M.Sc. in MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

3rd Semester

Prepared By

Paramedical & Allied Health Science Dept.

MMLT

MIDNAPORE CITY COLLEGE

CODE: MLT 395B

PAPER NAME: BACTERIOLOGY AND MYCOLOGY LAB

1. Isolation, characterization and identification of pathogens from clinical samples

Isolation of bacteria forms a very significant step in the diagnosis and management of the illness. Isolation of bacteria involves various steps –

a. Specimen collection
b. Preservation and transportation of specimen
c. Microscopic examination of sample
d. Various methods used for isolation of bacteria

Specimen collection

Many different specimens are sent for microbiological examination from patients with suspected bacterial infection. Common specimens include urine, faeces, wound swabs, throat swabs, vaginal swabs, sputum, and blood. Less common, but important specimens include cerebrospinal fluid, pleural fluid, joint aspirates, tissue, bone and prosthetic material (e.g. line tips).

Some types of specimen are normally sterile e.g. blood, CSF. These samples are usually obtained via a percutaneous route with needle and syringe, using appropriate skin disinfection and an aseptic technique. The culture of bacteria from such specimens is usually indicative of definite infection except if they are skin contaminants (bacteria inhabitants of normal skin).

In contrast, many microbiological specimens are obtained from non-sterile sites e.g. vaginal or throat swabs, urine sample, stool sample. Such samples often contain bacteria of no clinical relevance in addition to possible pathogens, making the interpretation of culture results more difficult. In general it is preferable to send samples from sterile sites if available.

It is preferred to obtain the samples for bacteriological culture before antibiotic therapy is started. This maximizes the sensitivity of the investigations and reduces false-negative results. Similarly, samples of tissue or pus are preferred over swabs, to maximize the recovery of bacteria in the laboratory.

Specimens must be accurately labelled and accompanied by a properly completed requisition form, indicating the nature of the specimen, the date of sample collection, relevant clinical information, the investigations required, and details of antibiotic therapy, if any.

This allows the laboratory to perform the correct range of tests, and helps in the interpretation of results and reporting. Along with clinical specimens, medical microbiology laboratories also process samples of food, water and other environmental samples (e.g. air sampling from operating theatres) as part of infection control procedures.

High-risk samples

Certain bacterial infections are a particular hazard to laboratory staff, and specimens that might contain these pathogens should be labelled as ‘high risk’ to allow for additional safety measures if necessary. For example - blood cultures from suspected typhoid (*Salmonella typhi*) or brucellosis (*Brucella* species), and samples from suspected Mycobacterium tuberculosis.

Preservation and Transport of specimen

Most specimens are sent to the laboratory in sterile universal containers. Swabs are placed in a suitable transport medium (eg. charcoal medium) otherwise it leads to false negative reporting.

Specimens should be transported as soon as possible to the laboratory. In case a delay is anticipated the specimen should be stored at 4° C.

Immediate transport is necessary in order to:
(i) Preserve the viability of the 'delicate' bacteria, such as *Streptococcus pneumoniae* or *Haemophilus influenzae* (delays in processing can cause false-negative culture results);
(ii) Minimize the multiplication of bacteria (e.g. coliforms) within specimens before they reach the laboratory. In particular urine and other specimens that utilize a semiquantitative culture technique for thier detection, as delays in transport can give rise to falsely high bacterial counts when the specimen is processed.

Microscopy

A Gram stain helps with the visualization of bacteria, and gives an indication of the type of bacteria present, based on the shape of the bacteria and the staining properties (Gram positive: purple; Gram negative: pink/red). A Gram stain also helps to identify mixtures of bacteria, helps to determine the appropriate range of agar plates to be used for subsequent culture, and helps with the interpretation of culture results.

For liquid specimens e.g. CSF, the sample is first centrifuged to concentrate any bacterial cells in the deposit, and Gram stain and culture is performed from the deposit after the supernatant is decanted. This helps increase the sensitivity of both microscopy and culture.

Ziehl-Neelsen (ZN) stain is used to demonstrate the presence of Mycobacteria. Mycobacteria can also be visualized using the fluorescent dye auramine and a fluorescence microscope. Direct immunofluorescence is employed to detect certain pathogens (e.g. *Legionella*, *Pneumocystis*) using specific antibodies conjugated to a fluorescent dye.

Another microscopic technique is dark ground microscopy. This is mainly used to detect the thin spirochaetal cells of *Treponema pallidum* (syphilis bacteria).

METHODS OF ISOLATION OF BACTERIA

Methods of isolation of bacteria can be broadly classified into two

- Culture methods
    - On Solid media
    - On Liquid media
    - Automated systems
- Non-culture methods

CULTURE ON SOLID MEDIA

The principal method for the detection of bacteria from clinical specimens is by culture on solid culture media. Bacteria grow on the surface of culture media to produce distinct colonies.

Different bacteria produce different but characteristic colonies, allowing for early presumptive identification and easy identification of mixed cultures. There are many different types of culture media.

Agar is used as the gelling agent to which is added a variety of nutrients (e.g. blood, peptone and sugars) and other factors (e.g. buffers, salts and indicators).

Some culture media are nonselective (e.g. blood agar, nutrient agar) and these will grow a wide variety of bacteria. While some e.g. MacConkey agar are more selective (in this case through the addition of bile salts selecting for the 'biletolerant' bacteria found in the large intestine such as *Escherichia coli* and *Enterococcus faecalis*). MacConkey agar also contains lactose and an indicator system that identifies lactose-fermenting coliforms (e.g. *Escherichia coli, Klebsiella*) from lactose-non fermenting coliforms (e.g. *Morganella Salmonella*).

Media can be made even more selective by the addition of antibiotics or other inhibitory substances, and sophisticated indicator systems can allow for the easy detection of defined bacteria from mixed populations.

Materials:

Glass petri plates, conical flasks (250 ml), pipettes (1 ml and 10 ml), spirit lamp, 70% alcohol, fuel alcohol, non-absorbent cotton, tissue paper, distilled water
Media Composition: As desired
Agar 20.0 gm
Distilled Water 1000 ml
Final pH 6.8 ± 0.2.

