutrophil shows increased basophilia, vacuolization and many small blue gray Döhle bodies (image courtesy of Ms. Tillie Laws). Right; similar changes but fewer, smaller Döhle bodies.

Blast cells

Blast cells in circulation can be an important indicator of underlying neoplasia, recurring neoplasia or stage of lymphoma. Note that with a severe inflammatory leukocytosis with a significant left shift, a few myeloblasts may be seen as part of the bone marrow response, but in the absence of these findings, or when blast cells are present in disproportionate numbers compared with other neutrophil stages, underlying neoplasia is a concern. Blasts are characterized by cells that are medium to large in size with pale chromatin, a visible nucleolus or nucleolar ring and, typically, a scant rim of cytoplasm. Other features may be present depending on the origin of the cell. Often small cytoplasmic fragments are also found in circulation. They are small platelet sized fragments of cytoplasm – they can be distinguished from platelets because they lack normal platelet granulation.

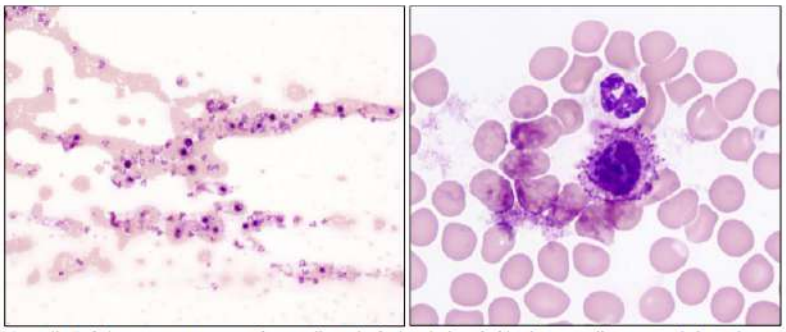


Blast cells. Left; the blast cells are large and variably shaped; they are seen molding to each other and to adjacent RBCs causing distortion of the cell shape. The chromatin is very pale and delicate. Nucleoli were faintly visible on higher power. Right; these blast cells are also large but exhibit less molding, have darker, coarser chromatin and more robustly visible nucleoli and nucleolar rings.

Mast cells

Mast cells can be found in circulation secondary to mast cell neoplasia or severe inflammation, especially of the GI tract. Often they are found on the feathered edge of the smear, but may be present in the monolayer when in high numbers. These are large cells with round nuclei that are filled with purple granules; in dogs, there is often a smudged purple ‘halo’ around the cells from partial degranulation, cats don’t tend to do this. Don’t mistake these cells for basophils. Basophils have

bandBlast cells. Left; the blast cells are large and variably shaped; they are seen molding to each other and to adjacent RBCs causing distortion of the cell shape. The chromatin is very pale and delicate. Nucleoli were faintly visible on higher power. Right; these blast cells are also large but exhibit less molding, have darker, coarser chromatin and more robustly visible nucleoli and nucleolar rings. like nuclei and few dark blue granules (dogs) or many pale gray-blue granules (cats). Remember mast cell granules may not stain with quick stains.

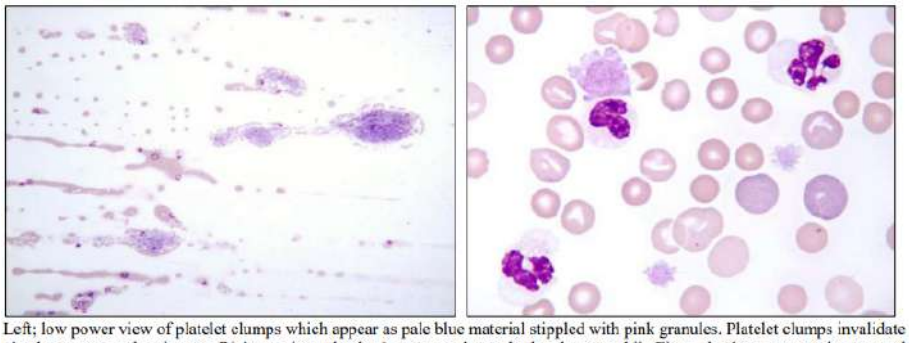


Mast cells. Left; low power appearance of mast cells on the feathered edge of a blood smear; cells appear as dark purple spots. Right; a high power image of a mast cell. These cells are characterized by a round nucleus and purple granules. Note the smudged purple 'halo' streaming away from the cell to the left; this is typical of canine mast cells seen on blood smears; cats do not tend to have a hale.

Platelets and miscellaneous

Confirm/estimate count

This is an important step, especially in animals with low platelet counts from a hematology analyzer. Most analyzers can't reliably detect platelet clumps and will give a falsely low number when clumps are present. Although this is more common in cats, it also happens in dogs. Clumps are easiest to identify on the feathered edge of the smear. They have a pale pink/blue stippled appearance. Platelet clumps always invalidate hematology analyzer or hemocytometer counts. If clumps are not present, you should perform an estimate in the monolayer region by averaging the number of platelets present in several 100x fields and multiplying that by 20,000.



Left; low power view of platelet clumps which appear as pale blue material stippled with pink granules. Platelet clumps invalidate platelet counts and estimates. Right; a giant platelet is present above the band neutrophil. Giant platelets may not be counted correctly; they suggest a regenerative response to thrombocytopenia.

MYELOPEROXIDASE STAIN

MYELOPEROXIDASE STAIN

Histochemical Method **100 Tests**

INTRODUCTION :

Myeloperoxidase is a lysosomal enzyme localized in the azurophil granules of neutrophils and monocytes. Azurophil granules in granulocytic cells correspond to the relatively large electron dense (primary) granules seen under the electron microscope. The secondary (specific) granules are less electron dense and appear at the myelocyte stage. In the monocytic series the azurophil granules are smaller and are not the first to appear during maturation in these cells. Thus the designation primary for them is not appropriate. The lysosomal granules present in early monocytic cells (monoblasts) are very small and have acid phosphatase but lack peroxidase activity .

Myeloperoxidase can also be demonstrated in the specific granules of eosinophils and basophils. In eosinophils the specific granules are not newly formed but derive from primary granules which are also myeloperoxidase positive. The eosinophil peroxidase has been shown by chemical, cytochemical and immunological methods to be different from that of neutrophils and probably to be under separate genetic control. The enzyme in eosinophils is cyanide-resistant and, in neutrophils, cyanide-sensitive.

Most of the early methods for the demonstration of peroxidase use benzidine and hydrogen peroxide. Probably safer substrates should be considered such as 3,3' –diamino benzidine (DAB) tetrahydrochloride. DAB is the substrate of choice for ultrastructural studies because its oxidized product is electron dense and can be intensified by post-fixation with osmium tetroxide. DAB is also frequently used to visualize the immunoperoxidase reaction. and found reliable in the diagnosis of acute myeloid leukaemia (AML).

REAGENTS :

1.	Fixative : formal-ethanol
2.	Incubation mixture (prepared just before use): a. DAB Content of one Vial b. Buffer 10 ml c. H_2O_2 (substrate) 0.1 ml Add the reagents in this order and mix well after each addition .
3.	Enhancer : Copper sulphate
4.	Counterstain : Methyl green

METHOD :

1. Fix fresh air - dry film of peripheral – blood or bone – marrow films for 1 min. using 20 drops of R1 and then rinse in D. water .
2. Put on the section 10 drops of the incubation mixture (R2) for 1 min. at room temperature .
3. Rinse briefly in D. water
4. Put on the sections 20 drops of the enhancer (R3) for two min.
5. Rinse in D. water .
6. Counterstain in methyl green,put on the section 10 drops of (R4) for 10 min. wash, dehydrate, clear and mount.

RESULT :

Cytoplasm : Dark brown
Nucleus : Green

REFERENCE:

Hanker, J.S., et al . Cancer Research, 39, 1635, (1979)

PAS (Periodic Acid Schiff) Staining Protocol

PAS (Periodic Acid Schiff) Staining Protocol

Used for the detection of glycogen in tissues such as liver, cardiac and skeletal muscle on formalin-fixed paraffin-embedded tissue sections, and may be used for frozen sections as well.

Solutions and Reagents:

0.5% Periodic Acid Solution

Periodic acid ---------- 0.5 g
Distilled water ---------- 100 ml

Schiff's reagent

Schiff's reagent: Fisher Scientific (cat: 50-301-27)

Test for Schiff's reagent: Pour 10 ml of 10% formalin to a beaker, add a few drops of the Schiff's reagent to be tested. Good Schiff's reagent will rapidly turn a red purple color. A deteriorating Schiff's reagent will give a delayed reaction and the color produced will be a deep blue-purple.

Mayer's hematoxylin

Mayer's modified hematoxylin: Fisher Scientific (cat: NC9220898)

Procedure:

1. Deparaffinize and hydrate to water.
2. Oxidize in 0.5% Periodic Acid solution for **5 minutes**.
3. Rinse in 3 changes of distilled water.
4. Place in Schiff's reagent for **15 minutes** (Sections become light pink color during this step).
5. Wash in tap water for 5 minutes (Sections immediately turn dark pink).
6. Counterstain in Mayer's hematoxylin for **1 minute**.
7. Wash in tap water for **5 minutes then rinse in distilled water.**
8. Dehydrate, coverslip and mount using Xylene based mounting media

Results:

Glycogen, mucin and some basement membranes -------- Red/ purple

Fungi ---------- Red/ purple

Background. ---------- Blue

SUDAN BLACK B STAIN

SUDAN BLACK B STAIN

Histochemical Method **100 Tests**

PRINCIPLE :

Sudan Black B was used to stain the granules of neutrophils , many of which appear to contain phospholipids . the close parallelism observed between sudanophilia and myeloperoxidase activity relates to the fact that both cytochemical reactions are positive in the azurophil granules of neutrophils and monocytes and in the specific eosinophil granules . the biochemical basis for the sudanophilia in this cells is poorly understood. One possible view is that Sudan Black B stains the lipid membrane of the granules which contain the enzyme myeloperoxidase. Another is that the dye stains through an enzymatic mechanism. Perhaps linked to myeloperoxidase , and not just by physical solution in the lipids. Both reactions are positive in mature and immature myeloid cells and thus are useful in the differential diagnosis and classification of the acute leukaemias. the simplicity of the Sudan Black B reaction makes its use mandatory in routine haematology laboratories.

REAGENTS :

1.	Dye	60 ml
2.	Buffer	40 ml

ADDITIONAL REAGENTS:

Fixative : Available on request .

- Formaldehyde Fixative . or
- Formaline – ethanol fixative

Counterstain : Methyl green or Giemsa

STABILITY :

Reagents are stable up to the expiration date given when stored at 4 °C

PROCEDURE:

Fixation:

1. Fix air – dried films of blood or bone marrow for 10 min in formalin vapour, formaldehyde fixative or formaline – ethanol fixative .
2. Wash gently in water for 5 – 10 min ;

Staining:

Working Reagent:

Mix Reagent R1 and Reagent R2 in ratio of 1. 5 : 1, Filter before use.

1. Incubate the fixed film in 20 drops working reagent for 10 min. in closed area to avoid evaporation.
2. Wash in 70 % ethanol by waving the slides in the alcohol for 3 - 5 min .
3. Wash with water for 2 min, dry .
4. Counterstain for 5 min and mount .

RESULT :

The reaction product in the cytoplasm is black; the nuclei stain blue or red depending on the counterstain used. (Giemsa, safranin or methyl green.)

REFERENCE :

Sheehan , H .L . and Story , G . W . (1977)
J . Path . and Bacter . 59 , 336 .

BMLT WBUHS: Paper 302

EXPEIMENT 1: Montouxe test.

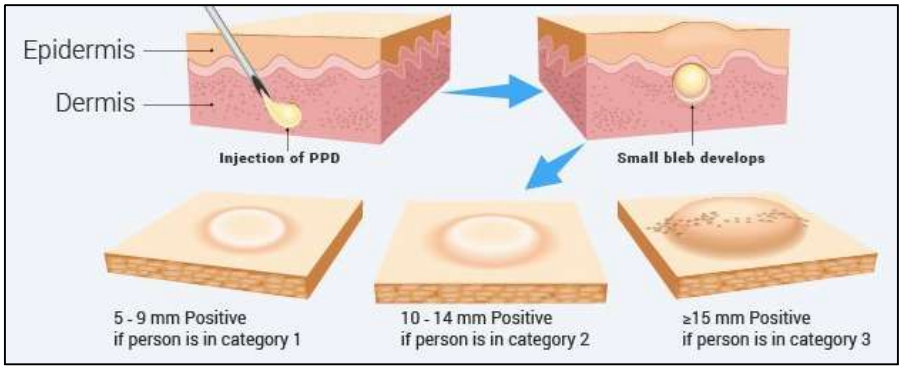
AIMS:
To rapid detect of tuberculosis.

INTRODUCTION:
The Mantoux test or Mendel–Mantoux test (also known as the **Mantoux** screening **test**, **tuberculin** sensitivity **test**, Pirquet **test**, or PPD **test** for purified protein derivative) is a tool for screening for tuberculosis (TB) and for tuberculosis diagnosis. It is one of the major tuberculin skin tests used around the world. The Mantoux test is endorsed by the American Thoracic Society and Centers for Disease Control and Prevention. It was also used in the USSR and is now prevalent in most of the post-Soviet states.

PRINCIPLE:
Tuberculin is a glycerol extract of the tubercle bacillus. Purified protein derivative (PPD) tuberculin is a precipitate of species-nonspecific molecules obtained from filtrates of sterilized, concentrated cultures. This active agent is tuberculin, a protein. A person who has been exposed to the bacteria is expected to mount an immune response in the skin containing the bacterial proteins. The response is a classical example of delayed-type hypersensitivity reaction (DTH), a type IV of hypersensitivities. T cells and myeloid cells are attracted to the site of reaction in the timeframe of 1-3 days and generate local inflammation. The reaction is read by measuring the diameter of induration (palpable raised, hardened area) across the forearm (perpendicular to the long axis) in millimeters. If there is no induration, the result should be recorded as "0 mm". Erythema (redness) should not be measured. In the Pirquet version of the test tuberculin is applied to the skin via scarification.

MATERIALS:
Tuberculin, Alcohol swab, Syringe, Scale.

PROCEDURE:
1. In the Mantoux test, a standard dose of 5 tuberculin units (TU - 0.1 ml), according to the CDC, or 2 TU of Statens Serum Institute (SSI) tuberculin RT23 in 0.1 ml solution, according to the NHS, is injected intra-dermally (between the layers of dermis) on the flexor surface of the left forearm, mid-way between elbow and wrist.
2. The injection should be made with a tuberculin syringe, with the needle bevel facing upward. Alternatively, the probe can be administered by a needle-free jet injector.
3. When placed correctly, injection should produce a pale wheal of the skin, 6 to 10 mm in diameter.
4. The result of the test is read after 48-96 hours but 72 hours (3rd day) is the ideal.
5. This intradermal injection is termed the **Mantoux technique**.



RESULTS & INTERPRETATION:

The person's medical risk factors determine at which increment (5 mm, 10 mm, or 15 mm) of induration the result is considered positive. A positive result indicates TB exposure.

According to the guidelines published by Centers for Disease Control and Prevention in 2005, the results are re-categorized into 3 parts based on their previous or baseline outcomes:

- Baseline test: ≥10 mm is positive (either first or second step); 0 to 9 mm is negative
- Serial testing without known exposure: Increase of ≥10 mm is positive
- Known exposure:
  - ≥5 mm is positive in patients with baseline of 0 mm
  - ≥10 mm is positive in patients with negative baseline or previous screening result of >0 mm

Diameter 5 mm or more is positive: An HIV-positive person; Persons with recent contacts with a TB patient; Persons with nodular or fibrotic changes on chest X-ray consistent with old healed TB; Patients with organ transplants, and other immunosuppressed patients.

Diameter 10 mm or more is positive: Recent arrivals (less than five years) from high-prevalence countries; Injection drug users; Residents and employees of high-risk congregate settings (e.g., prisons, nursing homes, hospitals, homeless shelters, etc.); Mycobacteriology lab personnel; Persons with clinical conditions that place them at high risk (e.g., diabetes, prolonged corticosteroid therapy, leukemia, end-stage renal disease, chronic malabsorption syndromes, low body weight, etc.); Children less than four years of age, or children and adolescents exposed to adults in high-risk categories.

Diameter 15 mm or more is positive: Persons with no known risk factors for TB.

A tuberculin test conversion is defined as an increase of 10 mm or more within a two-year period, regardless of age. Alternative criteria include increases of 6, 12, 15 or 18 mm.

Experiment 2: ELISA

Enzyme-linked immunosorbent assays (ELISAs) are plate-based assays for detecting and quantifying a specific protein in a complex mixture. The detection and quantification of target-specific protein in ELISA is accomplished by using highly specific antibodies that immobilizes the target protein (antigen) to the plate and indirectly detects the presence of the target protein. There are three types of ELISA being carried out.

Buffers and reagents:

Bicarbonate/carbonate coating buffer (100 mM): Antigen or antibody should be diluted in coating buffer to immobilize them to the wells: 3.03 g Na2CO3, 6.0 g NaHCO3 1000 ml distilled water pH 9.6, PBS:

1.16 g Na2HPO4, 0.1 g KCl, 0.1 g K3PO4, 4.0 g NaCl (500 ml distilled water) pH 7.4.

Blocking solution: Commonly used blocking agents are 1% BSA, serum, non-fat dry milk, casein, gelatin in PBS.

Wash solution: Usually PBS or Tris-buffered saline (pH 7.4) with detergent such as 0.05% (v/v) Tween20 (TBST).

Antibody dilution buffer: Primary and secondary antibody should be diluted in 1x blocking solution to reduce non-specific binding

Direct ELISA: Procedure:

Coating antigen to microplate

1. Dilute the antigen to a final concentration of 20 µg/ml in PBS or other carbonate buffer. Coat the wells of a microtiter plate with the antigen by pipeting 50 µl of the antigen dilution in the top wells of the plate. Dilute down the plate as required. Ensure the samples contain the antigen at a concentration that is within the detection range of the antibody.
2. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature, or 4oC overnight. The coating incubation time may require some optimization.
3. Remove the coating solution and wash the plate twice by filling the wells with 200 µl PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking:

4. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk/PBS, per well. Alternative blocking reagents include BlockACE or BSA.
5. Cover the plate with an adhesive plastic and incubate for at least 2 h at room temperature or, if more convenient, overnight at 4°C.
6. Wash the plate twice with PBS.

Incubation with the antibody:

7. Add 100 µl of the antibody, diluted at the optimal concentration (according to the manufacturer's instructions) in blocking buffer immediately before use.

8. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature. This incubation time may require optimization. Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often observed when incubated overnight at 4°C.

9. Wash the plate four times with PBS.

Detection

10. Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipet or a multipipet.

11. After sufficient color development (if it is necessary) add 100 µl of stop solution to the wells.

12. Read the absorbance (optical density) of each well with a plate reader. Note: some enzyme substrates are considered hazardous (potential carcinogens), therefore always handle with care and wear gloves.

Analysis of data

13. Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear). Interpolate the concentration of the sample from this standard curve

Procedure: Indirect ELISA protocol

Coating antigen to microplate

1. Dilute the antigen to a final concentration of 20 µg/ml in PBS or other carbonate buffer. Coat the wells of a PVC microtiter plate with the antigen by pipeting 50 µl of the antigen dilution in the top wells of the plate. Dilute down the plate as required.

2. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature, or 4°C overnight. The coating incubation time may require some optimization.

3. Remove the coating solution and wash the plate three times by filling the wells with 200 µl PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking

4. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk or 5% serum in /PBS, per well. Alternative blocking reagents include BlockACE or BSA.

5. Cover the plate with an adhesive plastic and incubate for at least 2 hrs at room temperature or, if more convenient, overnight at 4°C.

6. Wash the plate twice with PBS.

Incubation with primary and secondary antibody:

7. Add 100 µl of diluted primary antibody to each well.

8. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature. This incubation time may require optimization. Although 2 hours is usually enough to obtain a strong signal, if a weak signal is obtained, stronger staining will often observed when incubated overnight at 4°C.

9. Wash the plate four times with PBS.

10. Add 100 µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use.

11. Cover the plate with an adhesive plastic and incubate for 1-2 hrs at room temperature.

12. Wash the plate four times with PBS.

Detection

Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the two widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells) and this may result in non-specific signal. If necessary, perform an additional blocking treatment with Levamisol (for ALP) or with 0.3% solution of H_2O_2 in methanol (for peroxidase). **ALP substrate:** For most applications pNPP (p-Nitrophenyl-phosphate) is the most widely used substrate.The yellow color of nitrophenol can be measured at 405 nm after 15-30 min incubation at room temperature. (This reaction can be stopped by adding equal volume of 0.75 M NaOH).

HRP chromogens The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during reaction.

TMB (3,3',5,5'-tetramethylbenzidine): add TMB solution to each well, incubate for 15-30 min, add equal volume of stopping solution (2 M H_2SO_4) and read the optical density at 450 nm

13. Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipet or a multipipet.

14. After sufficient color development (if it is necessary) add 100 µl of stop solution to the wells. 15. Read the absorbance (optical density) of each well with a plate reader.

Analysis of data: Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear). Interpolate the concentration of the sample from this standard curve.

Sandwich ELISA:

The Sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two

antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect).

Procedure:

Coating with Capture antibody

1. Coat the wells of a PVC microtiter plate with the capture antibody at a concentration of 1-10 µg/ml in carbonate/bicarbonate buffer (pH9.6).

2. Cover the plate with an adhesive plastic and incubate overnight at 4°C.

3. Remove the coating solution and wash the plate twice by filling the wells with 200 µl PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking and Adding Samples

4. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk/PBS, per well.

5. Cover the plate with an adhesive plastic and incubate for at least 1-2 h at room temperature or, if more convenient, overnight at 4°C.

6. Add 100 µl of appropriately diluted samples to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy. Incubate for 90 min at 37°C.

7. Remove the samples and wash the plate twice by filling the wells with 200 µl PBS.

Incubation with Detection antibody and then Secondary antibody

8. Add 100 µl of diluted detection antibody to each well.

9. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

10. Wash the plate four times with PBS.

11. Add 100 µl of secondary antibody conjugated, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use.

12. Cover the plate with an adhesive plastic and incubate for 1-2 h at room temperature.

13. Wash the plate four times with PBS.

Detection:

Although many different types of enzymes have been used for detection, horse radish peroxidase (HRP) and alkaline phosphatase (ALP) are the two most widely used enzymes employed in ELISA assay. It is important to consider the fact that some biological materials have high levels of endogenous enzyme activity (such as high ALP in alveolar cells, high peroxidase in red blood cells) and this may result in a nonspecific signal. If necessary, perform an additional blocking treatment with Levamisol (for ALP) or with 0.3% solution of H2O2 in methanol (for peroxidase).

ALP substrate: For most applications pNPP (p-Nitrophenyl-phosphate) is the most widely used substrate. The yellow colour of nitrophenol can be measured at 405 nm after 15-30 min incubation at room temperature. (This reaction can be stopped by adding equal volume of 0.75 M NaOH).

HRP chromogens:

The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes colour during reaction. TMB (3,3',5,5'-tetramethylbenzidine) add TMB solution to each well, incubate for 15-30 min, add equal volume of stopping solution (2 M H2SO4) and read the optical density at 450 nm. OPD (o-phenylenediamine dihydrochloride) the end product is measured at 492 nm. Be aware that the substrate is light sensitive so keep and store it in the dark. ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt. The end product is green and the optical density can be measured at 416 nm.

14. Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipet or a multipipet.

Analysis of data:

Prepare a standard curve from the data produced from the serial dilutions with concentration on the x axis (log scale) vs absorbance on the Y axis (linear). Interpolate the concentration of the sample from this standard curve

Experiment 3. Immune-chromatographic devices for HIV/HCV/HBs Ag etc

To determine HIV-1/2 Ag/Ab Combo is not intended for newborn screening or for use with cord blood specimens or specimens from individuals less than 12 years of age.

INTRODUCTION:

HIV–1/2 Ag/Ab Combo is an *in vitro*, visually read, qualitative immunoassay for the simultaneous detection of Human Immunodeficiency Virus Type 1 (HIV-1) p24 antigen (Ag) and antibodies (Ab) to HIV Type 1 and Type 2 (HIV-1 and HIV-2) in human serum, plasma, capillary (fingerstick) whole blood or venipuncture (venous) whole blood. It is intended for use as a point-of-care test to aid in the diagnosis of infection with HIV-1 and HIV-2, including an acute HIV-1 infection, and may distinguish acute HIV-1 infection from

established HIV-1 infection when the specimen is positive for HIV-1 p24 antigen and negative for anti-HIV-1 and anti-HIV-2 antibodies. The test is suitable for use in multi-test algorithms designed for the statistical validation of rapid HIV test results. When multiple rapid HIV test are available, this test can be used in appropriate multi-test algorithms.

***PRINCIPLE*:**
HIV–1/2 Ag/Ab Combo is an immunochromatographic test for the simultaneous and separate qualitative detection of free HIV-1 p24 antigen and antibodies to HIV-1 and HIV-2. The test device is a laminated strip that consists of a Sample Pad containing monoclonal biotinylated anti-HIV-1 p24 antibody, a Conjugate Pad containing monoclonal anti-HIV-1 p24 antibody-colloidal selenium and HIV-1 and HIV-2 recombinant antigen-colloidal selenium, and a nitrocellulose membrane with an immobilized mixture of recombinant and synthetic peptide HIV-1 and HIV-2 antigens in the Lower Test Area, immobilized streptavidin in the Upper Test Area, and an immobilized mixture of anti-HIV-1 antibodies, HIV-1/2 antigens, and HIV-1 p24 recombinant antigen and anti-HIV-1 p24 monoclonal antibody in the Control Area.

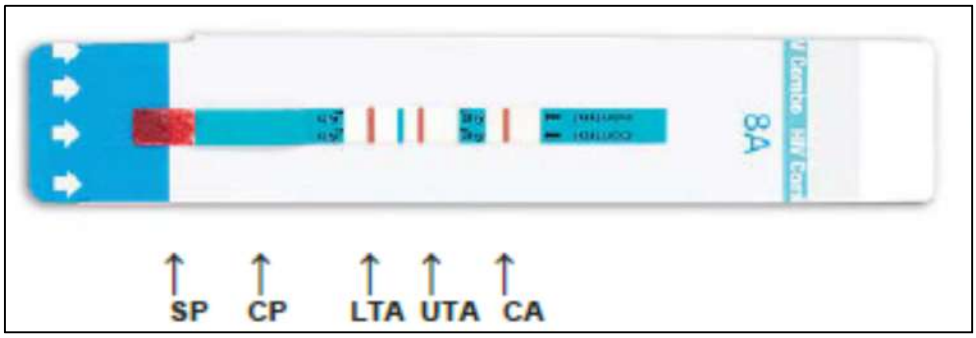
A specimen (venipuncture or capillary whole blood, serum, or plasma) is applied to the Sample Pad (followed by Chase Buffer for venipuncture or fingerstick whole blood specimens) and migrates by capillary action through the Conjugate Pad and then through the nitrocellulose membrane.

If HIV-1 p24 antigen is present in the specimen; it binds with the monoclonal biotinylated anti-HIV-1 p24 antibody from the Sample Pad and then with monoclonal anti-HIV-1 p24 antibody-colloidal selenium from the Conjugate Pad to form a complex (biotinylated antibody-antigen-colloidal selenium-antibody). This complex migrates through the solid phase by capillary action until it is captured by immobilized streptavidin at the Upper Test Area (labeled “Ag”) where it forms a single pink/red “Ag” line. If HIV-1 p24 antigen is not present in the specimen or is below the limit of detection of the test, no pink/red Ag line is formed. NOTE: The monoclonal biotinylated anti-HIV-1 p24 antibody used in this assay does not cross react with HIV-2 p26 antigen.

If antibodies to HIV-1 and/or HIV-2 are present in the specimen, the antibodies bind to recombinant gp41 (HIV-1) and gp36 (HIV-2) antigen-colloidal selenium conjugates from the Conjugate Pad. The complex migrates through the solid phase by capillary action until it is captured by immobilized HIV-1 and HIV-2 synthetic peptide antigens and recombinant gp41 antigen at the Lower Test Area (labeled “Ab”) and forms a single pink/red “Ab” line. If antibodies to HIV-1 and/or HIV-2 are absent or are below the detection limit of detection of the test, no pink/red Ab line is formed.

To ensure assay validity, a procedural “Control” line containing a mixture of anti-HIV-1 antibody, HIV-1/2 antigens, and HIV-1 p24 recombinant antigen and anti-HIV-1 p24 monoclonal antibody is incorporated in the nitrocellulose membrane. For a test result to be valid there must be a visible pink/red Control line. During the testing procedure the colloidal

selenium conjugates released from the Conjugate Pad will be captured by the antibodies and antigens immobilized in the Control Area and form a pink/red Control line for samples that are either positive or negative.



SP = Sample Pad; CP = Conjugate Pad; LTA = Lower Test Area; UTA = Upper Test Area; CA = Control Area

REAGENTS:

Materials:

1. HIV-1/2 Ag/Ab Combo Cards. Each Card consists of 5 or 10 Test Units which can be separated from each other by tearing along the perforated lines. Each Test Unit has a cover that is to be removed for sample application and visualization of test results.
2. Desiccant Package
3. Chase Buffer: Containing sodium chloride, disodium hydrogen phosphate, and Nipasept as a preservative.
4. Quick Reference Guide
5. Package Insert
6. Subject Information Notices: 25 in the 25 Test Units kit, and 100 in the 100 Test Units kit.
7. Customer Letter
8. Disposable Capillary Tubes: For collection and transfer of fingerstick samples.
9. Disposable Workstations: 25 in the 25 Test Units kit, and 100 in the 100 Test Units kit.

Accessory:

• Fingerstick Sample Collection Kit

• HIV–1/2 Ag/Ab Combo Controls:

HIV-1 p24 Antigen Control: 1.5mL, HIV-1 viral lysate in defibrinated pooled normal human plasma; negative for antibodies to HIV-1, HIV-2 and HCV; negative for HBsAg.

HIV-1 Reactive Control: 1.5mL, human plasma positive for anti-HIV-1 antibodies, diluted in defibrinated pooled normal human plasma; negative for antibodies to HIV-2 and HCV; negative for HBsAg.

HIV-2 Reactive Control: 1.5mL, human plasma positive for anti-HIV-2 antibodies, diluted in defibrinated pooled normal human plasma; negative for antibodies to HIV-1 and HCV; negative for HBsAg and HIV-1 p24.

Nonreactive Control: 1.5mL, defibrinated normal human plasma; negative for antibodies to HIV-1, HIV-2, and HCV; negative for HBsAg and HIV-1 p24.

Materials General

• Clock, watch, or other timing device.
• Precision pipette capable of delivering 50µL of sample with disposable tips.
• Disposable gloves
• Sterile gauze (for fingerstick whole blood specimens)
• Antiseptic wipes
• Biohazard disposal container
• Collection devices for specimens (other than fingerstick whole blood specimens)

PROCEDURE:

HIV-1/2 Ag/Ab Combo Controls should be tested prior to testing patient specimens when a new operator performs testing, a new test kit lot is to be used, a new shipment of test kits is received, and at periodic intervals indicated by the testing facility. Controls should be tested in the same manner as serum or plasma samples in the following Test Procedure.

Kit Component Preparation

1. Remove the desired numbers of test units from the 5- or 10-Test Unit Card by bending and tearing at the perforation.
2. Lay the Test Unit flat in the workstation and remove the protective foil cover from each Test
Unit. The test should be initiated within 2 hours after removing the protective foil cover from each Test Unit.

For serum or plasma samples:

1. Apply 50 µL of sample (precision pipette) to the Sample Pad (marked by the arrow symbol). Do not add Chase Buffer when using serum or plasma specimens.
2. Read the test result between 20 and 30 min after the addition of the Sample. Do not read test results after 30 min.

For whole blood (venipuncture) samples:

1. Using a precision pipette with a disposable tip, apply 50 µL of sample to the Sample Pad (marked by the arrow symbol).
2. Wait for one minute, then apply one drop of Chase Buffer to the Sample Pad.
3. Read the test result between 20 and 30 minutes after the addition of the Chase Buffer.

For whole blood (fingerstick) samples using the Disposable Capillary Tube:

1. Align the tip of the Capillary Tube containing the blood sample with the Sample Pad (marked by the arrow symbol) and gently squeeze the bulb. Avoid air bubbles. Wait until all the blood is transferred from the Capillary Tube to the Sample Pad.
2. Do not lift the Capillary Tube from the Sample Pad before all the blood has been transferred – a bubble may form which will prevent the complete transfer of sample.
3. Wait for one minute, then apply one drop of Chase Buffer to the Sample Pad.
4. Read the test result between 20 and 30 minutes after the addition of the Chase Buffer. Do not read Test Results after 30 minutes.
5. Discard the used pipette tips, Capillary Tube, Test Units and any other test materials into a biohazard waste container.

RESULTS & INTERPRETATION:

1. ANTIBODY REACTIVE (Two Lines - Control Line and Ab Line)

A pink/red Control line appears in the Control Area and a pink/red Ab line appears in the Lower Test Area of the Test Unit. The intensity of the Ab and Control lines may vary. Any visible pink/red color in both the Control and Lower Test Areas, regardless of intensity, is considered REACTIVE. A Reactive test result means that HIV-1 and/or HIV-2 antibodies have been detected in the specimen. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 and/or HIV-2 antibodies.

2. ANTIGEN (HIV-1p24) REACTIVE (Two Lines - Control Line and Ag Line)

A pink/red Control line appears in the Control Area and a pink/red Ag line appears in the Upper Test Area of the Test Unit. The intensity of the Ag and Control lines may vary. Any visible pink/red color in both the Control and Upper Test Areas, regardless of intensity, is considered REACTIVE. A Reactive test result means that HIV-1 p24 antigen has been detected in the specimen. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 p24 antigen.

3. ANTIBODY REACTIVE AND ANTIGEN (HIV-1 p24) REACTIVE (Three Lines - Control, Ab and Ag Lines)

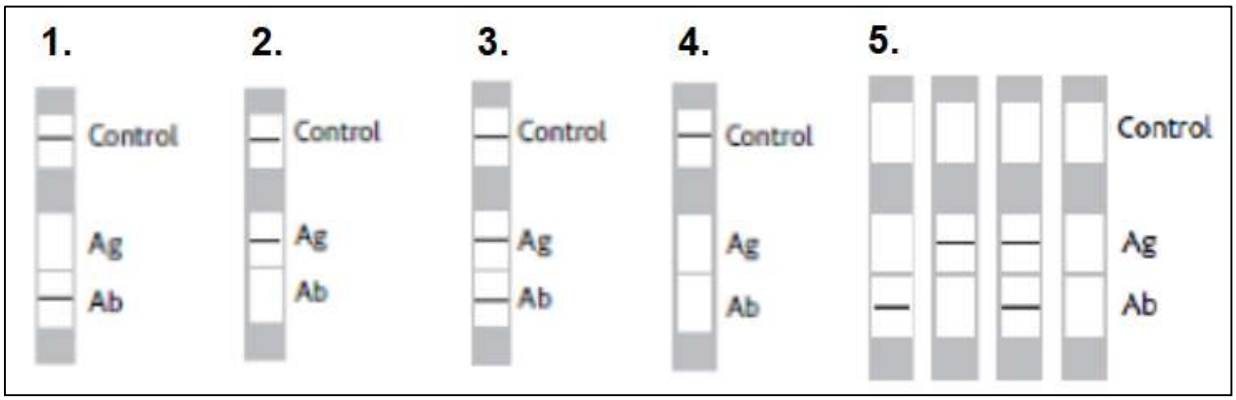
A pink/red Control line appears in the Control Area and a pink/red Ab line appears in the Lower Test Area and a pink/red Ag line appears in the Upper Test Area of the Test Unit. The intensity of the Ab, Ag and Control lines may vary. Any visible pink/red color in the Control Area, the Lower Test Area and the Upper Test Area, regardless of intensity, is considered REACTIVE. The test result is interpreted as PRELIMINARY POSITIVE for HIV-1 and/or HIV-2 antibodies and HIV-1 p24 antigen.