Procedure:

Media preparation:

Required amount of media was dissolved into 100 ml of distilled water kept in a conical flask.
pH was adjusted to 6.8 ± 0.2.
Then 2.0 gm of agar was added to 100 ml of nutrient broth.
The media was sterilized at 15 lb pressure and 121 ^{o}C for 15 min by using autoclave.
After autoclaving, the media was cooled down to 50 ^{o}C and poured in to the autoclaved petri plates.
Dilution preparation:

Nine milliliter of autoclaved distilled water was added to 6 test tubes.
One milliliter of water sample (Tap water) was mixed with 9 ml of distilled water and thus 10^{-1} dilution was prepared.
Then 1 ml of water from10^{-1} dilution was poured in another test tube containing 9 ml of autoclaved distilled water and thus 10^{-2} dilution was prepared.
Like this, upto 10^{-6} dilution was prepared.
Plating:

One hundred microliter of 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread on the solidified nutrient agar plates.
The plates were incubated at 37 ^{o}C for 24 h.

Result:

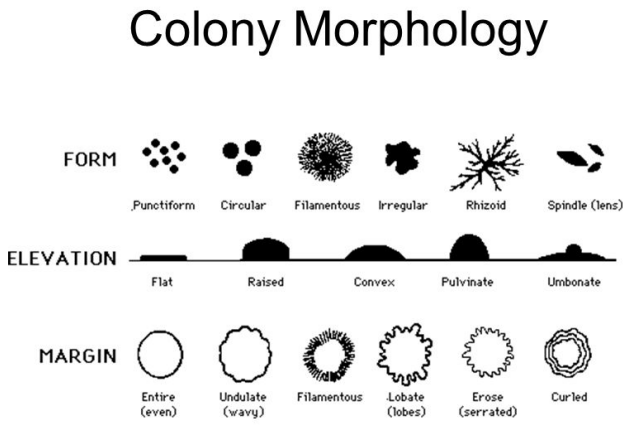
After 24 h, the colony appeared on the petri plates were enumerated and the colony characteristics were observed.

Enumeration of bacteria from water sample:

Sample number	Dilution no.	Sample added (ml)	CFU numbers/ 0.1 ml	CFU numbers/ 1 ml	CFU No./ml in original sample	Average number of CFU No./ml in original sample

Colony characteristics:

Colony number	Colour	Form	Elevation	Margin	Figure



2. Identification of *E. coli, P. aeruginosa, S. aureus, Salmonella sp.* by biochemical tests.

Indole Test

OBJECTIVES:

1) Determine the ability of bacteria to degrade the amino acid tryptophan.
2) Distinguish the bacteria based on the indole activity.

PRINCIPLE:

Tryptophan is an essential amino acid that can undergo oxidation by enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. The metabolic end products are indole, skatole and indole acetic acid. The ability to hydrolyse tryptophan with the production of indole is not a characteristic of all bacteria. Only some bacteria produce indole.

REQUIREMENTS:

Equipments: Incubator.

IReagents and lab wares: Peptone water / tryptone broth, Kovac's reagent, or Ehrlich's reagent, glass tubes and inoculating wire.

Kovac's reagent consists of para-dimethyl amino benzaldehyde, 5.0 gm; isoamyl alcohol, 75.0 ml; and concentrated hydrochloric acid, 25.0 ml. Ehrlich's reagent consists of p-dimethyl amino benzaldehyde, 4.0 gm; absolute ethyl alcohol, 380.0 ml; and concentrated hydrochloric acid, 80.0 ml.

Specimen: 24 hours to 48 hours peptone water culture of *Escherichia coli* incubated at 37°C.

PROCEDURE

1) Take 0.5 ml of 24 hours to 48 hours peptone water cultures of *E. coli* in a small test tube.
2) Add 0.2 ml of Kovac's reagent to the peptone water and shake.
3) Allow it to stand for few minutes and read the result.

OBSERVATION

In a positive test, a red-violet ring develops within minutes on addition of Kovac's reagent. In a negative test a yellow ring appears.

RESULTS AND INTERPRETATION

Positive indole test is indicated by the appearance of red-violet ring on adding the reagent. Negative reaction is indicated by developing a yellow ring. *E. coli* colonies tested are an indole producing bacteria. *K. pneumoniae* does not produce the indole.

List of Indole positive and negative bacteria

Indole positive bacteria	Indole negative bacteria
1. *Escherichia coli*	*1. Escherichia vulnaris*
2. *Klebsiella oxytoca*	2. *Klebsiella pneumoniae*
3. *Proteus vulgaris*	3. *Proteus mirabilis*
4. *Morganella morganii*	4. Salmonella Typhi
5. *Providencia rettgeri*	5. *Shigella sonnei*
6. *Aeromonas hydrophila*	
7. *Pasteurella multocida*	
8. *Vibrio cholerae*	
9. *Falvobacterium*	
10. *Plesiomonas shigelloides*	

Methyl Red Test

OBJECTIVES

1) Determine the ability of bacteria to oxidise glucose with the production of high concentrations of acidic end products by methyl red test.
2) Differentiate between all glucose oxidizing enteric bacteria particularly *Escherichia coli* and *Enterobacter aerogenes*.

PRINCIPLE

Methyl red is a pH indicator with a range between 6.0 (yellow) and 4.4 (red). The pH at which methyl red detects acid is considerably lower than the pH of other indicators used in bacteriologic culture media. Thus to produce a colour change, the test organism must produce large quantities of acid from the carbohydrate substrate being used.

The methyl red test is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic acid, acetic acid, formic acid) from glucose through the mixed acid fermentation pathway. Because many species of the Enterobacteriaceae may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phase of incubation, only organisms that can maintain this low pH after prolonged incubation (48–72 hours) overcoming the pH buffering system of the medium can be called methyl red positive.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating wire. Methyl red test broth. It consists of poly peptone, 7 gm; glucose, 5 gm; dipotassium phosphate, 5 gm; and distilled water, 1l at a pH of 6.9. Methyl red indicator. It consists of methyl red, 0.1 g in 300 ml of 95% ethyl alcohol.

Specimen: Culture of *E. coli, E. aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days.

PROCEDURE

1 Take 0.5 ml of broth cultures of *E. coli* in a small test tube.

2 Add five drops of 0.04% solution of methyl red directly to the broth culture and mix well.

3 Note any change in the colour of medium at once.

OBSERVATION

Look for the development of stable red colour on adding methyl red indicator.

RESULTS AND INTERPRETATION

The development of a stable red colour in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an intermediate orange colour between yellow and red may develop. This does not indicate a positive test. Yellow colour indicates a negative test.