4. NONREACTIVE (One Line – Control Line)

A pink/red Control line appears in the Control Area of the Test Unit, and no pink/red Ab or Ag line appears in the Lower Test Area and the Upper Test Area of the Test Unit, respectively. A NONREACTIVE test result means that HIV-1 or HIV-2 antibodies and HIV-1 p24 antigen were not detected in the specimen.

5. INVALID (No Control Line)

If there is no pink/red Control line in the Control Area of the Test Unit, even if a pink/red line appears in the Lower Test Area or the Upper Test Area of the Test Unit, the result is INVALID and the test should be repeated. If the problem persists, contact Alere Technical Support.



Experiment 4: VDRL test:

AIM:

To rapid detect the syphilis in patient sample.

INTRODUCTION:

The venereal disease research laboratory (VDRL) test is used to detect if a person has been infected with the bacteria causing syphilis, which is a sexually transmitted disease. The test detects the presence of antibodies against the bacteria *Treponema pallidum*. This test is only indicative, and if positive, it must be followed up with another blood test to make a definitive diagnosis of syphilis.

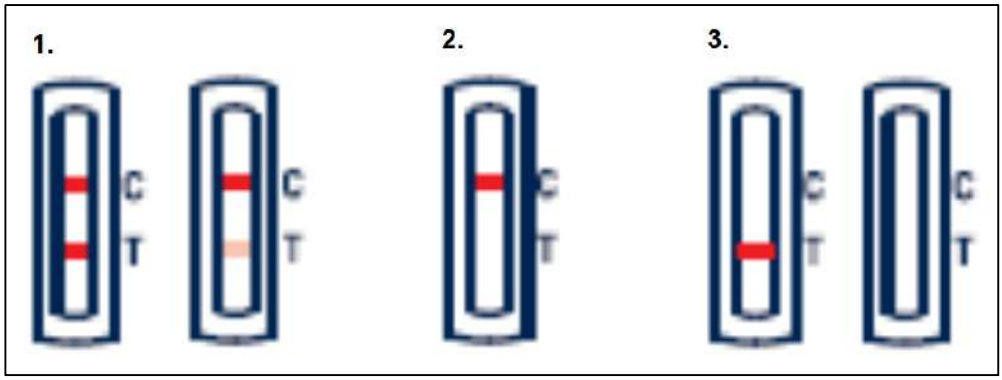
PRINCIPLE:

The Syphilis Ultra Rapid Test Device is a rapid chromatographic immunoassay for the qualitative detection of antibodies (IgG and IgM) to *Treponema pallidum* (TP) in whole blood, serum or plasma to aid in the diagnosis of Syphilis. Recombinant Syphilis antigen is immobilized in the test line region of the device. After a specimen is added to the specimen well of the device, it reacts with Syphilis antigen coated particles in the test. This mixture migrates chromatographically along the length of the test strip and interacts with the immobilized Syphilis antigen. The double antigen test format can detect both IgG and IgM in specimens. If the specimen contains TP antibodies, a colored line will appear in the test line region, indicating a positive result.

PROCEDURE:

1. Add two drops of serum or plasma into the sample window using provided disposable dropper.

2. For Venipuncture Whole Blood specimens: Add 1 drop (20µl) of whole blood to the specimen well (S), then add 2 drops of buffer.
3. For Finger stick Whole Blood: Allow 1 hanging drop of finger stick whole blood specimen to fall into the center of the specimen well (S), then add 2 drops of buffer.
4. Wait for the colored band(s) to appear. The result should be read at 20 minutes.



RESULTS & INTERPRETATION:

1. Positive: Two lines appear. One coloured line should be in the control line region (C) and another apparent coloured line should be in the test line region (T).
2. Negative: One coloured line appears in the control line region(C). No line appears in the test line region (T).
3. Invalid: No visible band at all or test band without control band.

Experiment 5: RPR test:

AIM:

Rapid Plasma Reagin (RPR) Test for the diagnosis of Syphilis.

INTRODUCTION:

Rapid plasma reagin (RPR) is macroscopic, non treponemal, flocculation card test used to screen for syphilis caused by *Treponema pallidum*. RPR is simple test can be done within few minutes. This test is less sensitive than treponemal test in early syphilis infection. Two types of antibodies are produced in syphilis i.e. autoantibody (cardiolipin) response and treponemal antibody response. Autoantibodies are produced in 2-3 weeks of treponemal infection due to tissue damage. These auto antibodies are often referred to as cardiolipin antibodies because they can be detected in serological test using cardiolipin antigen. This test doesn't look for antibodies against actual bacterium but rather for antibodies against substances released by cells they are damaged by *Treponema pallidum*. The anti-lipodial antibodies are antibodies that are not produce only in syphilis infection but also in other non treponemal disease of an acute and chronic nature in which tissues are damaged. RPR measures IgM and IgG antibodies to lipodial materials released from damaged host cells as well as lipoprotein like material and possibly cardiolipin released from treponems. Antigen used in RPR test contain cardiolipin lecithin, cholesterol, 10% choline chloride, EDTA, charcoal in buffer. This test

cannot be performed on CSF. Serum or plasma can be used for testing, serum not heated. This test tends to give negative results during late syphilis.

PRINCIPLE:

RPR is 18 mm circle card test is a macroscopic flocculation test for syphilis. The antigen is prepared from modified VDRL (Venereal Disease Research Laboratory), antigen suspension containing choline chloride and EDTA (ethylenediamine tetra acetic acid) to enhance stability of suspension, finely divided charcoal particles as visualizing agents. In this test antigen is mixed with unheated serum on plastic –coated card. This test measures IgM & IgG antibodies to lipodial material released from damaged host cells as well as possibly cardiolipin released from treponems. If antibodies are present, they combine with lipid particles of the antigen, causing them to agglutinate. The charcoal particles co-agglutinate with antibodies and shows black clumps on white cards. If antibodies are not present, the test mixture is uniformly gray

REAGENTS:

1. RPR antigen suspension: RPR antigen suspension is a stabilized combination of 0.003% cardiolipin, 0.020-0.022% lecithin, 0.09% cholesterol, 10% choline chloride, 0.0125M EDTA, 0.01875% charcoal, 0.01M Na2HP04, 0.01M KH2P04, 0.1% thimerosal in distilled water.

2. Control serum samples: Control serum samples are lyophilized reactive (R), minimally reactive (Rm), and nonreactive (N) control serum specimens on a card, or liquid or lyophilized serum samples of graded reactivity. If quantitative tests are to be performed, a control serum that can be titered to at least a 1:4 dilutions should be used.

3. 0.9% Saline: Add 0.9 gm of dry sodium chloride (ACS) to 100 ml of distilled water.

4. Diluent: Prepare a 2% solution of human serum in 0.9% saline, by diluting a human serum nonreactive for syphilis 1:50 in 0.9% saline.

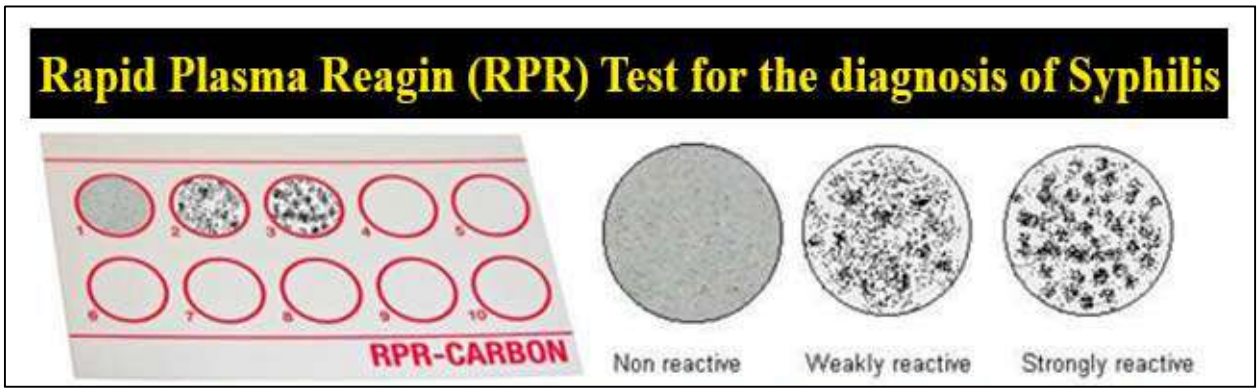
PROCEDURE:

Qualitative Test

1. Place 50µl of serum or plasma on 18mm circle of RPR test using a disposable dispenstirs or a safety pipetting device.
2. Spread serum or plasma to fill the entire circle. Don't spread the specimen beyond the confines of the circle.
3. Gently shake the antigen dispensing bottles to re-suspend the particle.
4. Dispense several drops of antigen (17µl of ag) suspension to each circle containing serum or plasma.
5. Mix the suspension well in one direction.
6. Rotate card for 4-8 mins and observed for flocculation.

Quantitative Test

1. Dilute the endpoint titre all serum specimen with rough non-reactive results in qualitative test. Test each specimen undiluted (1:1) and in 1:2, 1:4, 1:8, 1:16 dilution.
2. Place 50µl of 0.9% saline in circles. Don't spread saline.
3. Using safety pipette device, place 50µl of serum in circle labeled 1 and 50µl of serum in circle 2. Mix the saline and serum in circles.
4. Transfer 50µl from circle 2 (1:2) to circle3, & mix
5. Transfer 50µl from circle 3 (1:4) to circle 4 &Mix
6. Same way transfer 50µl from circle (1:8) to circle (1:16), mix and discard the last 50µl.
7. Spread the serum dilution using clean dispenstirs to fill entire circle.
8. Gently shake the dispensing bottles to re-suspend the antigen particles.
9. Add (17µl of ag) antigen suspension in each circle.
10. Place the card in rotator for 8 min at 100v 2rpm under humidifying cover.
11. Remove card from rotator and tilt the card by hand (three or four to and fro motions) to aid in differentiating non-reactive from minimally reactive results.



RESULTS AND INTERPRETATION:

Positive Result (Reactive): Clumping (Characteristic clumping ranging from marked and intense (reactive) to Reactive (R) slight but definite (minimally to moderately) reactive).

Negative Result (Non-Reactive): No Clumping or slight roughness.

Experiment 6: WIDAL test

AIM:

To rapid detect the typhoid from serum sample.

INTRODUCTION:

Widal Test is an agglutination test which detects the presence of serum agglutinins (H and O) in patients serum with typhoid and paratyphoid fever. When facilities for culturing are not available, the Widal test is the reliable and can be of value in the diagnosis of typhoid fevers

in endemic areas. It was developed by Georges Ferdinand Widal in 1896. The patient's serum is tested for O and H antibodies (agglutinins) against the following antigen suspensions (usually stained suspensions):
S. typhi 0 antigen suspension, 9, 12
S. typhi H antigen suspension, d
S. paratyphi A 0 antigen suspension, 1, 2, 12
S. paratyphi A H antigen suspension, a
S. paratyphi B 0 antigen suspension, 1, 4, 5, 12
S. paratyphi B H antigen suspension, b, phase 1
S. paratyphi C 0 antigen suspension, 6, 7
S. paratyphi C H antigen suspension, c, phase 1

Salmonella antibody starts appearing in serum at the end of first week and rise sharply during the 3rd week of endemic fever. In acute typhoid fever, O agglutinins can usually be detected 6–8 days after the onset of fever and H agglutinins after 10–12 days. It is preferable to test two specimens of sera at an interval of 7 to 10 days to demonstrate a rising antibody titre. *Salmonella* antigen suspensions can be used as slide and tube techniques.

PRINCIPLE:
Bacterial suspension which carry antigen will agglutinate on exposure to antibodies to *Salmonella* organisms. Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, coloured *Salmonella* antigens in a agglutination test. The main principle of widal test is that if homologous antibody is present in patients serum, it will react with respective antigen in the reagent and gives visible clumping on the test card and agglutination in the tube. The antigens used in the test are "H" and "O" antigens of *Salmonella typhi* and "H" antigen of *S. paratyphi*. The paratyphoid "O" antigen is not employed as they cross react with typhoid "O" antigen due to the sharing of factor 12. "O" antigen is a somatic antigen and "H" antigen is flagellar antigen.

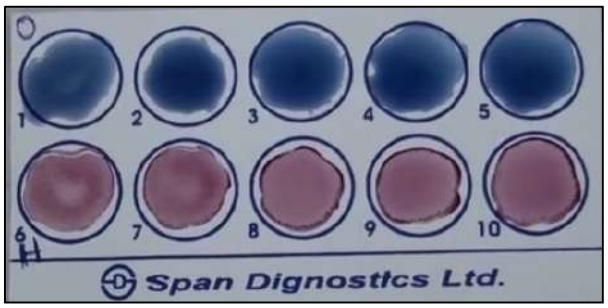
Preparation of Widal Antigens

- H suspension of bacteria is prepared by adding 0.1 per cent formalin to a 24 h broth culture or saline suspension of an agar culture.
- For preparation of O suspensions of bacteria, the organisms are cultured on phenol agar (1:800) to inhibit flagella.
- Standard smooth strains of the organism are used; *S* Typhi 901, O and H strains are employed for this purpose.
- 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:

SLIDE TEST

1. Place one drop of positive control on one reaction circles of the slide.
2. Pipette one drop of Isotonic saline on the next reaction cirlcle. (-ve Control).
3. Pipette one drop of the patient serum tobe tested onto the remaining four reaction circles.
4. Add one drop of Widal TEST antigen suspension 'H' to the first two reaction circles. (PC & NC).
5. Add one drop each of 'O', 'H', 'AH' and 'BH' antigens to the remaining four reaction circles.
6. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7. Rock the slide, gently back and forth and observe for agglutination macroscopically within one minute.

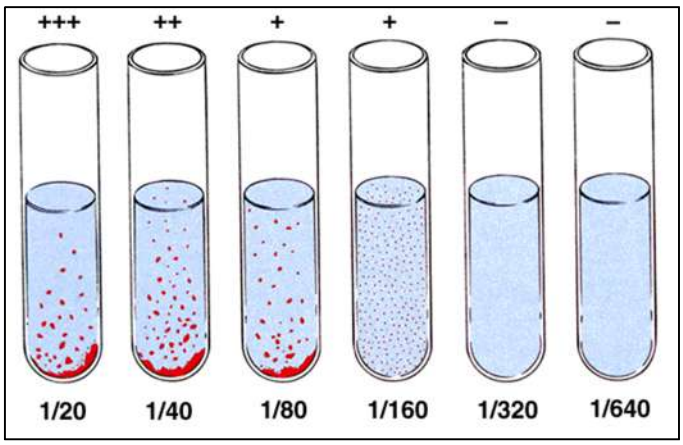


SEMI-QUANTITATIVE METHOD

1. Pipette one drop of isotonic saline into the first reaction circle and then place 5, 10, 20, 40, 80 ul of the test sample on the remaining circles.
2. Add to each reaction circle, a drop of the antigen which showed agglutination with the test sample in the screening method.
3. Using separate mixing sticks, mix the contents of each circle uniformly over the reaction circles.
4. Rock the slide gently back and forth, observe for agglutination macroscopically within one minute.

STANDARD TUBE TEST METHOD

In Widal Test, two types of tubes were originally used:
(1) Dreyer's tube (narrow tube with conical bottom) for H agglutination and
(2) Felix tube (short round-bottomed tube) for O agglutination.
Now a days 3 x 0.5 ml Kahn tubes are used for both types of agglutination.



1. Take 4 sets of 8 Kahn tubes/test tubes and label them 1 to 8 for O, H, AH and BH antibody detection.
2. Pipette into the tube No.1 of all sets 1.9 ml of isotonic saline.
3. To each of the remaining tubes (2 to 8) add 1.0 ml of isotonic saline.
4. To the tube No.1 tube in each row add 0.1 ml of the serum sample to be tested and mix well.
5. Transfer 1.0 ml of the diluted serum from tube no.1 to tube no.2 and mix well.
6. Transfer 1.0 ml of the diluted sample from tube no.2 to tube no.3 and mix well. Continue this serial dilution till tube no.7 in each set.
7. Discard 1.0 ml of the diluted serum from tube No.7 of each set.
8. Tube No.8 in all the sets, serves as a saline control. Now the dilution of the serum sample achieved in each set is as follows: Tube No. : 1 2 3 4 5 6 7 8 (control) Dilutions 1:20 1:40 1:80 1:160 1:320 1:640 1:1280.
9. To all the tubes (1 to 8) of each set add one drop of the respective WIDALTEST antigen suspension (O, H, AH and BH) from the reagent vials and mix well.
10. Cover the tubes and incubate at 37° C overnight (approximately 18 hours).
11. Dislodge the sedimented button gently and observe for agglutination.

INTERPRETATION:

SLIDE TEST

- Agglutination is a positive test result and if the positive reaction is observed with 20 ul of test sample, it indicates presence of clinically significant levels of the corresponding antibody in the patient serum.
- No agglutination is a negative test result and indicates absence of clinically significant levels of the corresponding antibody in the patient serum.

TEST TUBE

- The titre of the patient serum using Widal test antigen suspensions is the highest dilution of the serum sample that gives a visible agglutination.
- The sample which shows the titre of 1:100 or more for O agglutinations and 1:200 or more for H agglutination should be considered as clinically significant (active infection). Example: In the above figure, titre is 160.

- Demonstration of 4-fold rise between the two is diagnostic.
- H agglutination is more reliable than O agglutinin.
- Agglutinin starts appearing in serum by the end of 1st week with sharp rise in 2nd and 3rd week and the titre remains steady till 4th week after which it declines.

Experiment 7: RA (Rheumatoid Arthritis) test:

AIM:

To rapid test the qualitative detection of all subtypes of rheumatoid factor (RF) in human serum, plasma or whole blood at a sensitivity of 8 IU/mL. by a lateral flow immunoassay.

INTRODUCTION:

Rheumatoid factors are human auto-antibodies that bind to the Fc moieties of immunoglobulins leading to tissue damage. Elevated levels of RF are found in 70-90% of rheumatoid arthritis, 75-95% of Sjögren's syndrome, 95% of Felty's syndrome cases, as well as a number of connective tissue and inflammatory diseases including infectious mononucleosis, SLE, scleroderma and hepatitis. The majority of RF are of the IgM subtype, but it can also be of IgG, IgA, IgE or IgD subtypes. IgM RF, IgA RF and IgG RF are seen in 92%, 65% and 66% of RA patients respectively.

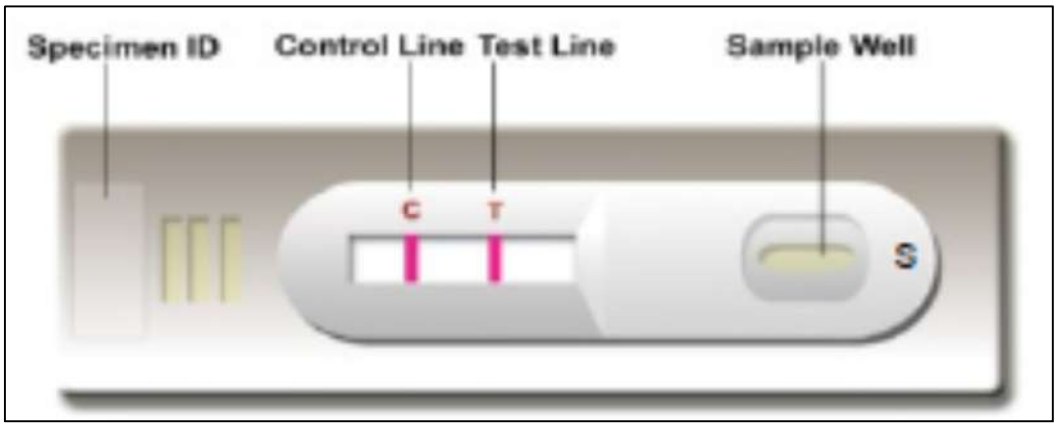
Therefore, detection of all the isotypes of RF is necessary. Pathological values of RF vary with subtypes, test methods and laboratories. Levels of IgM RF are reported to be ≥ 3-15 IU/mL, IgA RF are ≥ 4-15 IU/mL and IgG RF ≥ 6-60 IU/mL. RF is traditionally detected by agglutination assays. In recent years, many laboratories have begun to use nephelometry and enzyme-linked immunosorbent assays (ELISA) for the quantitative detection of RF.

The RF Rapid Test is a lateral flow immunoassay for the qualitative detection of all subtypes of RF in serum, plasma or whole blood. The RF Rapid Test can be performed within 10 minutes by minimally skilled personnel and without the use of laboratory equipment.

PRINCIPLE:

The RF Rapid Test is a lateral flow chromatographic immunoassay. The test cassette consists of: 1) a burgundy colored conjugate pad containing immunoglobulins conjugated with colloidal gold (Ig conjugates) and a control antibody conjugated with colloidal gold, 2) a nitrocellulose membrane strip containing a test line (T line) and a control line (C line). The T line is pre-coated with another immunoglobulin for the detection of rheumatoid factor, and the C line is pre-coated with a control line antibody.

When an adequate volume of test specimen is dispensed into the sample well of the test cassette, the specimen migrates by capillary action across the cassette. RF, if present in the specimen, will bind to the Ig conjugates. The immunocomplex is then captured on the membrane by the pre-coated immunoglobulin forming a burgundy colored T line, indicating a level of RF greater than or equal to 8 IU/mL. Absence of the T line suggests that the RF level in the specimen is lower than 8 IU/mL. The test contains an internal control (C line) which should exhibit a burgundy colored line of the immunocomplex of the control antibodies regardless of any color development on the T line. If the C line does not develop, the test result is invalid, and the specimen must be re-tested with another device.



MATERIALS:

REAGENTS PROVIDED

1. Individually sealed foil pouches containing: a. One cassette device; b. One desiccant
2. 5 µL capillary tubes
3. Sample diluent (5 mL/bottle)
4. One package insert (instruction for use)
5. Clock or timer
6. Lancing device for whole blood test
7. RF Positive Control
8. RF Negative Control

All reagents are ready to use as supplied. Store unused test devices unopened at 2-30°C. If stored at 2-8°C, ensure that the test device is brought to room temperature before opening. The test device is stable through the expiration date printed on the sealed pouch. Do not freeze the kit or expose the kit to temperatures above 30°C.

SAMPLE COLLECTION AND HANDLING:

Consider any materials of human origin as infectious and handle them using standard bio-safety
procedures.

Plasma/Serum:

Step 1: Collect blood specimen into collection tube containing EDTA, citrate or heparin for plasma or collection tube containing no anticoagulants for serum by venipuncture.
Step 2: To make plasma specimen, centrifuge collected specimens and carefully withdraw the plasma into a new pre-labeled tube.
Step 3: To make serum specimen, allow blood to clot, then centrifuge collected specimens and carefully withdraw the serum into a new pre-labeled tube.

Test specimens as soon as possible after collecting. Store specimens at 2-8°C, if not tested immediately. The specimens can be stored at 2-8°C for up to 5 days. The specimens should be frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. Prior to testing,

bring frozen specimens to room temperature slowly and mix gently. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Whole Blood:
Drops of whole blood can be obtained by either fingertip puncture or venipuncture. Collect blood specimen into a collection tube containing EDTA, citrate or heparin. Do not use hemolyzed blood for testing. Whole blood specimens should be stored in refrigeration (2-8°C), if not tested immediately. The specimens must be tested within 24 hours of collection.

PROCEDURE:
1: Bring the specimen and test components to room temperature if refrigerated or frozen. Once thawed, mix the specimen well prior to assay.

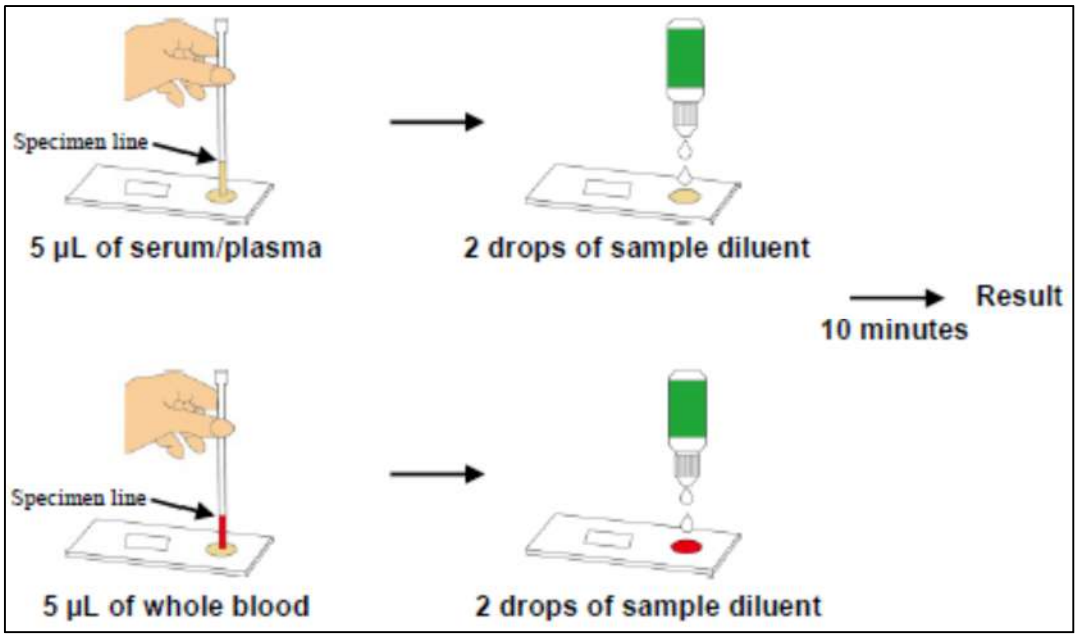
2: When ready to test, open the pouch at the notch and remove the device. Place the test device on a clean, flat surface.

3: Be sure to label the device with the specimen ID number.

4: Using a squeezing motion, fill the capillary tube with specimen (about 5 µL) not to exceed the specimen line. Holding the capillary tube vertically, dispense the entire amount of specimen into the center of the sample well making sure that there are no air bubbles. Immediately add 2 drops (about 60-80 µL) of sample diluent to the sample well with the bottle positioned vertically.

Step 5: Set up timer.

Step 6: Results should be read at 10 minutes. Positive results may be visible in as short as 1 minute. Negative results must be confirmed at the end of the 15 minutes only.

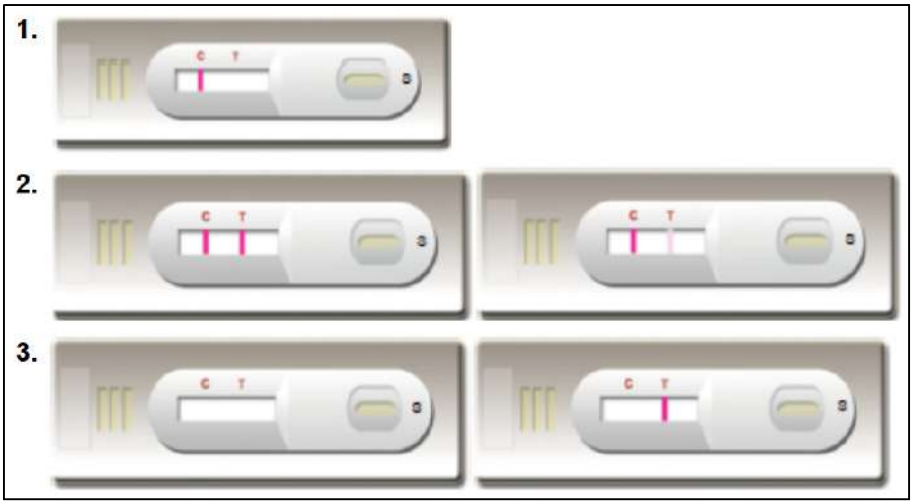


RESULT & INTERPRETATION:

1. **NEGATIVE RESULT:** If only the C line develops, the test indicates that the level of rheumatoid factor is less than 8 IU/mL. The result is negative or non-reactive.

2. **POSITIVE RESULT:** If both the C and T lines develop, the test indicates that the level of rheumatoid factor is greater than or equal to 8 IU/mL. The result is positive or reactive.

3. **INVALID:** If no C line develops, the assay is invalid regardless of color development on the T line as indicated below. Repeat the assay with a new device.



Defined as the 95% detection level, the limit of detection or sensitivity for the RF Rapid Test is 8 IU/mL. Serum RF levels greater than or equal to 8 IU/mL routinely test positive. Samples containing RF less than 8 IU/mL may also produce a very faint positive line.

Experiment 8. Detection of C-reactive protein (CRP).

AIM:
Rapid detection of CRP in patient sample

INTRODUCTION:
C-reactive protein (CRP) is an acute-phase protein found in concentrations of up to 5µg/ml in the serum of healthy persons. However, during an increase by as much as one thousand fold. This increase in CRP levels in serum can be used to monitor certain diseases. The changes in concentration of CRP usually be demonstrated in cases of acute myocardial infarction, rheumatoid arthritis, bacterial and viral infections

PRINCIPLE:

This test is based on the immunologic reaction between CRP as an antigen and latex particles have been coated with mono specific anti- human CRP and sensitized to detect levels greater than 6µg/ ml CRP. The latex slide test has the advantage of rapid performance in comparison to other tests for detection of CRP.

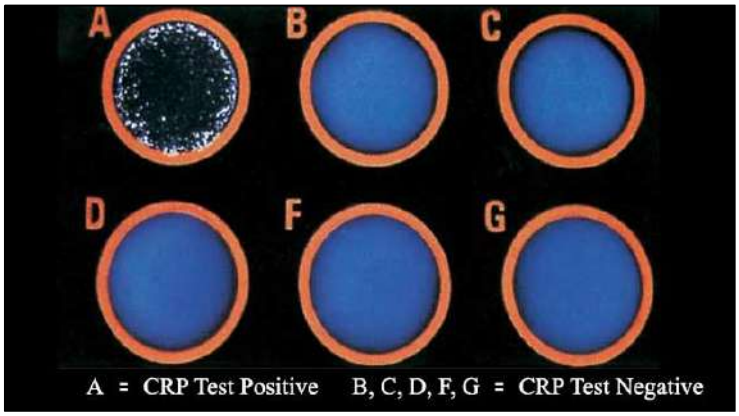
PROPCEDURE:

1. Test serum has to be used undiluted.
2. Using the disposable plastic dropper, place one drop of test serum within the circled area on the special slide provided in the kit.
3. Add one drop of latex CRP Reagent (shake well the vial gently immediately before use) above the test serum drop and mix well with a disposable applicator stick & spread out in the test area.
4. Rock the slide gently to and for 2 minutes and examine for macroscopic agglutination under direct light source. Do not examine beyond 2 minutes.

OBSERVATION AND RESULT:

Formation of coarse agglutination shows strong positive, whereas finer agglutination shows weakly positive and negative result indicates smooth suspension without any noticeable change.

Dilution	CRP (ug/ml) in undiluted sample
1:2	14
1:4	28
1:8	56
1:16	112
1:32	224
1:64	448



Positive: Agglutination of latex particles, indicating the presence of C – reactive protein at a significant and detectable level.

Negative: No Agglutination.

For Semi-Quantitative Test Results, the last dilution of serum with visible agglutination is the CRP titre of the serum.

CALCULATION OF TITRE:

CRP ug/ml = 7 x D, where D is the highest dilution of serum showing agglutination and 7 is the sensitivity in ug/ml.

Experiment 9. ASO test:

AIM:

For the qualitative and semi-quantitative measurement of antibodies to streptococcal exoenzymes in human serum.

INTRODUCTION:

The group A β-hemolytic streptococci produces various toxins that can act as antigens. One of these exotoxins Streptolysin O was discovered by Todd in 1932. A person infected with group A β-hemolytic streptococci produces specific antibodies against these exotoxins, one of which is anti-streptolysin O (ASO). The quantity of this antibody in a patient's serum will establish the degree of infection due to the β-hemolytic streptococcal. The usual procedure for the determination of the antistreptolysin titer is based on the inhibitory effect that the patient's serum produces on the hemolytic power of a pretitrated and reduced Streptolysin O. However, the antigen-antibody reaction occurs independently of the hemolytic activity of Streptolysin O. This property enables the establishment of a qualitative and quantitative test for the determination of the ASO by agglutination of latex particles on slide.

PRINCIPLE:

ASO test 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 without previous sample dilution.

REAGENTS & SPECIMEN/SAMPLE:

1. ASO Latex Reagent: A suspension of polystyrene particles coated with streptococcal exoenzymes. MIX WELL BEFORE USING.
2. ASO Positive Control: A stabilized human serum containing at least 200 IU/mL of ASO reactive with the test reagent. Ready for use; do not dilute.
3. ASO Negative Control: A stabilized human serum containing less than 200 IU/mL of ASO non- reactive with the test reagent. Ready for use; do not dilute.
4. Glycine-Saline Buffer (20x) pH = 8.2 ± 0.1. A diluent containing 0.1 M glycine and 0.15 M NaCl. Dilute buffer according to instructions on the label. All reagents contain 0.1% (w/v) sodium azide as a preservative. Store all reagents at 2 - 8°C.

REAGENT STORAGE AND STABILITY

1. Reagents are stable until stated expiration date on bottle label when stored refrigerated (2 - 8°C).
2. The ASO Latex Reagent, once shaken, must be uniform without visible clumping. When stored refrigerated, a slight sedimentation may occur and should be considered normal.
3. Do not use the latex reagent or controls if they become contaminated.

SPECIMEN COLLECTION AND STORAGE

1. Use fresh serum collected by centrifuging clotted blood. Patient may be instructed not to eat (fast) six hours before the test.
2. If the test cannot be carried out on the same day, the serum may be stored between 2 - 8°C for no longer than 48 hours after collection. For longer periods the sample must be frozen.
3. As in all serological tests, hemolytic or contaminated serum must not be used.
4. DO NOT USE PLASMA.

PRECAUTIONS

1. Reagents containing sodium azide may combine with copper and lead plumbing to form highly explosive metal azides. Dispose of reagents by flushing with large amounts of water to prevent azide build-up.
2. For in vitro diagnostic use.
3. Positive and negative controls predated using human sera found negative for hepatitis B surface antigen (HBsAg) and HIV, however, handle controls as if potentially infectious.

MATERIALS AND REAGENTS PROVIDED

1. ASO Latex Reagent.
2. ASO Positive Control.
3. ASO Negative Control.
4. Glycine - Saline Buffer.
5. Reaction Slide.
6. Pipette/Stir Sticks.

OTHER MATERIALS REQUIRED:

1. Timer
2. Test Tubes, Rack
3. Serological Pipettes

PROCEDURE:

Qualitative Test:

4. Bring reagents and specimens to room temperature before use.

2. Place one drop (50 µl) of ASO Positive Control on field #1 of the reaction slide. Place one drop (50 µl) of the ASO Negative Control on field #2 of the reaction slide. Use pipette/stir stick to deliver 1 drop (50µl) of undiluted test serum sample to field #3. Continue likewise with additional unknowns. Retain pipette/stir sticks for mixing step.