List of MR positive and negative bacteria

MR positive bacteria	MR negative bacteria
1. *E. coli*	1. *K. pneumoniae*
2. *K. ozaenae*	2. *Enterobacter* spp
3. *K. rhinoscleromatis*	
4. *K. ornitholytica*	
5. Edwardsielleae	
6. Salmonellae	
7. Citrobacter	
8. Proteae	
9. Yersinia	

Voges-Proskauer Test

PRINCIPLE

The Voges-Proskauer test determines the capability of some bacteria to produce non-acidic or neutral end products such as acetyl methyl carbinol from the organic acids produced as a result of glucose metabolism. Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose is further metabolised through various metabolic pathways, depending on the enzyme systems possessed by different bacteria.

One such pathway results in the production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. Enteric bacteria such as members of the Klebsiella-EnterobacterHafnia-Serratia group produce acetoin as the chief end products of glucose metabolism and form smaller quantities of mixed acids.

The test depends on the production of acetyl methyl carbinol from pyruvic acid, as an intermediate product in its conversion to 2: 3 butylene glycol. In the presence of atmospheric oxygen and alkali (40% potassium hydroxide), the small amount of acetyl methyl carbinol present in the medium is converted to diacetyl, which reacts with the peptone of the broth to produce a red colour.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop. VP broth. It consists of polypeptone, 7 gm; glucose,5 gm; dipotassium phosphate, 5 gm and distilled water, 1 litre at a pH of 6.9. 5% a naphthol. It consists of a naphthol, 5 gm; and absolute ethyl alcohol, 100 ml. It serves as the colour intensifier. 40% potassium hydroxide. It consists of 40 gm potassium hydroxide in 100 ml distilled water. It serves as the oxidising agent.

Specimen: Culture of *Escherichia coli, Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 30°C for five days or 37°C for 48 hours.

PROCEDURE

1) Take 1 ml of broth cultures of *E. coli* in a small test tube.
2) First add 40% KOH and then add 0.6 ml of a 5% solution of α-naphthol in ethanol to the broth culture and shake gently. It is essential that the reagents are added in this order.
3) Note any change in the colour of medium within 2-5 minutes.

OBSERVATIONS

Look for the development of pink colour 15 minutes or more after addition of the reagents.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a pink colour 15 minutes or more after addition of the reagents, deepening to magenta or crimson in half an hour. This indicates the presence of diacetyl, the oxidation product of acetoin. A negative test is indicated by colour less reaction for half an hour. The test should not be read after standing for over 1 hour because negative VP test may produce a copper-like colour, leading to a false positive interpretation.

VP positive and negative bacteria

VP positive bacteria	VP negative bacteria
1. *Klebsiella pneumoniae*	1. *Escherichia coli*
2. *Enterobacter cloacae*	2. *Edwardsiella tarda*
3. *Cedicia netri*	3. Salmonellae
4. *Ewingella americana*	4. Proteae
5. *Serratia marcescens*	5. Yersinieae
6. *Aeromonas sobria*	
7. *Vibrio cholerae*	
8. *Chryseomonas luteola*	
9. *Flavimonas oryzihabitans*	
10. *Sphingomonas paucinobilix*	

Citrate Utilisation Test

OBJECTIVES

Differentiate certain enteric organisms on the basis of their ability to utilize citrate as a sole source of carbon.

PRINCIPLE

In the absence of fermentable glucose or lactose, some bacteria are capable of using citrate as a sole source of carbon for their energy. This ability depends on the presence of the enzyme, a citrate permease that facilitates the transport of citrate in the cell.

Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide (CO_2). During this reaction the medium becomes alkaline because the CO_2 that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the indicator, bromo thymol blue present in the medium from green at pH 6.9 to deep Prussian blue at pH 7.6. Simmon's citrate and Koser's citrate are two examples of different types of citrate media used in the test.

REQUIREMENTS

Equipments: Incubator.

Reagents and lab wares: Inoculating loop, Simmon's citrate medium (It consists of ammonium dihydrogen phosphate, 1 gm; dipotassium phosphate, 1 gm; sodium chloride, 5 gm; sodium citrate, 2 gm; magnesium sulfate, 0.20 gm; agar, 15 g; bromo thymol blue, 0.08 gm and distilled water 1 litre) pH adjusted to 6.9. The medium is poured into a tube on a slant.

Specimen: Culture of *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in glucose phosphate medium incubated at 37°C for 48 hours.

PROCEDURE

1) Using sterile technique, inoculate each bacteria into its appropriately labeled tube by means of a stab and streak inoculation.
2) Incubate all cultures for 24 hours to 48 hours at 37°C.

OBSERVATIONS

Look for the development of deep blue colour within 24-48 hours of incubation of the inoculated tube.

RESULTS AND INTERPRETATION

A positive test is represented by the development of a deep blue colour within 24 hours to 48 hours, indicating that the test organism has been able to utilize the citrate contained in the medium, with the production of alkaline products. A negative test is indicated by no change of colour of the citrate medium.

List of citrate positive and negative bacteria

Citrate positive bacteria	Citrate negative bacteria
1. *Klebsiella pneumoniae*	*1. Escherichia coli*
2. *Citrobacter diversus*	2. Salmonella Typhi
3. *Enterobacter cloacae*	3. Salmonella Paratyphi A
4. *Serratia marcescens*	4. *Shigella* species
5. *Providencia alcalifaecians*	5. *Yersinia enterocolitica*
6. *Euringella americana*	6. *Edwardsiella tarda*
7. *Acroncobacter oxylosoxidans*	7. *Vibrio holisae*
8. *Vibrio vulnificus*	

3. Staining of bacteria: Endospore and acid fast staining

Endospore staining

PRINCIPLE

Malachite green stain, also known as Schaeffer-Fulton Method for bacterial endospores uses two different reagents: primary stain (malachite green) and counter stain (0.5% safranine or 0.05% basic fuchsin).Ordinary tap water acts as decolourising agent. Unlike most of the vegetative cells that are stained by common procedures, the spore, because of its impervious coats, are not stained by the primary stain easily. The application of heat facilitates penetration of the primary stain, malachite green. After the primary stain is applied and the smear is heated, both the vegetative cell and spore appear green. Once the spore is stained with the malachite green, it cannot be decolourised by tap water, which removes only the excess primary stain. The spore remains green. On the other hand, water removes the stain from the vegetable cells, because the stain does not demonstrate a strong affinity for the vegetative cell components and these vegetable cells therefore become colourless.
Red coloured-safranine as counterstain is used as the second reagent to colour the decolourised vegetative cells, which will absorb the counterstain and appear red. The spores retain the green of the primary stain.

REQUIREMENTS

I Equipments: Compound light microscope.