3. Gently resuspend the ASO Latex Reagent and add one drop to each test field.

4. Mix well with the flat end of the pipette. Gently rock the slide for two (2) minutes and read immediately under direct light.

Semi-quantitative Test:

1. Set up at least five test tubes: 1:2, 1:4, 1:8, 1:16, 1:32, etc. and dilute samples according to dilution factors on each test tube with diluted saline solution NOTE: Saline solution has to be diluted with distilled water before use.

2. Place one drop each of positive and negative controls onto the slide rings. Place one drop of each dilution on successive fields of the reaction slides.

3. Gently resuspend the ASO Latex Reagent and add one drop to each test field.

4. Mix well with the flat end of the pipette. Gently rock the slide for two (2) minutes and read immediately under direct light.

Note:

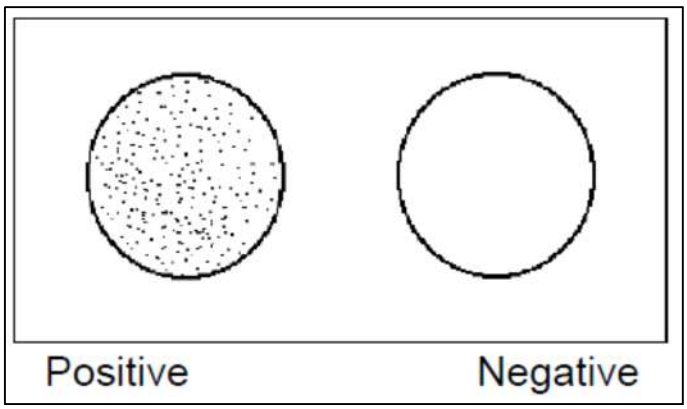
1. Positive and negative controls should be included in each test batch.
2. Acceptable performance is indicated when a uniform milky suspension with no agglutination is observed with the ASO Negative Control and agglutination with large aggregates is observed with the ASO Positive Control.
3. Results should be read two (2) minutes after the mixing of the reagent on the slide. A reading obtained after this period of time may be incorrect.

RESULTS:

Qualitative Test:

1. ***Negative reaction*****:** Uniform milky suspension with no agglutination as observed with the ASO
Negative Control.

2. *Positive reaction*: Any observable agglutination in the reaction mixture. A positive reaction indicates that the concentration of ASO in the specimen is equal or greater than 200 IU/mL. The specimen reaction should be compared to the ASO Negative Control.



Semi-quantitative Test:

A positive reaction is indicated by any observable agglutination in the reaction mixture. Record the last dilution showing a positive reaction. Concentration of ASO can be determined by multiplying the last positive dilution factor of the sample with the concentration of the positive control (200 IU/ml).

The titer of the serum is the reciprocal of the highest dilution, which exhibits a positive reaction.

IU/ml of sample = Conc. of positive control x reciprocal

DILUTION	RECIPROCAL	IU/ml
1/2	2	400
1/4	4	800
1/8	8	1600

EXPECTED VALUES

1. Normal values can vary with age, season of the year and geographical area, the "upper limit of normal" ASO titers for preschool children is less than 100 IU/ml and in school age children or young adults is usually between 166 and 250 IU/ml. In any case, the average can be established at less than 200 IU/ml.
2. Following acute streptococcal infection, the ASO titer will usually rise after one week, increasing to a maximum level within 3 to 5 weeks and usually returning to the pre-infection levels in approximately 6 to 12 months.

INFERENCE:

The ASO titer normal range is one below 200 in case of adults while an ASO test value below 100 is considered normal for kids below age 5. However, results may slightly vary from lab to lab and your doctor will consider this before suggesting aso titre treatment for any complications. The test may have to be repeated after two weeks in order to confirm the diagnosis since ASO antibodies are produced with a week of the infection. As a thumb rule, increasing antibodies indicates that the infection is recent while decreasing antibodies show that the infection is reducing.

Experiment 10. Lowenstein-Jensen (LJ) Medium

Lowenstein-Jensen (LJ) is the selective medium which is used for the cultivation and isolation of *Mycobacterium* species. It was developed by **Lowenstein** who incorporated congo red and malachite green to inhibit unwanted bacteria. The present formulation, **a glycerated egg-based medium**, is based upon **Jensen's modification**. Jensen's version eliminates congo red and uses a moderate concentration of malachite green to prevent growth of the majority of contaminants surviving decontamination of the specimen. This formulation also encourages the earliest possible growth of mycobacteria.

Composition of LJ Medium

Ingredients	Amount
Potato Flour (Potato Starch)	30.0 gm
L-Asparagine	3.6 gm
Monopotassium Phosphate	2.4 gm
Magnesium Citrate	0.6 gm
Malachite Green	0.4 gm
Magnesium Sulfate	0.24 gm
Glycerol	12 ml
Egg suspension	1000 ml
Distilled Water	600 ml

For cultivation of *M. bovis*, **glycerol** is omitted and **sodium pyruvate** is added.

Principle of LJ Medium

L-Asparagine and **Potato Flour** are sources of nitrogen and vitamins. **Monopotassium Phosphate** and **Magnesium Sulfate** enhance organism growth and act as buffers. **Malachite green,** prevent the growth of the majority of contaminants surviving decontamination of the specimen while encouraging the growth of Mycobacteria. **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.

Uses of LJ Medium

1. It is used for the diagnosis of Mycobacterial infections.
2. It is used for testing antibiotic susceptibility of isolates.
3. It is also used for differentiating different species of mycobacterium (by colony morphology, growth rate, biochemical characteristics and microscopy).

Preparation of LJ Medium

1. Dissolve 37.3 gm of the medium in 600 ml of distilled water containing 12 ml of glycerol.
2. Heat if necessary to dissolve the medium completely.
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.
7. Place the tubes in a slanted position and heat at 85°C for 45 minutes.

Colony Morphology on LJ Medium

Cultures should be read within 5– to 7 days after inoculation and once a week thereafter for up to 8 weeks.

Typical non-pigmented, rough, dry colonies are seen on LJ medium. The green color of the medium is due to the presence of malachite green which is one of the selective agents to prevent growth of most other contaminants.

Limitations of LJ Medium

1. It is recommended that biochemical and/or serological tests be performed on colonies from pure culture for complete identification.
2. LJ Media require incubation in a 5-10% CO2 atmosphere in order to recover mycobacteria. Mycobacteria, for unknown reasons, are not recovered well from candle extinction jars.
3. Negative culture results do not rule-out active infection by mycobacteria.
4. Due to nutritional variation, some strains may be encountered that grow poorly or fail to grow on this medium. Further tests are necessary for confirmation of *Mycobacterium* spp.

Experiment 11. Antibiotic sensitivity test

Antibiotic Sensitivity Assay (Kirby-Bauer Method)

Principle: The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum of activity, effective against only one group of microorganisms. Others exhibit broad-spectrum activity against a range of microorganisms. The drug susceptibilities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice.

A standardized diffusion procedure with filter-paper discs on agar, known as the Kirby-Bauer method, is frequently used to determine the drug susceptibility of microorganisms isolated from infectious processes. This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. In this

procedure, filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the organism to be tested. The medium of choice is Mueller-Hinton agar, with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5 mm. The plates are then heavily inoculated with a standardized inoculum by means of a spreader to ensure the confluent growth of the organism. The discs are aseptically applied to the surface of the agar plate at well-spaced intervals. Once applied, each disc is gently touched with a sterile applicator stick to ensure its firm contact with the agar surface. Following incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a drug is assessed by the size of this zone, which is affected by other variables such as the following:

1. The ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism.

2. The number of organisms inoculated.

3. The growth rate of the organism.

Requirements: Muller-Hinton Agar, Antibiotics disc, Forceps, Inoculating loop, Cultures, Spreader.

Procedure:

1. Label the bottom of each of the agar plates with the name of the test organism to be inoculated.

2. Using aseptic technique, inoculate all agar plate with the test organism and properly spread it over the plates.

3. Allow all culture plates to dry for about 5 minutes.

4. Using sterilized forceps the antibiotic discs were placed over the agar surface and pressing the disc slightly.

5. Examine all plate cultures for the presence or absence of a zone of inhibition surrounding each disc and carefully measure each zone of inhibition in millimetre scale.

Result and Observation: A measurement of the diameter of the zone of inhibition in millimeters is made, and its size is compared with that contained in a standardized chart. Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antibiotic.

Table 42.2 Zone Diameter Interpretive Standards for Organisms Other Than *Haemophilus* and *Neisseria gonorrhoeae*

		ZONE DIAMETER, NEAREST WHOLE MM		
ANTIMICROBIAL AGENT	DISC CONCENTRATION	RESISTANT	INTERMEDIATE	SUSCEPTIBLE
Ampicillin				
when testing gram-negative bacteria	10 μg	≤13	14–16	≥17
when testing gram-positive bacteria	10 μg	≤28	—	≥29
Carbenicillin				
when testing *Pseudomonas*	100 μg	≤13	14–16	≥17
when testing other gram-negative organisms	100 μg	≤19	20–22	≥23
Cefoxitin	30 μg	≤14	15–17	≥18
Cephalothin	30 μg	≤14	16–17	≥18
Chloramphenicol	30 μg	≤12	13–17	≥18
Clindamycin	2 μg	≤14	15–20	≥21
Erythromycin	15 μg	≤13	14–22	≥23
Gentamicin	10 μg	≤12	13–14	≥15
Kanamycin	30 μg	≤13	14–17	≥18
Methicillin when testing staphylococci	5 μg	≤9	10–13	≥14
Novobiocin	30 μg	≤17	18–21	≥22
Penicillin G				
when testing staphylococci	10 units	≤28	—	≥29
when testing other bacteria	10 units	≤14	—	≥15
Rifampin	5 μg	≤16	17–19	≥20
Streptomycin	10 μg	≤11	12–14	≥15
Tetracycline	30 μg	≤14	15–18	≥19
Tobramycin	10 μg	≤12	13–14	≥15
Trimethoprim/sulfamethoxazole	1.25/23.75 μg	≤10	11–15	≥16
Vancomycin				
when testing enterococci	30 μg	≤14	15–16	≥17
when testing *Staphylococcus* spp.	30 μg	—	—	≥15
Sulfonamides	250 or 300 μg	≤12	—	≥17
Trimethoprim	5 μg	≤10	—	≥16

Source: Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, Tenth Edition, 2008.

Experiment 12. Minimum Inhibitory Concentration Determination

Principle: Due to emergence of many antibiotic resistant strains of bacteria, antimicrobial susceptibility testing is done in order to determine which antimicrobial agent to use against a specific strain of bacteria. The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum while others have a wide spectrum of activities against bacteria. The bacterial strains isolated from clinical samples should be tested for antimicrobial sensitivity because it gives the clinician an idea as to what antimicrobial therapy should be started to the patients. The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of the test microorganism. Quantitative data of this nature may be used by a clinician to establish effective antimicrobial regimens for the treatment of a bacterial infection in a host. These data are of particular significance when the toxicity of the antibiotic is known to produce major adverse effects in host tissues. Agar dilution is a quantitative method for determining the minimum inhibitory concentration of the antibiotics against bacteria to be tested.

Requirements:

I. Reagents and lab wares: 0.5 McFarland standard, sterile Mueller Hinton agar (pH 7.2-7.4), sterile Mueller Hinton broth, antibiotic powder, sterile test tubes, pipettes, screw capped

flat bottomed bottles (25 ml capacity) and Petri dishes (90 mm diameter). These also include sterile saline (0.85 %) and stock solution of antibiotic.

II. Preparation of stock solutions of antibiotics: The required dilutions of the antibiotics are made as per the table 33-1. Prepare a stock solution containing 2000 µg / ml of the antibiotic to be tested. For example, weigh 200 mg of the antibiotic powder and dissolve in 5 ml of distilled water / appropriate solvent. Mix 0.5 ml of this solution with 9.5 ml distilled water (working solution contains antibiotics at a strength of 200 µg / ml-solution A)

III. Specimens: Preparation of suspension of bacteria: Approximately, 4-5 well isolated colonies of the bacterial strain to be tested are transferred to Tryptic soy broth or BHI broth. The turbidity of the suspension is adjusted to match 0.5 McFarland standards (106 organisms/ml).

Procedure:

Preparation of the agar plate with different concentration of the antibiotics

1. Dispense 2 ml of the diluted antibiotic solution into each of the marked sterile screw capped bottle.

Note: It is advisable to start with the highest dilution so that single pipette can be used to dispense all the dilutions prepared.

2. Sterile Muller-Hinton agar is cooled and maintained at 50°C – 55°C in a water bath.

3. Pour this medium (18 ml) into the screw capped bottle containing the different concentration of antibiotic, shake well and pour into sterile petri dish.

Note: By this method, exact volume of medium (22.6 ml) is delivered into the screw capped bottles without the danger of the molten agar jellifying during transfer into dilution of the antibiotic

4. Keep the poured plates at 4°C for setting.

5. After the plates have set, dry the plates well in an incubator at 37°C for 30-60 mins. The plates must be dry before performing the test.

Test procedure

1. A grid is marked on the bottom of the plates containing antibiotics.

Note: 20 – 25 strains can be tested in plate including the control.

2. A loopful of inoculating loop is calibrated to deliver 0.001 –0.002 ml (1-2 µl) of the culture.

3. Inoculate the culture on the surface of the medium, indicated by the square marked below. In each case 104 bacteria are delivered to a spot 5 – 8 mm in diameter.

Note: Inoculation is done starting with the plates containing highest dilution of the antibiotic.

4. Inoculate a control plate without antibiotics simultaneously as control.

Experiment 13. Bacteriological analysis of water for faecal coliforms, coliform count.

MPN Test

Principle: The three basic tests to detect coliform bacteria in water are presumptive, confirmed, and completed. The tests are performed sequentially on each sample under analysis. They detect the presence of coliform bacteria (indicators of fecal contamination), through the fermentation of lactose that will produce acid and gas that is detectable following a 24-hour incubation period at 37°C.

The Presumptive Test: The presumptive test is specific for detection of coliform bacteria. Measured aliquots of the water to be tested are added to a lactose fermentation broth

containing an inverted gas vial. Because these bacteria are capable of using lactose as a carbon source (the other enteric organisms are not), their detection is facilitated by the use of this medium. In this experiment, you will use lactose fermentation broth containing an inverted Durham tube for gas collection. Tubes of this lactose medium are inoculated with 10-ml, 1-ml, and 0.1-ml aliquots of the water sample. The series consists of at least three groups, each composed of five tubes of the specified medium. The tubes in each group are then inoculated with the designated volume of the water sample, as described under "Procedure: Lab One." The greater the number of tubes per group, the greater the sensitivity of the test. Development of gas in any of the tubes is presumptive evidence of the presence of coliform bacteria in the sample. The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the most probable number (MPN) test. The MPN is estimated by determining the number of tubes in each group that show gas following the incubation period.

The Confirmed Test: The presence of a positive or doubtful presumptive test immediately suggests that the water sample is nonpotable. Confirmation of these results is necessary because positive presumptive tests may be the result of organisms of noncoliform origin that are not recognized as indicators of fecal pollution. The confirmed test requires that selective and differential media (e.g., eosin–methylene blue (EMB) or Endo agar) be streaked from a positive lactose broth tube obtained from the presumptive test. The nature of the differential and selective media was discussed in Experiment 14 but is reviewed briefly here. Eosin–methylene blue contains the dye methylene blue, which inhibits the growth of gram-positive organisms. In the presence of an acid environment, EMB forms a complex that precipitates out onto the coliform colonies, producing dark centers and a green metallic sheen. The reaction is characteristic for *Escherichia coli*, the major indicator of fecal pollution. Endo agar is a nutrient medium containing the dye fuchsin, which is present in the decolorized state. In the presence of acid produced by the coliform bacteria, fuchsin forms a dark pink complex that turns the *E. coli* colonies and the surrounding medium pink.

The Completed Test: The completed test is the final analysis of the water sample. It is used to examine the coliform colonies that appeared on the EMB or Endo agar plates used in the confirmed test. An isolated colony is picked up from the confirmatory test plate, inoculated into a tube of lactose broth, and streaked on a nutrient agar slant to perform a Gram stain. Following inoculation and incubation, tubes showing acid and gas in the lactose broth and presence of gram-negative bacilli on microscopic examination is further confirmation of the presence of *E. coli*, and they are indicative of a positive completed test.

Materials: Lactose broth, MacConkey agar, nutrient agar, test tubes, Petridish, slide, crystal violet, safranin, gram's iodine, 95% ethanol.

Procedure:

Presumptive Test

1. Examine the tubes from your presumptive test after 24 and 48 hours of incubation. Your results are positive if the Durham tube fills 10% or more with gas in 24 hours, doubtful if gas develops in the tube after 48 hours, and negative if there is no gas in the tube after 48 hours.

2. Determine the MPN using standard table, and record your results in the Lab Report.

Confirmed Test

1. Using a positive 24-hour lactose broth culture from the sewage water series from the presumptive test, streak the surface of one EMB or one Endo agar plate, to obtain discrete colonies.

2. Incubate all plate cultures in an inverted position for 24 hours at 37°C.

3. Examine all the plates from your confirmed test for the presence or absence of *E. coli* colonies.

4. Based on your results, determine whether each of the samples is potable or nonpotable. The presence of *E. coli* is a positive confirmed test, indicating that the water is nonpotable. The absence of *E. coli* is a negative test, indicating that the water is not contaminated with fecal wastes and is therefore potable.

Completed Test

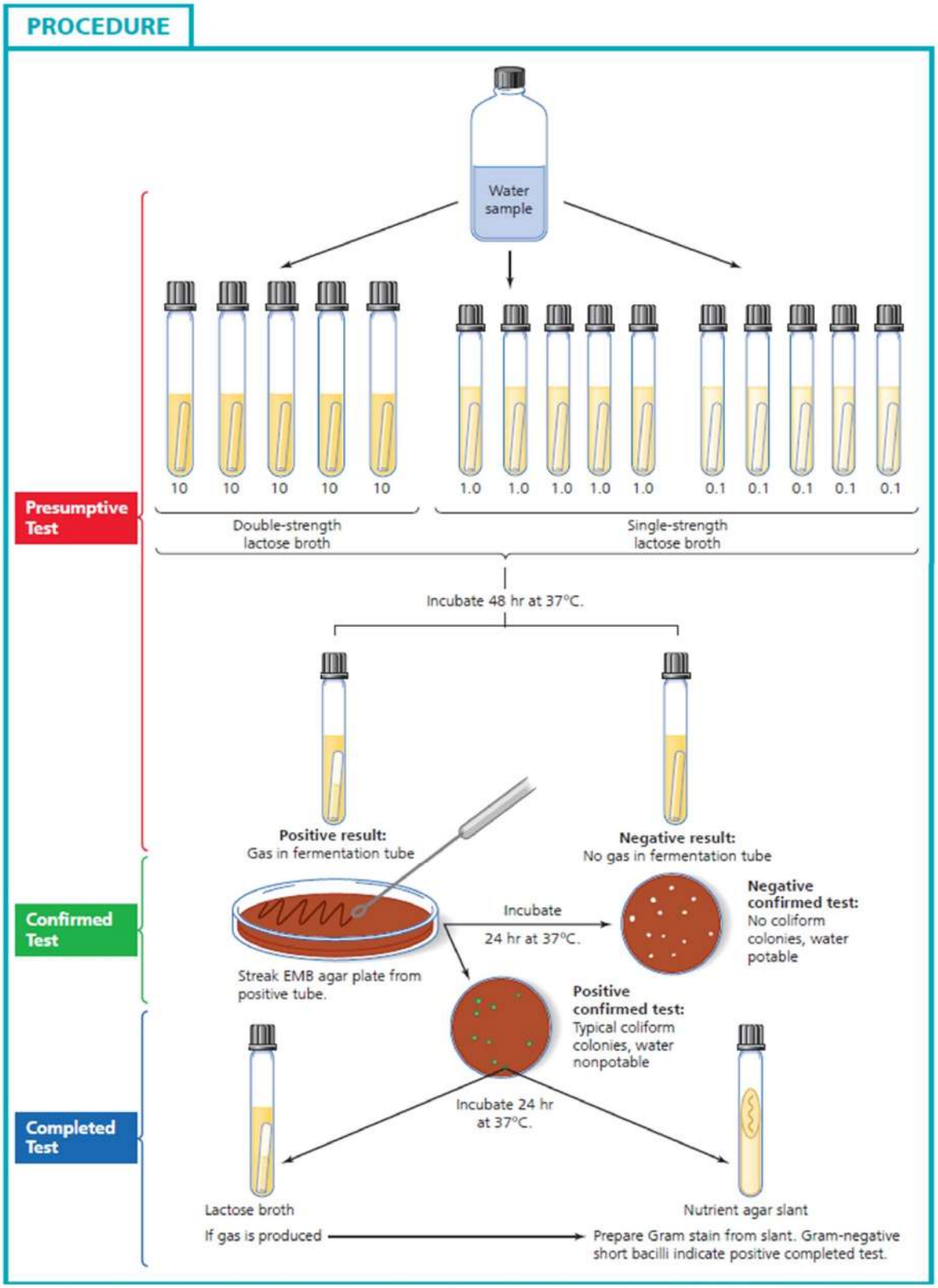
1. Label each tube of nutrient agar slants and lactose fermentation broths with the source of its water sample.

2. Inoculate one lactose broth and one nutrient agar slant with a positive isolated *E. coli* colony obtained from each of the experimental water samples during the confirmed test.

3. Incubate all tubes for 24 hours at 37°C.

4. Prepare a Gram stain, using the nutrient agar slant cultures of the organisms that showed a positive result in the lactose fermentation broth.

5. Examine the slides microscopically for the presence of gram-negative short bacilli, which are indicative of *E. coli* and thus nonpotable water.



Observation and Result: MPN index

NUMBER OF TUBES WITH POSITIVE RESULTS						NUMBER OF TUBES WITH POSITIVE RESULTS					
FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS		FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	95% CONFIDENCE LIMITS	
				LOWER	UPPER					LOWER	UPPER
0	0	0	<2	0	6	4	2	1	26	7	67
0	0	1	2	<0.5	7	4	3	0	27	9	78
0	1	0	2	<0.5	7	4	3	1	33	9	78
0	2	0	4	<0.5	11	4	4	0	34	11	93
1	0	0	2	0.1	10	5	0	0	23	7	70
1	0	1	4	0.7	10	5	0	1	31	11	89
1	1	0	4	0.7	12	5	0	2	43	14	100
1	1	1	6	1.8	15	5	1	0	33	10	100
1	2	0	6	1.8	15	5	1	1	46	14	120
2	0	0	5	<0.5	13	5	1	2	63	22	150
2	0	1	7	1	17	5	2	0	49	15	150
2	1	0	7	1	17	5	2	1	70	22	170
2	1	1	9	2	21	5	2	2	94	34	230
2	2	0	9	2	21	5	3	0	79	22	220
2	3	0	12	3	28	5	3	1	110	34	250
3	0	0	8	2	22	5	3	2	140	52	400
3	0	1	11	4	23	5	3	3	180	70	400
3	1	0	11	5	35	5	4	0	130	36	400
3	1	1	14	6	36	5	4	1	170	58	400
3	2	0	14	6	36	5	4	2	220	70	440
3	2	1	17	7	40	5	4	3	280	100	710
3	3	0	17	7	40	5	4	4	350	100	710
4	0	0	13	4	35	5	5	0	240	70	710
4	0	1	17	6	36	5	5	1	350	100	1100
4	1	0	17	6	40	5	5	2	540	150	1700
4	1	1	21	7	42	5	5	3	920	220	2600
4	1	2	26	10	70	5	5	4	1600	400	4600
4	2	0	22	7	50	5	5	5	≥2400	700	– – –

Experiment 14 Drawing of blood from animals

Collection of blood from small laboratory animals is necessary for a wide range of scientific research and there are a number of efficient methods available for that. It is important that blood sample collection from experimental animals should be least stressful because stress will affect the outcome of the study. Various regulatory agencies and guidelines have restricted the use of animals and the techniques used for blood collection in laboratory animals. This article deals with the approved blood collection techniques for laboratory animals like rodents, lagomorphs and nonrodents. Permission of the Institute

Animal Ethics Committee has been obtained for the use of animals for demonstrating the techniques.

Guidelines:

As a general rule, 1% of an animal's body weight (measured in grams) can be collected in blood (measured in milliliters) within a 24-hour period, every 14 days. For example, 0.3 ml can be collected once every two weeks from a 30-gram mouse. Alternatively, 0.05 ml of blood can be collected hourly for 6 consecutive hours from a 30-gram mouse, every two weeks (0.05 × 6 = 0.3 ml). Although blood volume is rapidly restored in an animal after blood collection, a two-week "rest period" is needed for blood constituents (e.g., red blood cells, platelets, clotting factors) to be regenerated by the body. If blood samples need to be collected once a week, it is recommended that not more than 0.5% of the animal's body weight in blood be removed within a 24-hour period. For example, 0.15 ml can be collected once a week from a 30-gram mouse. This volume can be further divided if blood samples need to be collected more frequently. For example, 0.03 ml can be collected once a day for five days from a 30-gram mouse, provided the mouse is given a one week (or greater) "rest period" before blood is collected again. The key to determining how much and how frequently blood can be drawn depends on the "rest period" between blood collections.

Typical venipuncture sites may include but are not limited to jugular, cephalic, lateral saphenous (dogs), femoral (cats, non-human primates), tail vein (rodents, cattle) and auricular (rabbit, swine) sites. Blood collection frequency must take into account maximum blood volumes collected with an intended rest period.

Table 1: Blood Collection Sites for small mammals

Location	Anesthesia	Frequency	Comments
Retro-orbital sinus	Yes, general anesthesia	Same eye, once every 2 weeks; can be performed on same eye if blood is collected within 30 minutes of the first sample	Good for large blood collection on a weekly (monthly) basis
Saphenous vein	No	Multiple	Good for multiple collection of small volumes
Dorsal pedal vein	No	Multiple	Good for single collection of a small volume in smaller species; good for multiple collection of small volumes in larger species
Tail vein/nick	No	Multiple	Good for multiple

			collection of small volumes
Tail clipping	No, for mice and rats ≤17 days of age Yes, for mice and rats >17 days of age; analgesia is also required	Multiple (small volumes) e.g.: glucose measurements	<1mm (mice), <2 mm (rat) distal tail the first time; for subsequent collections, only the scab/clot should be gently removed
Submandibular nick (Facial vein)	No	Multiple	Good for multiple small or single large blood collection volumes
Gingival vein	Yes, general anesthesia	Multiple	Good for multiple collection of small volumes
Cardiac puncture	Yes, general anesthesia, terminal procedure only (Cardiac puncture in snakes is not considered a terminal procedure. Sedation or anesthesia of the snake is required for this blood collection technique.)	Not applicable	Good for large, one-time collection

Table 2: Total Blood and Blood Sample Volumes

Species (weight)	Blood Volume (mL)	7.5% of blood volume (mL)	10% of blood volume (mL)	15% of blood volume (mL)
Mouse (25 g)	1.8	0.13	0.18	0.27
Rat (250 g)	16	1.2	1.6	2.4
Syrian Hamster (115 g)	8.4	0.6	0.8	1.2
Gerbil (75 g)	5	0.37	0.5	0.75
Guinea Pig (850 g)	64	4.8	6.4	9.6
Ferret (1 kg)	75	5.6	7.5	11
Rabbit (4 kg)	224	16.8	22	33.6
Cat (4 kg)	160	12	16	24
Dog (10 kg)	850	63.7	85	127.5
Macaque, Rhesus (5 kg)	280	21	28	42
Macaque, Cynomolgus (5 kg)	325	24.3	32.5	48.7
Marmoset (350 g)	25	1.8	2.5	3.7
Swine (150 kg)	9,750	731	975	1,462
Goat/Sheep (30 kg)	2,400	180	240	360
Horse (500 kg)	35,000	2,625	3,500	5,250
Passerine Bird, ex. Zebra Finches (15 g)	1.5 mL	0.11	0.15	0.22
Small Psittaccine Bird, ex. Cockatiels (100 g)	10 mL	0.75	1	1.5
Large Psittacine Bird, ex. Amazons (400 g)	40 mL	3	4	6
Recovery period for single sampling		1 week	2 weeks	4 weeks
Recovery period for multiple sampling		1 week	2 weeks	2 weeks

Blood Collection methods in Mice and Rats (unanesthetized):

Superficial temporal (a.k.a. "Submandibular" or "Facial" vein/artery) Sampling:

- Obtainable blood volumes: medium to large.
- Repeated sampling is possible by alternating sides of the face.
- Sample may be a mixture of venous and arterial blood.
- Requires less hands-on training than tail or retro-orbital sampling to reliably withdraw a reasonable quantity of blood.
- Perform on awake animals to achieve proper restraint, which in turn results in proper site alignment and venous compression for good blood flow.
- Can be performed rapidly and with a minimal amount of equipment, allowing for rapid completion.
- Sample volume can be partially controlled with the size of needle (20 gauge or smaller) or lancet (4 mm) used to puncture the site.
- Use of a lancet is recommended to control depth of puncture and reduce potential for complications. These can be significant if puncture is too deep or homeostasis is not assured prior to returning the mouse to its cage (5).

Saphenous Sampling (medial or lateral approach):

- Obtainable blood volumes: small to medium.
- Can be used in both rats and mice by piercing the saphenous vein with a needle or lancet.
- Variable sample quality.
- The procedure is customarily done on an awake animal but effective restraint is required.
- Requires more hands-on training than tail or retro-orbital sampling to reliably withdraw more than a minimal amount of blood.
- Although more aesthetically acceptable than retro-orbital sampling, prolonged restraint and site preparation time can result in increased animal distress when handling an awake animal.
- Temporary favoring of limb may be noted following the procedure.
- Application of sterile petroleum jelly to the site facilitates blood droplet formation, which can enhance the total blood volume captured.
- The clot/scab can be gently removed for repeated small samples if serial collection is required

Lateral Tail Vein:

- Obtainable blood volumes: small to medium.
- Can be used in both rats and mice by cannulating the blood vessel or by superficially nicking the vessel perpendicular to the tail.
- Sample collection by nicking the vessel is easily performed in both species, but produces a sample of variable quality that may be contaminated with tissue and skin products. Sample quality decreases with prolonged bleeding times and "milking" of the tail.

- Sample collection using a needle (cannulation) minimizes contamination of the sample, but is more difficult to perform in the mouse.
- Repeated collections possible. With tail nicking, the clot/scab can be gently removed for repeated small samples if serial testing is required (e.g., glucose measures, etc.)
- In most cases warming the tail with the aid of a heat lamp or warm compresses will increase obtainable blood volume.
- Cannulation and tail nicking are routinely done without anesthesia, although effective restraint is required.

Jugular Sampling (typically limited to the rat, but can be performed in mice as well (6):

- Obtainable blood volumes: medium to large.
- May be needed when a larger blood volume withdrawal and survival are needed.
- Results in high quality sample.
- Jugular sampling can be conducted without anesthesia in rats, although the use of anesthesia greatly facilitates the procedure and reduces the potential for injury to the animal as well as the individual (e.g., bites, needle stick).
- Does not easily lend itself to repeated serial sampling

ANESTHETIZED COLLECTION METHODS
Blood Collection methods in Mice and Rats (anesthetized):

The following methods require anesthesia (local and/or general depending on species and technique) to relieve pain and distress associated with the technique or for restraint. Use of these methods may require scientific justification for why less painful techniques cannot be used in the animal care and use protocol.

Retro-orbital Sinus/Plexus Sampling:

- Obtainable volume: medium to large.
- Retro-orbital sampling can be used in both mice and rats (though not a preferred method in the rat) by penetrating the retro-orbital sinus in mice or plexus in rats with a capillary tube or Pasteur pipette.
- Rapid – large number of animals can be bled within a short period of time.
- Good sample quality. Potential contamination with topical anesthetic, if used, should be taken into account.
- A minimum of 10 days should be allowed for tissue repair before repeat sampling from the same orbit. Otherwise the healing process may interfere with blood flow.
- Alternating orbits should not be attempted until the phlebotomist is proficient in obtaining samples from the orbit accessed most readily by the dominant hand ie a right handed individual should gain proficiency withdrawing samples from the right orbit before attempting to obtain samples from the left orbit.
- In the hands of an unskilled phlebotomist, retro-orbital sampling has a greater potential than other blood collection routes to result in complications.
- General anesthesia must be used unless scientific justification is provided and approved by the IACUC. In addition, a topical ophthalmic anesthetic, e.g. proparacaine or tetracaine drops, is recommended prior to the procedure.
- In both mice and rats, care must be taken to ensure adequate hemostasis following the procedure.

- Use of sterile capillary tubes and pipettes are recommended for use to help avoid periorbital infection and potential long-term damage to the eye. The edges of the tubes should be checked for smoothness to also decrease likeliness of eye damage.
- Animals must be monitored following collection for damage to the orbit or globe of the eye. If damage is noted, veterinary staff should be contacted for treatment options up to and including euthanasia.

Tail Clip Sampling:

- Obtainable volume: small.
- Can be used in both rats and mice by clipping (e.g. amputating) no more than 1mm of the distal tail in mice or 2 mm in rats
- Produces a sample of variable quality that may be contaminated with tissue and skin products. Sample quality decreases with prolonged bleeding times and "milking" of the tail.
- Obtainable volume: small.
- Repeated collections possible. The clot/scab can be gently removed for repeated small samples if serial testing is required (e.g., glucose measures, etc.)
- In most cases warming the tail with the aid of a heat lamp or warm compresses will increase obtainable blood volume.
- When performing tail clipping, consideration should be given to anesthesia/analgesia, particularly if the tail has been previously clipped for genotyping. If a topical hypothermic anesthetic is used, blood will flow as the tail re-warms. If a local anesthetic is applied, adequate contact time should be allowed for it to take effect.

ANESTHETIZED TERMINAL
Blood Collection methods in Mice and Rats (anesthetized and terminal):

Cardiac Puncture:

- Obtainable Volume: medium – large.
- Cardiac puncture is done only as a terminal procedure and always under anesthesia as evidenced by lack of response to a painful stimulus (e.g., toe pinch)
- The use of a 1cc syringe with a 25 gauge needle is recommended. Find the xiphoid process as a reference point. Insert the needle at a 35-40 degree angle just under and to the left of the xiphoid process. As the needle is inserted into the chest, gently aspirate until blood begins to flow.
- Ensure death of the animal after collection. This determination may be made by auscultation for cessation of both heartbeat and respiration by a qualified individual in larger animals or by utilizing an unequivocal secondary means of ensuring death (decapitation, opening thoracic cavity, etc.) following euthanasia with an inhalant agent (anesthetic overdose or CO2)

Over all Monitoring

If the animal is being bled routinely, the red blood cell packed volume (PCV) should be checked weekly to determine when blood collection should be suspended in order for the animal to recover from potential anemia. While healthy adult animals can recover their blood volume within 24 hours, it may take up to 2 weeks for all the other blood constituents (i.e. cells, proteins) to be replaced.

By monitoring the hematocrit (Hct or packed cell volume- PCV) and/or hemoglobin of the animal, it is possible to evaluate whether the animal has sufficiently recovered from a single or multiple blood draws. After a sudden or acute blood loss, it takes up to 24 hours for the hematocrit and hemoglobin to reflect this loss. In general, if the animal's hematocrit is less than 35% or the hemoglobin concentration is less than 10 g/dl, it is not safe to remove blood. Please contact a DLAR Veterinarian if you need assistance with monitoring PCVs in animals.