II Reagents and lab wares: Bunsen burner, beaker of boiling water, staining tray, glass slides, inoculating loop, malachite green and safranine.
Preparation of malachite green stain: This stain is prepared by dissolving 5 gram of malachite green in 100 ml of distilled water.
Preparation of safranine stain: This stain is prepared by dissolving 0.5 gram of safranine in 100 ml distilled water.

III Specimen: Smear collected from 48 hours to 72 hours nutrient agar slant culture of *Bacillus cereus*/ thioglycollate culture of *Clostridium butyricum.* On a clean glass slide, a smear from the culture is made in saline, then air dried and fixed with heat.

PROCEDURE

1. Heat fix the smears by passing the slide 2–3 times gently over the flame with the smear side up. Allow the smear to be air dried.
2. Put the slide with the smear over a beaker of boiling water, resting it on the run with the bacterial film upper most. When, within several seconds, large droplets have condensed on the underside of the slide, flood the smear with 5% acqueous solution of malachite green and allow acting for 1 minute, while the water continues to boil.
3. Wash the smears with cold water.
4. Then cover the smear with 0.5% safranine or 0.05% basic fuchsin. Allow it to act for 30 seconds.

5. Rinse the smears again under tap water and blot those dry.
6. Observe the smear first under low power (10x) objective, and then under oil immersion (100x) objective.
7. Record the observations in the note book.

OBSERVATION

Bacterial endospores stain green, and vegetative bacilli stain red.

RESULTS AND INTERPRETATION

A 2 – 3 µm red coloured rod-shaped structure seen along with an intracellular 0.5 µm sized spherical green coloured structure.

It represents red coloured vegetative bacilli with green coloured spores by the malachite green staining method. The sample may be sporebearing bacilli (eg. *Bacillus* species or *Clostridium* species).

Acid fast staining

PRINCIPLE

Acid fastness of acid-fast bacilli is attributed to the presence of large quantities of unsaponifiable wax fraction called mycolic acid in their cell wall and also the intactness of the cell wall. The degree of acid fastness varies in different bacteria.

In this staining method, application of heat helps the dye (a powerful staining solution containing carbol fuchsin and phenol) to penetrate the tubercle bacillus. Once stained, the stain cannot be easily removed. The tubercle bacilli resist the decolourizing action of acid-alcohol which confers acid fastness to the bacteria. The other microorganisms, which are easily decolourised by acid-alcohol, are considered non-acid fast. The non-acid fast bacilli readily absorb the colour of the counter stain (methylene blue) appearing blue, while the acid-fast cells retain the red colour of primary stain (carbol fuchsin).

REQUIREMENTS

I Equipments

Compound light microscope.

II Reagents and glass wares

Bunsen flame/ torch soaked in methylated spirit, loop wire, glass slides, slide rack, strong carbol fuchsin, acid-alcohol (3 ml HCl + 97 ml ethanol) (decolourising agent), and Loeffler's methylene blue (counter stain).

Preparation of strong carbol fuchsin: This solution is prepared by dissolving 5 grams basic fuchsin powder in 25 grams crystalline phenol by placing them in a 1 litre flask. The flask containing solution

is kept over a boiling water-bath for about 5 minutes, shaking the contents from time to time. When the solution is complete, 50 ml of 95% alcohol or 100% ethanol is added to the solution and mixed thoroughly. Then 500ml of distilled water is added to it and the mixture is filtered before use.

Preparation of 20% sulphuric acid : 800ml of water is collected in a large flask. The 200ml concentrated sulphuric acid (about 98% or 1.835g / ml)) is poured slowly down the side of the flask into the water, about 50 ml at a time. The mixture becomes hot. Remainder of acid is added in same manner.

Note: The acid must be added to the water. It is dangerous to add the water to the acid. Great care must be taken to avoid spilling the acid on skin, clothing or elsewhere.

Preparation of 95% alcohol : This is prepared by adding 95 ml of ethanol and adding water to it to make 100ml.

Preparation of acid-alcohol decolouriser: This solution contains 75 ml concentrated hydrochloric acid (HCl) and 25 ml of industrial methylated spirit. Methylated spirit is poured into a large flask. The flask is placed in cold water in the sink. Then hydrochloric acid is added slowly and the top of the flask is covered to stop the fumes from escaping. It is left for 10 minutes. It is then decanted into a labeled bottle for use. The final concentration of HCl is 3%.

III Specimen

Sputum smear positive for tubercle bacilli / culture smear of *Mycobacterium* species.

PROCEDURE

1 Heat fixes the smears by passing the slide 2–3 times gently over the flame with the smear side up. Allow the smear to be air dried.

2 Put the smears on a slide rack and cover the smears with strong carbol fuchsin. Allow it to stain for 5 minutes.

3 During this period, heat the slides from below intermittently by Bunsen flame or torch soaked in methylated spirit without boiling the solution, until the steam rises. Do not allow the stain to dry on the slide, and if necessary add more carbol fuchsin to cover the smear.

4 Rinse the smears gently under tap water.

5 Cover the smear with 20% sulphuric acid for at least 10 minutes for decolourisation.

6 Wash the slides thoroughly with water to remove all traces of acid.

Note: Decolourisation with 95% alcohol for 2 minutes isonly optional and may be omitted.

7 Cover the smear with Loeffler’s methylene blue for 15–20 seconds.

8 Rinse the smears again under tap water and air dry it.

9 Observe the smear first under low power (10x) objective, and then under oil immersion (100x) objective.

Note: The smear should be examined following a zig-zag pattern for at least 10 minutes or 300 fields, before declaring the smear negative.

10 Record the observations in the note book. Findings are recorded, together with grading of the positive smear.

QUALITY CONTROL

A positive control sputum smear from a known case of tuberculosis patient, stained with Z-N stain is compared with the stained test smear for appropriate morphology and staining appearance.

With appropriate staining, the acid-fast bacillus appears pink against blue background of pus cells.

OBSERVATION

Presences of pink coloured slender rod shaped structures are seen with curved ends, and are scattered amidst blue coloured round cells with darkly stained multilobed nucleus.

RESULTS AND INTERPRETATION

The stained smear contains pink coloured acid fast bacilli seen among the blue coloured multilobed pus cells. The smear is positive for acid fast bacilli. Probably, the smear contains Mycobacterium tuberculosis

4. Antibiotic sensitivity test. Determination of Minimum Inhibitory Concentration (MIC) and MBC of antibiotics.

OBJECTIVES

Determine antibacterial sensitivity of bacterial isolates by Kirby –Bauer disc diffusion method.