Fluid replacement

Lactated Ringer's Solution (LRS) is the recommended balanced crystalloid solution for fluid replacement. Alternatively, 0.9% sterile isotonic saline may be used. For adult mice, 1.0 ml of warmed LRS or isotonic saline can be given by IP or SC administration. For adult rats, administer 5 -10 ml warmed LRS or 0.9% saline (½ of the total volume via IP and ½ via SC routes).

Paper-303

SGPT (ALAT) KIT

(Modified IFCC Method)

Summary:

SGPT is found in a variety of tissues but it is mainly found in liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

Principle:

SGPT (ALAT) catalyzes the transfer of amino group between L Alanine and α-Ketoglutarate to form Pyruvate and Glutamate. The Pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGPT (ALT) activity in the sample.

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

Expected values:

Serum (Males): upto 40 U/L at 37°C

(Females): upto 31U/L at 37°c

Procedure:

Wavelength/filter: 340 nm

Temperature: 37°C/30°C/25°C R.T.

Light path: 1 cm

Substrate Start Assay:

Pipette into a clean dry test tube labelled as Test (T):

Addition Sequence	**(T) 25°C/30°C**	**(T) 37°C**
Enzyme Reagent (L 1)	0.8 ml	0.8 ml
Sample	0.2ml	0.2ml

Incubate at the assay temperature for 1 min. and add

Starter Reagent (L2)	0.2 ml	0.2 ml

Mix well and read the initial absorbance A, after 1 min. and repeat the absorbance reading after every 1, 2, & 3 mins. Calculale the mean absorbance change per min. (ΔA /min).

Calculations

Substrate /Sample start

SGPT (ALAT) Activity in U/L 25°C/30°C = ΔA/min x 952

SGPT (ALAT) Activity in U/L 37°C = ΔA/min. x 1746

SGOT (ASAT) KIT

((Modified IFCC Method)

Summary:

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injuny to these tissues results in the release of the enzyme in blood. Elevated levels are found in myocardial infarction, Cardiac operations, Hepatitis, Cirrhosis, acute pancreatitis, acute renal diseases, primary muscle diseases. Decreased levels may be found in Pregnancy, Beri Beri and Diabetic ketoacidosis.

Principle:

SGOT (ASAT) catalyzes the transfer of amino group between L-Aspartate and α-Ketoglutarate to form Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with NADH in the presence of Malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGOT (ASAT) activity in the sample.

$$\text{L-Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+$$

Expected values:

Serum (males): upto 37 U/L at 37°C

(Females): upto 31 U/L at 37°C

Procedure:

Wavelength/filter: 340 nm

Temperature: 37°C/30°C/25°C R.T.

Light path: 1 cm

Substrate Start Assay

Serum Pipette into a clean dry test tube labelled as Test (T)

Addition Sequence	(T) 25°C/30°C	(T) 37°C
Enzyme Reagent (L1)	0.8 ml	0.8 ml
Sample	0.2 ml	0.1ml
Incubate at the assay temperature for 1 min. and add		
Starter Reagent (L2)	0.2 ml	0.2 ml

Mix well and read the initial absorbance A, after 1min. & repeat the absorbance reading after every 1, 2, & 3 mins. Calculate the mean absorbance change per min. (ΔA /min.).

Sample Start Assay:

Pipette into a clean dry test tube labelled as Test (T)

Addition Sequence	**(T) 25°C/30°C**	**(T) 37°C**
Working Reagent	1.0 ml	1.0 ml

Incubate at the assay temperature for 1 min and add

Sample	0.2 ml	0.1 ml

Mix well and read the initial absorbance A, after 1 min. & repeat the absorbance reading after every 1, 2, & 3 mins. Calculate the mean absorbance change per min. (ΔA/min.)

Calculations:

Substrate/Sample start

SGOT (ASAT) Activity in U/L 25C/30°C = ΔA /min. x 952

37C °C = ΔA /min. x 1746

ALPHA AMYLASE (SINGLE REAGENT)

Introduction

α-Amylase is derived mainly from the salivary glands and the exocrine pancreas. α-Amylase catalyses the hydrolysis of α-1-4 glucosidic linkages of starch and other related polysaccharides to produce maltose and other oligosaccharides. The enzyme is a relatively small molecule which is rapidly cleared by the kidneys and excreted in the urine.

Principle:

2-Chloro-4-nitrophenol-β -1- 4 galactopyranosylmaltotrioside (CNP-G) is a direct substrate for determination of α-amylase activity, which does not require the presence of ancillary enzymes. The rate of 2-chloro-4-nitrophenol formation can be monitored at (400-420) nm and is proportional to the α-amylase activity.

Gal – G2 - α-CNP → Gal – G2 + CNP

Expected values at 37°C

Serum: up to 80 U/l

Urine: up to 500 U/l

Assay procedure

Wavelength: 405 (400 – 420) nm

Cuvette: 1cm

Working solution	1000 µl
Sample	20 µl

Mix, incubate 1 min. at 37°C and then measure the initial absorbance of calibrator and sample against reagent blank. Measure the absorbance change exactly after 1, 2 and 3 min. Calculate 1 minute absorbance change (ΔA/min).

CALCULATION:

1. Amylase activity (U/l) = $\frac{\Delta A sam/min}{\Delta A cal/min} \times$ Concentration of Cal

Ccal = calibrator concentration

2. Using factor: Amylase activity (U/l) = f x ΔA/min

f = factor, f = 3128 (at 405 nm)

Clinical significance:

α-Amylase is derived mainly from the salivary glands and the exocrine pancreas. α-Amylase catalyses the hydrolysis of α-1-4 glucosidic linkages of starch and other related polysaccharides to produce maltose and other oligosaccharides. The enzyme is a relatively small molecule which is rapidly cleared by the kidneys and excreted in the urine.

α-Amylase is most frequently measured in the diagnosis of acute pancreatitis when serum levels may be grossly elevated. In acute pancreatitis α-amylase starts to rise approximately 4 hours after the onset of pain, reaches a peak at 24 hours and remains elevated for 3-7 days. Hyperamylasamia is also associated with other acute abdominal disorders, biliary dysfunction, salivary gland disorders, ruptured ectopic pregnancy and macroamylasamia.

CREATINE KINASE

Quantitative determination of creatine kinase (CK)

Introduction

Creatine kinase is a cellular enzyme with wide tissue distribution in the body. Its physiological role is associated with adenosine triphosphate (ATP) generation for contractile or transport systems.

Principle of the method

Creatine kinase (CK) catalyses the reversible transfer of a phosphate group from phosphocreatine to ADP. This reaction is coupled to those catalysed by hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH).

$$\text{Phosphocreatine} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-phosphate}$$

$$\text{G6P} + \text{NADP} \xrightarrow{\text{G6P-DH+}} \text{Phosphogluconate} + \text{NADPH} + \text{H}+$$

The rate of NADPH formation, measured photometrically, is proportional to the catalytic concentration of CK present in the sample.

Procedure

Assay conditions:

Wavelength: 340 nm

Cuvette: 1 cm light path

Constant temperature: 25°C / 30°C / 37°C

	25 - 30°C	**37°C**
WR (ml)	1.0	1.0
Sample (µL)	40	20

i) Mix, incubate for 2 minutes.

ii) Read initial absorbance (A) of the sample, start the stopwatch and read absorbances at 1 minute intervals thereafter for 3 minutes.

iii) Calculate the difference between absorbances and the average absorbance differences per minute (ΔA/min).

Calculations:

25°- 30°C ΔA / min x 4127 = U/L CK

37°C ΔA / min x 8095 = U/L CK

Reference values:

Men, up to

25°C	**30°C**	**37°C**
80 U/L	130 U/L	195 U/L

Women, up to

70 U/L	110 U/L	170 U/L

Clinical Significance:

Elevated CK values are observed in diseases of skeletal muscle and after myocardial infarction.

LDH

Kinetic UV method

Principle

Kinetic determination of the lactate dehydrogenase according to the following reaction.

Pyruvate + NADH + H+ → L-Lactate + NAD+

Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to lactate with simultaneous oxidation of NADH to NAD+. The rate of NADH oxidation can be measured as a decrease in absorbance at 340nm.

This rate is directly proportional to LDH activity in serum.

Procedure

Wavelength	Hg 340 nm (334 - 365 nm)
Spectrophotometer	340 nm
Cuvette	1 cm light path
Temperature	37°C
Reaction	Kinetic

Assay: Incubate Working Reagent at 37 °C before use.

Sample	20 µl
Working Reagent	1000 µl

Mix, incubate for 30 sec. at 37 °C, then read change in the absorbance per 1 min. for 3 min. Determine the mean absorbance change per 1 min (ΔA/min).

Calculation:

LDH activity (U/L) = (ΔA /min.) X Factor

F = Conc. Calibrator/ Δ/min Calibrator

Factor

Wavelength	334 nm	340 nm	365 nm
Factors at 37°C	8252	8095	15000

Reference range

1 d.		< 1327	U/L
2 – 5 d.		< 1732	U/L
6 d. – 6 mth.		< 975	U/L
7 – 12 mth.		< 1100	U/L
1 – 3 yr.		< 850	U/L
4 – 6 yr.		< 615	U/L
7 – 12 yr.	women	< 580	U/L
	men	< 764	U/L
13 – 17 yr.	women	< 436	U/L
	men	< 683	U/L
Adults		< 480	U/L

LIPASE

(Methyl Resorufin)

Introduction: Lipases are glycoproteins with a molecular weight of 47000 Daltons. Lipases hydrolyzes the ester linkages. Specifically, lipase catalyzes the partial hydrolysis of dietary triglycerides in the intestine to the 2-monoglyceride intermediate, with the production of long chain fatty acids.

Principle:

Enzymatic color test.

Colorimetric substrate 1, 2-o-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin)-ester is cleaved by pancreatic lipase and the resulting dicarboxilic acid ester is hydrolysed under the alkaline test conditions to yield the chromophore methylresorufin. The kinetic of colour formation at 580 nm is monitored and it is proportional to lipase activity in sample.

Assay Procedure:

	Calibrator	**Sample**
Reagent 1	1000 µL	1000 µL
Calibrator	10 µL	-
Sample	-	10 µL

Mix and after 60 seconds incubation, measure the change in absorbance for 60 seconds at 37°C.

Determine the ΔAbs,

Calculation:

Lipase activity (U/I) = **(**ΔAbs. of sample/ΔAbs. of calibrator) x Concentration of calibrator

Reference normal value:

Serum lipase activity: 13-60 U/l

Clinical Significance:

Determination of lipase is used for investigation of pancreatic disorders. In acute pancreatitis the lipase concentrations rise to 2-50 fold to upper reference limit within 4-8 hours after begin of abdominal pain peaking at 24 hours and decreasing within 8 to 14 days. Elevated lipase values can also be observed in chronic pancreatitis and obstruction of the pancreatic duct.

T3 ELISA

Microwell ELISA Immunoassay for the Quantitative detection of tridothyronin (t3) in Human Serum/Plasma.

Introduction

Triiodothyronin (T3) is a thyroid hormone with molecular weight of 651 dalton. T3 affects almost every physiological function of body including growth, development, metabolism, body temperature and heart rate. T3 circulate in blood as an equilibrium mixture of iron and protein bound hormone in blood. Binding of T3 is detected by Enzyme conjugate. Incubation is followed by a washing step to remove unbound components. The colour reaction is started by addition of substrate and stopped after a defined time. The colour intensity is inversely proportional to the concentration of T3 in the sample.

Principle

T3 Quanti Microlisa is an enzyme immuno assay based on competitive ELISA. Microwells a Coated with anti-triiodothyronin antibodies. Sample is added to the microwell followed by additon of enzyme conjugate (T3 labelled with HRPO). Binding of T3 is detected by Enzyme Conjugate. Incubation is followed by a washing step to remove unbound components. Color reaction is started by addition of substrate and stopped after a defined time.

Summary of procedure

Prepare working enzyme conjugate	
Add standards and samples	50 µl
Add Enzyme conjugate	100 µl
Cover the plate & incubate	60 mins. at 37°C
Wash	3 Cycles
Prepare TMB Substrate	
Add Substrate	100 µl
Incubate in dark	15 mins. at room temp.
Add stop solution	50 µl
Read results	In ELISA Reader at 450 nm and 630 nm

Calculations:

i) Calculate the mean absorbance values for each set of standards and samples.

ii) Construct a best fit curve by plotting the absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (x) axis.

iii) Using the absorbance value for each sample determine the corresponding concentration from the best fit curve.

iv) Automated Method: The results have been calculated automatically using curve regression mode which is the preferred method. Other data reduction functions may give slightly different results.

v) The triiodothyronine in human concentration of the sample can be read directly from the best fit curve. Sample with concentrations higher than that of the highest standard have to be

further diluted ((1:0.5 or 1:1 with Standard-1) or reported as >7.5 ng/ml. For the calculation of the concentration, this dilution factor has to be taken into account.

vi) For subsequent run, once master curve has been established in an ELISA reader. Calculate the results with stored master curve and absorbance of 3 standard with necessary data analytics.

Expected values:

Distribution of normal Values ranges from 0 52 to 1.85 ng/ml.

T4 ELISA

Introduction

Thyroxine (T4) is one of the main thyrold hormones, and has a molecular weight of 777 Da. T4 enable to estimulate synthesis and energy metabolism, to increase basal metabolic rate and oxygen consumption, and to stimulate growth and development. In blood, 99.97% of T4 binds to thyroxine binding globlin (TBG) and thyroxine binding prealbumin (TBPA). 14'S synthesis and decomposition are controlled by hypothalamus and pituitary. T4 is one of the indicators of diagnostics and efficacy evaluation of hyperthyroidism and hypothyroidism.

Principle:

T4 Quanti Microlisa is an enzyme immune assay based on competitive ELISA. Microwells are coated with anti-thyroxine antibodies. Sample is added to the microwell followed by addition of enzyme conjugate (74 labelled with HRPO). Binding of T4 is detected by Enzyme Conjugate. Incubation is followed by a washing step to remove unbound components. The color reaction is started by addition of substrate and stopped after a defined time. The color intensity is inversely proportional to the concentration of T4 in the sample.

Test Procedure:

i) Fit the stripholder with the required number of T4 Quanti Microlisa coated microwell strips.

ii) Prepare working enzyme conjugate as specified in preparation of reagents.

iii) Add 25 µl of each standards and samples in respective wells. Use a separate tip for each sample and then discard as biohazardous waste.

iv) Add 100 ul of woking enzyme conjugate to each well.

v) Gently mix the microplate for 20-30 seconds and cover.

vi) Cover the plate and incubate in an incubator at 37°C ± 1°C for 60 minutes.

vii) Dilute the wash buffer concentrate with distilled water to 1:25 dilution.

viii) Wash with working wash buffer.

.

Washing: Washing can be performed either with washer or manually as follows:

i) Empty the wells.

ii) Add 300-350 ul of working washing solution into each well.

iii) Empty the wells.

iv) Wash each well 3 times in total.

v) After the third wash, tap dry the Microwells a few times on an absorbent tissue.

vi) Add 100 ul working substrate solution in each well.

vii) Incubate at room temperature (20-30°C) in dark for 15 mins. and do not expose to light.

viii) Add 50 ul of stop solution to each well.

ix) Read the absorbance at 450 & 630 nm within 15 minutes in ELISA reader.

Expected values:

Distribution of normal values ranges from 4.4 to 10.8 ug/dl in Male and from 4.8 to 11.6 ug/dl in Female.

Calculation of results:

i) Calculate the mean absorbance values for each set of standards and samples.

ii) Construct a best fit curve by plotting the absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

iii) Using the absorbance value for each sample determine the corresponding concentration from the best fit curve.

iv) Automated Method: The results have been calculated automaticaly using 4 PL (4 parameter logistics) curve fit which is the preferred method. Other data reduction functions may give slightly different results.

v) The concentration of the sample can be read directly from the best fit curve. Sample with concentrations higher than that of the highest standard have to be futher diluted (1:0.5 or 1:1 with Standard-1) or reported as > 25 to ug/dl. For the calculation of the concentration, this dilution factor has to be taken into account.

vi) For subsequent run, once master curve has been established in a Reader, Calculate the results with stored master curve and absorbance of 3 standards with necessary data analytics.

LH (HUMAN) ELISA KIT

Introduction

Luteinizing hormone (LH) is produced in both men and women from the anterior pituitary gland in response to luteinizing hormone-releasing hormone (LH-RH or Gn-RH), which is released by the hypothalamus. LH, also called interstitial cell-stimulating hormone (ICSH) in men, is a glycoprotein with a molecular weight of approximately 30,000 daltons. It is composed of two non-covalently associated dissimilar amino acid chains, alpha and beta. The alpha chain is similar to that found in human thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG).

The differences between these hormones lie in the amino acid composition of their beta subunits, which account for their immunological differentiation. The basal secretion of LH in men is episodic and has the primary function of stimulating the interstitial cells (Leydig cells) to produce testosterone. The variation in LH concentrations in women is subject to the complex ovulatory cycle of healthy menstruating women, and depends upon a sequence of hormonal events along the gonado-hypothalamic-pituitary axis.

Principle of the Assay

The LH ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA). The assay system utilizes mouse monoclonal anti-α-LH for solid phase (microtiter wells) immobilization, and a mouse monoclonal anti-β-LH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45 minute incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. A

solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of stop solution, and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of LH is directly proportional to the color intensity of the test sample.

Assay Procedure:

1. Secure the desired number of coated wells in the holder.
2. Dispense 50 µl of standards, specimens, and controls into appropriate wells.
3. Dispense 100 µl of Enzyme Conjugate Reagent into each well.
4. Gently mix for 30 seconds. It is very important to have complete mixing in this setup.
5. Incubate at room temperature (18-25°C) for 45 minutes.
6. Remove the incubation mixture by flicking plate contents into sink.
7. Rinse and flick the microtiter wells 5 times with distilled or dionized water. (Please do not use tap water.)
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 100µl of TMB Reagent into each well. Gently mix for 10 seconds.
10. Incubate at room temperature, in the dark, for 20 minutes.
11. Stop the reaction by adding 100µl of Stop Solution to each well.
12. Gently mix for 30 seconds. It is important to make sure that all of the blue color changes completely to yellow color completely.
13. Read optical density at 450nm with a microtiter plate reader within 15 minutes.

Calculations:

- Calculate the average absorbance value (A450) for each set of reference standards, controls and samples.
- Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in mIU/ml on linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of LH in mlU/ml from the standard curve.

Expected Values:

Each laboratory should establish its own normal range based on patient population.

Adult	mIU/ml
Male	1.24-7.8
Female	
Follicular phase: Ovulatory peak: Luteal phase: Postmenopausal:	1.68-15 21.9-56.6 0.61-16.3 14.2-52.3

FSH (HUMAN) ELISA KIT

Introduction

Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are intimately involved in the control of the growth and reproductive activities of the gonadal tissues, which synthesize and secrete male and female sex hormones. The levels of circulating FSH and LH are controlled by these sex hormones through a negative feedback relationship.

FSH is a glycoprotein secreted by the basophilic cells of the anterior pituitary. Gonadotropin-releasing hormone (GnRH), produced in the hypothalamus, controls the release of FSH from the anterior pituitary. Like other glycoproteins, such as LH, TSH, and HCG, FSH consists of subunits designated as alpha and beta. Hormones of this type have alpha subunits that are very similar structurally; therefore the biological and immunological properties are dependent on the unique beta subunits.

In the female, FSH stimulates the growth and maturation of ovarian follicles by acting directly on the receptors located on the granulosa cells; follicular steroidogenesis is promoted and LH production is stimulated. The LH produced then binds to the theca cells and stimulates steroidogenesis. Increased intraovarian estradiol production occurs as follicular maturation advances, thereupon stimulating increased FSH receptor activity and FSH follicular binding. FSH, LH, and estradiol are therefore intimately related in supporting ovarian recruitment and maturation in women.

Principle of the Assay

The Abnova FSH EIA Test is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay system utilizes a mouse monoclonal anti-α-FSH for solid phase (microtiter wells) immobilization, and mouse monoclonal anti-β –FSH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the FSH molecules

being sandwiched between the solid phase and enzyme-linked antibodies. After a 45-minute incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. A solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop solution, and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of FSH is directly proportional to the color intensity of the test sample.

Assay Procedure

1. Secure the desired number of coated wells in the holder.
2. Dispense 50µL of standards, specimens, and controls into appropriate wells.
3. Dispense 100µL of Enzyme Conjugate Reagent into each well.
4. Thoroughly mix for 30 seconds. It is very important to have a complete mixing in this setup.
5. Incubate at room temperature (18-25°C) for 45 minutes.
6. Remove the incubation mixture by flicking plate contents into a waste container.
7. Rinse and flick the microtiter wells 5 times with distilled or dionized water. (Please do not use tap water.)
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 100µl of TMB Reagent into each well. Gently mix for 10 seconds.
10. Incubate at room temperature, in the dark, for 20 minutes.
11. Stop the reaction by adding 100µl of Stop Solution to each well.
12. Gently mix for 30 seconds. It is important to make sure that all of the blue color changes to yellow color completely.
13. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

Calculation of Results

- Calculate the mean absorbance value (A450) for each set of reference standards, specimens, controls and patient samples.
- Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in mIU/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of FSH in mlU/ml from the standard curve.

Normal Range:
During puberty: 0.3 to 10.0 mIU/mL
Adult: 1.5 to 12.4 mIU/mL

INSULIN ELISA

Summary and explanation

Insulin is the principal hormone responsible for the control of glucose metabolism. It is synthesized in the ß-cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain and B chain (21 and 30 amino acids respectively). The two chains are linked together by two inter-chain disulphide bridges. There is also an intra-chain disulphide bridge in the A chain. Insulin concentrations are severely reduced in insulin-dependent diabetes mellitus (IDDM) and some other conditions such as hypopituitarism. Insulin levels are raised in non-insulin-dependent diabetes mellitus (NIDDM), obesity, insulinoma and some endocrine dysfunctions such as Cushing's syndrome and acromegaly.

Principle of the test

The Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with enzyme (HRP)-conjugated anti-insulin antibody and anti-insulin antibody bound to micro-titration well. A simple washing step removes unbound enzyme labeled antibody. The bound HRP complex is detected by reaction with TMB substrate. The reaction is stopped by adding acid to give a colorimetric endpoint that is read using ELISA reader.

Assay procedure

1. Place the desired number of coated strips into the holder
2. Pipette 25 µl of Insulin standards, control and patient's sera into appropriate wells.
3. Add 100µl of working Insulin Enzyme Conjugate to all wells.
4. Thoroughly mix for 10 sec., it is important to have a complete mixing in this step.
5. Incubate for 60 minutes at room temperature (18-26° C).
6. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbent paper towels.
7. Add 100 µl of TMB substrate to all wells.
8. Incubate for 15 minutes at room temperature.
9. Add 50µl of stop solution to all wells. Shake the plate gently to mix the solution.
10. Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculations:

The standard curve is constructed as follows:

1. Check Insulin standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit.

2. To construct the standard curve, plot the absorbance for the insulin standards (vertical axis) versus the insulin standard concentrations in µIU/ml (horizontal axis) on a linear graph paper. Draw the best curve through the points.
3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.
4. Value above the highest point of the standard are retested after diluting with "0" standard.

Normal range: 5 – 25 µIU/mL

GCG (HUMAN) CELL-BASED ELISA KIT

Introduction

The GCG (Human) Cell-Based ELISA Kit is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor Glucagon protein expression profile in cells. The kit can be used for measuring the relative amounts of Glucagon in cultured cells as well as screening for the effects that various treatments, inhibitors (ie. siRNA or chemicals), or activators have on Glucagon.

Principle of the Assay

The Colorimetric Cell-Based ELISA Kit allows for the detection of various target proteins and the effects that certain stimulation conditions have on target protein expression in different cell lines. Qualitative determination of target protein concentration is achieved by an indirect ELISA format. In essence, the target protein is captured by target-specific primary (1°) antibodies while the HRP-conjugated secondary (2°) antibodies bind the Fc region of the 1° antibody. Through this binding, the HRP enzyme conjugated to the 2° antibody can catalyze a colorimetric reaction upon substrate addition. Due to the qualitative nature of the Cell-Based ELISA, multiple normalization methods are described: 1) a monoclonal antibody specific for human GAPDH is included to serve as an internal positive control in normalizing the target absorbance values. 2) Following the colorimetric measurement of HRP activity via substrate addition, the crystal violet whole-cell staining method is used to determine cell density. After staining, the results can be analyzed by normalizing the absorbance values to cell amounts, by which the plating difference can be adjusted. 3) If a phosphorylated target is being detected, an antibody against the non- phosphorylated counterpart will be provided for normalization purposes. The absorbance values obtained for the non-phosphorylated target can be used to normalize the absorbance values for the phosphorylated target.

Assay Procedure

1. Seed 200 µl of 20,000 adherent cells in culture medium in each well of a 96-well plate. The plates included in the kit are sterile and treated for cell culture. For suspension cells and loosely attached cells, coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine (not included) to each well of a 96-well plate for 30 minutes at 37°C prior to adding cells.

2. Incubate the cells for overnight at 37°C, 5% CO2.

3. Treat the cells as desired.

4. Remove the cell culture medium and rinse with 200 µl of 1x PBS, twice.

5. Fix the cells by incubating with 100 µl of Fixing Solution for 20 minutes at room temperature. The 4% formaldehyde is used for adherent cells and 8% formaldehyde is used for suspension cells and loosely attached cells. During the incubation, the plates should be sealed with Parafilm. Note: Fixing Solution is volatile. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

6. Remove the Fixing Solution and wash the plate 3 times with 200 µl 1x Wash Buffer for five minutes each time with gentle shaking on the orbital shaker. The plate can be stored at 4°C for a week. Note: For all wash steps, tap the plate gently on absorbent papers to remove the solution completely.

7. Add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.

8. Wash the plate 3 times with 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

9. Add 200 µl of Blocking Buffer and incubate for 1 hour at room temperature.

10. Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

11. Add 50 µl of 1x primary antibodies (Anti-Glucagon Antibody and/or Anti-GAPDH Antibody) to the corresponding wells, cover with parafilm and incubate for 16 hours (overnight) at 4°C. If the target expression is known to be high, incubate for 2 hours at room temperature with gentle shaking on the shaker.

12. Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

13. Add 50 µl of 1x secondary antibodies (HRP-Conjugated Anti-Rabbit IgG and/or HRP-Conjugated Anti-Mouse IgG) to corresponding wells and incubate for 1.5 hours at room temperature with gentle shaking on the shaker. Note: Add HRP-Conjugated Anti-Rabbit IgG to the wells incubated with AntiGlucagon (rabbit, polyclonal) and add HRP-Conjugated Anti-Mouse IgG to the wells incubated with Anti-GAPDH Antibody (mouse, monoclonal).

14. Wash 3 times with 200 µl of 1x Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker.

15. Add 50 µl of TMB One-Step Solution to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking on the shaker. Note: TMB is a light-sensitive reagent. Keep away from light.

16. Add 50 µl of Stop Solution to each well and read OD at 450 nm immediately using the microplate reader.

Calculations:

GAPDH Normalization

The OD_{450} values obtained for the target protein can be normalized using the OD_{450} values obtained for GAPDH.

Normal range: 50 to 100 pg/mL

TOTAL ESTROGENS ELISA

Principle:

The total estrogens ELISA is a competitive immunoassay. Competition occurs between total estrogens (estrone, estradiol, and estriol) present in standards, controls and patient samples and an enzyme-labelled antigen (conjugate) for a limiting number of anti-estrogen antibody binding sites on the microplate wells. After a washing step that removes unbound materials the enzyme substrate is added and approximately 15–20 minutes later the enzymatic reaction is terminated by addition of stopping solution. The resulting optical density (OD), measured with a microplate reader, is inversely proportional to the concentration of total estrogens in the sample. A standard curve is plotted with a provided set of standards to calculate directly the concentration of total estrogens in patient samples and controls.

Assay procedure:

1. Prepare the working solution of wash buffer.

2. Remove the required number of microplate strips. Reseal the bag and return unused strips to the refrigerator.

3. Pipette 25 µL of each calibrator, control and specimen sample into planned wells in duplicate.

4. Pipette 150 µL of the Estrogen-HRP conjugate into each well. We recommend using a multichannel pipette.

5. Gently shake the plate by hand for ten seconds (or tap it on the side with your hand) to mix the contents of the wells.

6. Incubate for 2 hours at room temperature (no shaking). Cover the plate to avoid any contamination.

7. Wash the wells 3 times with 350 µL of diluted wash buffer per well. Tap the plate firmly against absorbent paper to ensure that no droplets remain in the wells. The use of a microplate washer is recommended. If a washer is not available, ensure the wash buffer reaches the top edge of the wells and no liquid remains in the plate after the final washing.)

8. Pipette 150 µL of TMB substrate into each well at timed intervals.

9. Incubate for 15 to 20 minutes at room temperature (or until calibrator A attains dark blue colour).

10. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 8. Gently tap the side of the microplate to mix the contents of the wells.

11. Read in a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.

Calculations:

1. Calculate the mean optical density of each calibrator duplicate.
2. Use a 4-parameter curve fit with immunoassay software to generate concentration results.
3. If no software is available draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis.
4. Read the values of the unknowns off the calibrator curve.
5. If a sample reads more than 10,000 pg/mL dilute it with calibrator A at a dilution of no more than 1:10. The result obtained must be multiplied by the dilution factor.

Normal range: 30 to 400 pg/mL for premenopausal women
0 to 30 pg/mL for postmenopausal women

Clinical applications

Total estrogens comprise the total quantity of estrone, estradiol, and estriol. The estrogens are involved in the development of female sex organs and secondary sex characteristics. Before the ovum is fertilized the main action of the estrogens is on the growth and function of the reproductive tract to prepare it for the fertilized ovum.

During the follicular phase of the menstrual cycle the total estrogens level shows a slight increase. The production of total estrogens then increases markedly to peak at around day 13. The peak is of short duration and by day 16 of the cycle levels will be low. A second peak occurs at around day 21 of the cycle; if fertilization does not occur, the production of total estrogens decreases.

In post-menopausal women the concentration of all estrogens decreases substantially and estrone becomes the predominant estrogen. In pregnant women the concentration of all estrogens escalates and estriol becomes the predominant estrogen.

A total estrogens test is commonly indicated to:
Aid in diagnosis of sex steroid metabolism related conditions, for example, premature or delayed puberty, and aromatase and 17 alpha-hydroxylase deficiencies.

- Assess fracture risk in postmenopausal women and, to a lesser degree, older men.

- Follow-up female hormone replacement therapy in postmenopausal women.
- Prognose antiestrogen therapy, for example, aromatase inhibitor therapy.

PROGESTERONE ELISA

Principle

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of progesterone in the sample. A set of standards is used to plot a standard curve from which the amount of progesterone in patient samples and controls can be directly read.

Assay procedure

1. Prepare working solutions of the progesterone-HRP conjugate and wash buffer.

2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.

3. Pipette 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

4. Pipette 100 µL of the conjugate working solution into each well. (We recommend using a multichannel pipette.)

5. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

6. Wash the wells 3 times with 300 µL of diluted wash buffer per well and tap the plate fi rmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)

7. Pipette 150 µL of TMB substrate into each well at timed intervals.

8. Incubate on a plate shaker for 10–20 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).

9. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 7.

10. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution. * If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

Calculations:

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 60 ng/mL then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

Normal range:

Female (pre-ovulation): less than 1ng/mLor 3.18 nmol/L
Female (mid-cycle): 5 to 20 ng/mL or 15.90 to 63.60 nmol/L
Male: less than 1 ng/mL or 3.18 nmol/L.

Clinical applications

Progesterone is a C-21 female sex steroid hormone with a variety of physiological effects. In the follicular phase of the menstrual cycle, progesterone is produced in low levels. It increases to the LH peak and then sharply rises 3 to 4 days later to higher levels, remaining elevated through the 10th to 12th days after the LH peak. Then there is a sharp decline to the low levels of the follicular phase. It is responsible for the induction of the cyclic changes in the endometrium of the uterus allowing implantation and successful growth of the fertilized ovum and maintenance of pregnancy.

Progesterone measurements are used in documenting ovulation and in the management of diffi culties during the fi rst trimester of pregnancy. Levels of progesterone may be useful in the evaluation of sterility due to luteal phase defects, prediction of impending abortion, and the diagnosis of ectopic pregnancy.

Normal values of progesterone may be affected by drugs such as, oral contraceptives, superovulatory drugs, estrogen replacement therapy medication, and GnRH analogues. The

removal of ovarian function following surgical oophorectomy or chemotherapy may influence serum progesterone values.

PROLACTIN (PRL) ELISA

Principle:

The principle of the following enzyme immunoassay test follows a typical one-step capture or 'sandwich' type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specifi c for prolactin is immobilized onto the microplate and another monoclonal antibody specifi c for a different region of prolactin is conjugated to horse radish peroxidase (HRP). Prolactin from the sample and standards are allowed to bind simultaneously to the plate and to the HRP conjugate. The washing and decanting steps remove any unbound HRP conjugate. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed by the enzymatic reaction is directly proportional to the concentration of prolactin in the sample. A set of standards is used to plot a standard curve from which the amount of prolactin in patient samples and controls can be directly read.

Assay procedure:

1. Prepare working solutions of the conjugate and wash buffer (refer to reagents provided section).

2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.

3. Pipette 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

4. Pipette 100 µL of the conjugate working solution into each well. (We recommend using a multichannel pipette.)

5. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

6. Wash the wells 3 times with 300 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)

7. Pipette 150 µL of TMB substrate into each well at timed intervals.

8. Incubate on a plate shaker for 10–15 minutes at room temperature (or until calibrator F attains dark blue colour for desired OD).

9. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 7.

10. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution. * If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

Calculations:

1. Calculate the mean optical density of each calibrator duplicate.
2. Calculate the mean optical density of each unknown duplicate.
3. Subtract the mean absorbance value of the "0" calibrator from the mean absorbance values of the calibrators, controls and serum samples.
4. Draw a calibrator curve on log-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
5. Read the values of the unknowns directly off the calibrator curve.
6. If a sample reads more than 3200 µIU/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

Normal range:

Men**:** less than 20 ng/mL
Nonpregnant women: less than 25 ng/mL
Pregnant women: 80 to 400 ng/mL

Clinical applications

Prolactin (PRL) is a polypeptide hormone synthesized by the lactotropic cells of the anterior pituitary gland. Structurally, it is similar to two other polypeptide hormones namely, growth hormone and placental lactogen. PRL is a polypeptide containing 199 amino acids, while growth hormone and placental lactogen each have 191 amino acids. There is approximately 100 µg of prolactin in the human pituitary gland, which is a very small amount when compared to growth hormone, which is present at 8–10 mg.

The target organ of prolactin is the breast (mammary gland). Its main physiological action is not only to initiate but also to sustain lactation. The hypothalamus secretes dopamine, which has a direct effect of inhibition of the secretion of PRL.

If dopamine is not available or absent the secretion of PRL is autonomous. Clinical trends-
• In patients where tumours secrete prolactin there is a remarkable increase in the PRL level, which then decreases the secretion of gonadotropin resulting in infertility.
• If the pituitary gland is defi cient it leads to failure of lactation.
• In Sheehan's syndrome the pituitary gland is deficient, therefore the PRL level is reduced.
• A few conditions where increases in prolactin levels are found include: hyperprolactinemia, adenomas of the pituitary gland, sleep, pregnancy, hypothyroidism, prolactinomas and stress.

• Prolactinomas are pituitary tumours secreting prolactin, found most frequently in females. In females they lead to amenorrhoea, which could be primary or secondary and give rise to a decrease in gonadotropin secretion by the pituitary. In men some degree of impotency accompanied by a low testosterone level occurs, followed by azospermia.