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.

Kirby – Bauer method is a method of determination of antibiotic sensitivity of the bacteria by disc diffusion method. In this method, standard suspension of bacteria to be tested are inoculated on the surface of Mueller Hinton agar plates. Filter paper discs containing specific concentration of antimicrobial agents are pressed on to the surface and incubated at 35℃ overnight (18-24 hr.). After incubation, the zone of inhibition of growth of bacteria around each disc is measured and the susceptibility is determined.

REQUIREMENTS

Equipment: Incubator.

Reagents and lab wares: 0.5 McFarland standard, Mueller Hinton agar plates (pH 7.2-7.4), peptone water, filter paper discs impregnated with appropriate concentration of antibiotics, sterile cotton swabs, millimeter ruler, forceps and inoculating wire.

Preparation of 0.5 McFarland standard: Solution A is prepared by adding barium chloride ($BaCl_2$, 2H2O) to 100 ml distilled water. Solution B is prepared by adding 1 ml of sulphuric acid (H_2SO_4 (0.36N) to 100 ml of distilled water. Then 0.5 ml of solution A is added to 99.5 ml of solution B, mixed well and distributed in test tubes with a screw cap. The cap is closed tightly to avoid evaporation. The mixture is stored in the dark. The solution is agitated vigorously before using it.

Specimens: *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, and Pseudomonas aeruginosa*.

Preparation of suspension of bacteria: Approximately, 4-5 well isolated colonies of the bacterial strain to be tested are inoculated into 5 ml of peptone water, and is incubated at 37 ℃ for 3-4 hours. The turbidity of the suspension is adjusted to match 0.5 McFarland standards. If the density is more it is diluted with sterile saline. The comparison is made against a white back ground with a contrasting black line.

PROCEDURE

1) After standardisation of bacterial suspension, immerse a sterile cotton swab in it and rotate the swab several times with firm pressure on the inside wall of the tube to remove excess fluid.
2) Prepare a Mueller Hinton agar (MHA) plate (pH 7.2-7.4) with a depth of 4 mm.
3) Inoculate the dried surface of the MHA agar plate by streaking the swab three times over the entire agar surface. It is streaked in three directions by rotating the plate 60° after each streak.
4) Place the appropriate antimicrobial impregnated discs on the surface of the agar using sterile forceps.
5) Gently press each disc onto the agar to provide uniform contact. Do not move the disc once it has contacted the agar because some of the antibiotics diffuse almost immediately Discs must be placed in such a way that they are at least 20 mm from one another.
   Note: 6 antibiotic discs may be put in an 85 mm plate.
6) Invert the plates and incubate at 35 ℃ -37℃ for 16-18 hr.

OBSERVATIONS

1) Examine the plates for the presence and size of inhibitory zones.
2) The diameter of the inhibitory zone including the diameter of the disc is measured by using a millimeter scale upto the nearest millimeter.
3) All measurements are made with unaided eye while viewing the back of the petri dish with reflected light against a black non-reflecting background.
4) Measure the inhibitory zones for each antimicrobial agent, compare with the standard Kirby-Bauer's chart and interpret the zone of inhibition as sensitive, intermediate or resistant.

RESULTS AND INTERPRETATION

Each antibiotics produces a specific zone size for each bacteria tested. Depending on the zone size, the bacteria are classified as follows:

Sensitive (S): Infection treatable with normal dosage of the antibiotic.

Intermediate (I): Infection may respond to therapy with higher dosage.

Resistant (R): Unlikely to respond to the antibiotic at the usual dosage.

Determination of Minimum Inhibitory Concentration (MIC)

PRINCIPLE

A stock solution of antimicrobial agent to be tested is prepared. Two fold dilutions of this solution is prepared in suitable broth. A standard suspension of the organism is inoculated into the medium with one antimicrobial agent -free medium as control. The inoculated media are inoculated at 35-37°C for 18-24 hr. and examined for growth. MIC is taken as the lowest concentration of antimicrobial agent which completely inhibits the growth.

REQUIREMENTS

I Equipments

Water bath

II Reagents and lab wares

0.5 McFarland standard, sterile Mueller Hinton broth, antibiotic powder, sterile test tubes. sterile pipettes of 10ml, 5 ml, 2 ml and 1 ml, sterile capped tubes and test tube rack.

These also include stock solution of antibiotic.

Preparation of stock solutions of antibiotics: The required dilutions of the antibiotics are made as per the table 34-1. 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 (stock 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 are soy broth or BHI broth. The turbidity of the suspension is adjusted to match 0.5 McFarland standards.

PROCEDURE

1 Serial dilutions of the antimicrobial agent are made in broth and are kept in test tubes.

2 The last tube is kept free of antibiotic and serves as a growth control.

3 Arrange the test tubes in a rack.

4 Standardised suspension of the microorganisms to be tested is inoculated into the tubes.

5 Tubes are incubated at 35-37°C for 18 hours.

OBSERVATIONS

At the end of the incubation period the tubes are examined for turbidity. Cloudiness indicates that bacterial growth has not been inhibited by the concentration of antibiotic present in the medium.

RESULTS AND INTERPRETATION

Minimum inhibitory concentration (MIC) is defined as the highest dilution which inhibits growth judged by lack of turbidity in the tube. The main advantage of the broth dilution method for MIC determination is that it can readily be converted to determine the minimum bactericidal concentration (MBC) also. The highest dilution showing at least 99% inhibition is taken as MBC. The tubes not showing visible growth are subcultured on solid medium and incubated at 37°C overnight.

5. Identification of pathogenic fungi *Aspergillus niger* and *Candida albicans*.

Aspergillus niger

Colonies: White, fluffy, reverse buff colored, covered with black spores.

Conidial head: large black to brownish, initially globose, become radiate then splits into divergent spore columns.

Conidiophore: Variable in size, thick smooth walls, conidiophore hyaline and brownish near the vesicle.

Vesicle: Globose, concave under surface, brownish sterigmata produced in two series, septate primary sterigmata and short secondary sterigmata.

Conidia: Globose, echinulate.

Candida albicans

PRINCIPLE

Germ tube is an initial hypha from a sprouting conidia, spore or yeast. Formation of germ tube can be demonstrated by inoculating rabbit, fetal calf or human serum with a small quantity of growth of Candida species. The suspension is then incubated at 37°C for a minimum of 1½–2 hours, after which a drop is examined under the microscope for the germ tube.

Germ tubes are produced by *C. albicans, C. stellatoidea* and rarely *C. tropicalis.* At times, some strains of *C. albicans* isolated from the patients with antifungal drugs or patients with cancer do not produce germ tubes.

REQUIREMENTS

I Equipments

Microscope.

II Reagents and glass wares

Standard laboratory glassware, and test tubes 12×75 mm, human, foetal calf or rabbit serum.

III Specimens

24 hour culture of suspected fungal colony on Sabouraud's dextrose agar to be tested.

24 hour culture of known strains of *C. albicans* and *C. parapsilosis* colony on Sabouraud's dextrose agar.

PROCEDURE

1 Take three test tubes and label as 1, 2 and 3.

2 Add 0.5ml. of serum to each of the test tube.

3 Take a half of a single colony to be tested by using a sterile loop, and mix with serum in the test tube 1.

4 Similarly, take a half of *C. albicans* single colony by using a sterile loop, and mix it with serum in the test tube 2.

5 Similarly, take a half of *C. parapsilosis* single colony by using a sterile loop, and mix it with serum in the test tube 3.

6 Incubate all the tubes at 37°C for a maximum of 1½ hrs.

7 Place one drop of suspension from tube 1, 2, and 3 onto 3 different slides and place cover slips over the drops.

8 Examine the slide under low (10x) and high power (40x) magnifications.

OBSERVATIONS

Under the microscope, the whole field under the cover slip is examined for any cell showing production of germ tube.

Germ tubes are seen as long tube like projections extending from yeast cells. This should be differentiated from pseudohyphae.

RESULTS AND INTERPRETATION

Tube 2 will show production of germ tube and Tube 3 will not.

The drop from tube 1 should be read and compared with these controls.

Tube 2 contains *C. albicans* and hence shows germ tube production while tube 3 does not show production of germ tube since it contains C. parapsilosis. Tube 1 should be interpreted with care by observing for the presence or absence of germ tube and should be compared with tube 2 and tube 3.

6. Common serological tests for the diagnosis of bacterial infections

WIDAL ANTIGEN SET / ANTIGENS FOR SLIDE AND TUBE TESTS

SUMMARY

Enteric fever occurs when pathogenic microorganisms like *S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C* infect the human body. During the course of disease, the body responds to this antigenic stimulus by producing antibodies whose titre rises slowly in early stages, to a maxima and then slowly falls till it is undetectable. Antibodies to Salmonella organisms may be detected in the patient serum from the second week after onset of infection. Information regarding the titres and whether or not they are rising or falling can be obtained by performing serological tests using TYDAL® antigen suspensions. Usually tube titres of 1:80 and above are taken as diagnostically significant, however for endemic areas higher cut-offs may need to be established.

REAGENT

TYDAL® contains ready to use concentrated, smooth antigen suspensions of the bacilli; *S. typhi 'O', S. typhi 'H', S. paratyphi 'AO', S. paratyphi 'BO', S. paratyphi 'AH', S. paratyphi 'BH', S. paratyphi 'CH', S. paratyphi 'CO'* and / or polyspecific positive control reactive with these antigens.

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

REAGENT STORAGE AND STABILITY

1. Store the reagents at 2-8°C. DO NOT FREEZE.
2. The shelf life of reagents is as per the expiry date mentioned on the reagent vial labels. Do not use beyond expiry date.
3. Once opened the shelf life of the reagent vial is as described on the reagent vial label provided it is not contaminated.

PRESENTATION

Contents		REF	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF
		105200045	105200085	105210025	105220005	105230005	105240005	105250005	105280005	105260005	105270005	105290005
Ag		O, H, AH, BH	O, H, AH, BH, CH, AO, BO, CO	O, H	O	H	AO	BO	CO	AH	BH	CH
Control	+	0.4 ml	2.0 ml	0.4ml								
Control	-		2.0 ml									
MIXING STICKS LADDER		4	4									
DISPENSER PP TUBES		50	50									
RUBBER TEAT		1	1									
SLIDE		1	1									
PACKAGE INSERT		1	1	1	1	1	1	1	1	1	1	1

ADDITIONAL MATERIAL REQUIRED

Slide test method: Stop watch, Variable Micropipettes.
Quantitative method: Timer, Kahn tubes / test tubes, Pipettes (0.1ml, 1ml), Physiological saline, Incubator (37°C), Test tube rack.

PRINCIPLE

When the coloured, smooth, attenuated TYDAL® antigen suspensions are mixed / incubated with patient serum, anti-salmonella antibodies present in the patient serum react with the antigen suspensions to give agglutination.

Agglutination is a positive test result, indicating presence of anti-salmonella antibodies in the patient serum. No agglutination is a negative test result indicating absence of anti-salmonella antibodies.

NOTE

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The *S. typhi 'O', S. paratyphi 'CO'* reagents contains 0.5% Phenol, *S. typhi 'H', S. paratyphi 'AH', S. paratyphi 'BH', S. paratyphi 'CH'* reagents contain 0.3% Formaldehyde and *S. paratyphi 'AO', S. paratyphi 'BO'* reagents contain 0.7% Ethanol along with 0.1% Sodium azide as preservatives. Avoid contact with skin and mucosa. Do not breathe vapour. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Sodium azide may react with lead and copper in plumbing and form highly explosive metal oxides, on disposal flush with large quantities of water.
3. The reagent can be damaged due to microbial contamination or on exposure to extreme temperatures. It is recommended that the performance of the reagent be verified with the positive and negative controls. Positive control provided with the kit only for TYDAL® 4 x 5 ml set (REF.: 105200045) & 2 x 5 ml set (REF.: 105210025) and 8 x 5 ml set (REF.: 105200085). Negative Control provided with the kit only for TYDAL® 8 x 5 ml set (REF.: 105200085).
4. Shake the reagent vials well before use to disperse the antigen suspension uniformly and improve test readability.
5. Only clean and dry slides / tubes must be used. Clean the slide / tube with distilled water and dry.
6. It is necessary to use the calibrated dropper provided in the reagent vial to dispense a reagent drop.
7. TYDAL® antigen suspensions are not from human sources hence contamination due to HBsAg and HIV is practically excluded.
8. Accessories provided with the kit only must be used for optimum results. (Applicable only for TYDAL® 4 x 5 ml set, REF: 105200045) and TYDAL® 8 x 5 ml (REF.: 105200085).
9. Do not use damaged or leaking reagents.

SAMPLE COLLECTION AND STORAGE

1. No special preparation of the patient is required prior to sample collection by approved techniques. Do not use haemolysed and turbid samples.
2. Clean and dry glassware free from detergents must be used for sample collection.
3. Do not heat inactivate the serum.
4. Though freshly collected serum is preferable, store samples at 2-8°C in case of delay in testing, for upto 72 hours.

TEST PROCEDURE

Bring reagents and samples to room temperature before testing.

Shake and mix antigens well before dispensing.

Slide Screen Method

1. Place one drop of positive control onto a reaction circle of the slide.
2. Place 50 µl of physiological saline onto the next reaction circle of the slide.
3. Place one drop of patient's serum to be tested onto each of the required number of reaction circles.
4. Add one drop of appropriate TYDAL® antigen suspension to the reaction circles containing Positive control & physiological saline.
5. Add one drop of appropriate TYDAL® antigen suspensions to the reaction circles containing the patient's serum.
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 at one minute**.

Slide Semi-Quantitative Method

1. Using a pipette place 80 µl, 40 µl, 20 µl, 10 µl, and 5 µl of patient serum to be tested on 5 different reaction circles on the slide. The corresponding titres obtained will be 1:20, 1:40, 1:80, 1:160, & 1:320 respectively.
2. Follow step No. 5-7 of slide screen method.

Note: This method is recommended for obtaining quick approximate titres only.

Quantitative Method

Tube-test Procedure

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

INTERPRETATION OF RESULTS

Slide Screen Method

Agglutination is a positive test result and indicates presence of the corresponding antibody in the patient's serum.
No agglutination is a negative test result and indicates absence of the corresponding antibody in the patient serum.

Slide Semi-Quantitative Method

Agglutination is a positive test result. The titre of the patient serum corresponds to the visible agglutination in the test circle with the smallest amount of serum sample.

Quantitative Method

The titre of the patient serum using TYDAL® antigen suspensions is the highest dilution of the serum sample that gives a visible agglutination.

REMARKS

1. Positive results obtained in the slide test should be confirmed with the tube test to establish whether the titres are diagnostically significant or not.
2. TAB vaccinated patients may show a high titre of antibodies to each of the antigens. Similarly, an amnestic response to other vaccines and unrelated fevers in case of patients who have had prior infection or immunization may give a false result.
3. Agglutinins usually appear by the end of the first week of infection, blood sample taken earlier may give a negative result.
4. A rising titre is more significant than a single high titre. It is therefore necessary to evaluate two or more serum samples taken at 4- 6 days intervals after the onset of the disease.
5. 'O' being a somatic antigen brings about a coarse, compact, granular agglutination whereas 'H' being a flagellar antigen brings about larger, loose, flocculant agglutination.
6. While the 'O' antigen is species specific, the 'H' antigen is specific to the serotype.
7. Serological findings are not intended as a substitute for culture. An appropriate attempt should be made to recover and identify the etiologic organisms through various culture and biochemical tests.
8. Generally antibody titres of 1:80 or more are considered clinically and diagnostically significant. However the significant titre may vary from population to population and needs to be established for each area.
9. False positive results are likely if the test is read more than one minute after mixing on the slide test.
10. Any deviation in test procedure could result in variable results.
11. Since techniques and standardization vary from lab to lab one tube difference in tube titres can be expected.
12. Use a separate disposable tip for each sample to prevent cross contamination.
13. After usage the antigen suspension should be immediately recapped and replaced at 2-8°C.
14. It is recommended that results of the tests should be correlated with clinical findings to arrive at the final diagnosis.
15. The performance of the reagents should be validated occasionally using the positive control provided. Good physiological saline may be used as a negative control.

PERFORMANCE CHARACTERISTICS

1. The positive control antisera should produce 1+ or greater agglutination at 1: 80 in the slide and tube test when tested with the TYDAL® antigen suspensions.
2. The negative control should show no agglutination with any of the TYDAL® antigen suspensions.
3. Generally accepted performance characteristic of this type of test is 70% specificity and sensitivity.
4. Reproducibility of TYDAL® antigen suspensions is 100% (+/- one double dilution).

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.

BIBILIOGRAPHY

1. Cruickshank R., (1982), Medical Microbiology, 12th Edition, 403.
2. Felix A., (1942), Brit. Med. J., 11, 597.
3. Data on file: Tulip Diagnostics (P) Ltd.

CE

2°C 8°C Store at 2-8°C	Manufacturer	REAGENT	Description of reagent
Use by	Consult Instructions for use	CONTROL +	Positive control
Date of Manufacture	REF Catalogue Number	CONTROL -	Negative control
LOT Batch Number	IVD In vitro Diagnostic Device	WIDAL Ag SET	Salmonella S. typhi 'O', S. typhi 'H', S. paratyphi 'AH', S. paratyphi 'BH', S. paratyphi 'CH', S. paratyphi 'AO', S.paratyphi 'BO' & S. paratyphi 'CO' antigens

Xn

Harmful if swallowed
Do not breathe vapour
May cause sensitization by skin contact
If swallowed, seek medical advice immediately and show this container or label
Avoid release to the environment. Refer to special instructions
Keep locked up and out of reach of children
Wear suitable protective clothing and eye/face protection
In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
In case of an accident or if you feel unwell, seek medical advice immediately (show the label where possible)
Use only in well ventilated areas

Nak., HCHO
R22, R43, S(H 2), S23,S24, S36/37/39, S45/46, S51,S61

S. typhi Ag SET
Salmonella S. typhi 'O' and S. typhi 'H'

This way up

EC REP
Authorised Representative

ASO Antistreptolysin 'O' Latex Test Kit

Latex-agglutination Slide Test for The Qualitative And Semi-Quantitative Determination of Antistreptolysin-O.

REF : 41LA300-20, 41LA300-50
: 20, 50

IVD +2°C +8°C STERILE A

INTENDED USE

The ASO Antistreptolysin 'O' Latex Test Kit is latex agglutination assay for the qualitative and semi-quantitative detection of antistreptolysin 'O' antibodies (ASO) in human serum. It is intended for in vitro diagnostic use by trained healthcare professionals.

INTRODUCTION

Most of the strains of streptococci which are pathogenic for human beings belong to Group-A. All such organisms produce an exotoxin known as Streptolysin 'O' (SLO) besides other toxins and enzymes.[1] When infected with streptococcal infection, a patient will produce an antibody, anti-Streptolysin 'O' (ASO) that is detectable in serum. Increased ASO titres may be associated with Rheumatic fever and Glomerulonephritis. An elevated ASO titre of more than 200 IU/mL indicates past or present acute streptococcal infection.[2] The titre of ASO should be observed repeatedly over a time of 4 to 6 weeks to follow the course of an injection.

ASO Antistreptolysin 'O' Latex Test Kit provides a simple screening method of detecting an elevated ASO titre and obviates the necessity of performing a cumbersome and time hemolytic inhibition test in all patients.

PRINCIPLE [3]

Antistreptolysin 'O' (ASO) test kit is based on the following principle:

Polystyrene latex particles are coated with purified and stabilised Streptolysin O (antigen), which reacts with its corresponding Antistreptolysin O (antibody) in the test sample resulting in the agglutination of latex particles. The reaction beetween antistreptolysin 'O' in serum and streptolysin 'O' coated to latex particles starts with the formation of web between them, the latex suspension changes its uniform appearance and clear agglutination shall appear. Results are expressed in IU/mL of anti-Streptolysin 'O' (ASO) based on the WHO International Standard.[4]

KIT CONTENTS

REAGENTS

Reagent No.	Reagent Name	Content
Reagent 1	ASO Latex Reagent	Suspension of latex particles coated with streptolysin O in buffer
Reagent 2	Positive Control Serum	Stabilised serum containing 0.1% Sodium Azide
Reagent 3	Negative Control Serum	Stabilised serum containing 0.1% Sodium Azide

ACCESSORIES (*QS - Quantity Sufficient)

Glass Slide
Disposable Applicator Sticks
Disposable Plastic Droppers
Rubber Teats

MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

Normal Saline (0.9% Sodium chloride), only for semi-quantitative test
Timing device
Mechanical rotator (optional)

REAGENT STORAGE & STABILITY

The reagents and the control sera are stable at +2 °C to +8 °C till the expiry date mentioned on the individual label. **Do not freeze**.

BIOSAFETY

1. Handle all samples with care, as they can be potentially infectious.
2. Wear disposable gloves throughout the test procedure and dispose them off as biohazard waste.
3. Wear protective laboratory clothing while performing the test.
4. Do not smoke, eat or drink in area where samples are being handled.
5. Technicians with wound, cut or skin abrasions on the hand must refrain from performing the test without proper precautions.
6. Avoid spilling of samples or solutions containing samples. In case of spillage, immediately clean it with 1:10 dilution of 5% freshly prepared sodium hypochlorite solution and dispose off the cleaning material by a suitable method.
7. Reagents contain 0.1% Sodium Azide as preservative. Avoid contact with skin and mucosa.
8. Remnants of samples after performing test should be collected in a waste container. Discard them as biohazard waste, as per local environmental regulations, in a suitable container. The containers should be finally disinfected and autoclaved at 121 °C for 1 hour.
9. Wash hands thoroughly with disinfectant after completion of the test.

SPECIMEN COLLECTION AND HANDLING

It is recommended that the test be performed only on serum. Store test sera in a refrigerator(+2 °C to +8 °C) or a deep freezer (-20 °C), if delay is anticipated before testing. Avoid repeated freezing and thawing of the test specimen.

PRECAUTIONS

1. Use clean and dry glass slide which has been previously rinsed in distilled water.
2. Do not heat inactivate test sera or controls.
3. Bacterial contaminated sera and observation beyond 2 minutes may lead to false positive results.
4. Lipemic serum should not be used because of the possibility of nonspecific results.
5. Cross contamination must be avoided by ensuring that the droppers for latex and controls are not interchanged.
6. Drying of the mixture at the periphery may lead to erroneous results. Hence the test should not read beyond 2 minutes.
7. While performing the test, it is advisable not to keep the ASO Latex Reagent vial open for any length of time. It must be tightly closed soon after use in order to prevent drying and formation of flaky particles in the antigen suspension.

PROCEDURE [5]

A. QUALITATIVE SLIDE TEST

1. Bring test sera and reagents to room temperature (+15 °C to +30 °C) before testing and mix gently prior to use.
2. With the help of disposable plastic dropper place one drop of undiluted test serum within the circled area on the special slide provided in the kit.
3. Add one drop of well mixed ASO Latex Reagent.
4. Mix the reagent and serum using the applicator stick and spread within the entire circle of the glass slide.
5. Slowly rock the slide and observe for macroscopic agglutination. Do not observe for longer than 2 minutes.

B. SEMI-QUANTITATIVE SLIDE TEST

1. All samples giving positive result in the qualitative test should be tested by semi-quantitative test or Hemolysin titration test.
2. Prepare dilutions of the serum sample with normal saline as suggested in the following table

Serum Dilution	Corresponding IU/mL
Neat	200
1 : 2	400
1 : 4	800
1 : 8	1,600
1 : 16	3,200

3. With the help of disposable plastic dropper, dispense one drop of diluted serum into one of the circled area on the special slide provided.
4. Add one drop of well mixed ASO Latex Reagent

5. Mix the reagent and the drop of diluted serum using the applicator stick and spread within the entire circle of the glass slide
6. Slowly rock the slide for macroscopic agglutination. Do not observe for longer than 2 minutes

INTERPRETATION OF RESULTS

A. CALCULATION:
The approximate ASO concentration in the patient sample is calculated as follows: 200 X ASO dilution = IU/mL

B. QUALITATIVE SLIDE TEST
Agglutination visible within 2 minutes is to be interpreted as a positive test result corresponding to ASO titre of more than 200 IU/mL. A test is considered negative when there is absence of agglutination indicating ASO titre of less than 200 IU/mL.

C. SEMI-QUANTITATIVE SLIDE TEST
ASO titre of the test sample is indicated by the corresponding value in IU/mL indicated against highest serum dilution which gives positive agglutination with 2 minutes

D. REFERENCE VALUES
Up to 200 IU/mL for adults and 100 IU/mL for children <5 years old.[6]
Each laboratory should establish its own reference range.

E. INTERFERENCE
Bilirubin(20 mg/dL), haemoglobin (10 mg/dL), lipids (10g/dL), rheumatoid factors (300 IU/mL) do not interfere. Other substances may interfere.[7]

LIMITATIONS

1. False positive results may be obtained in conditions such as rheumatoid arthritis, scarlet fever, tonsilitis, several streptococcal infections and healthy carriers.
2. Early infections and children from 6 months to 2 years may cause false negative results.
3. A single ASO determination does not produce much information about actual state of the disease. Titrations at biweekly intervals during 4 or 6 weeks are advisable to follow the disease evolution.
4. Clinical diagnosis should not be made on findings of a single test result, but should integrate both clinical and laboratory data.
5. Extremely high levels of antibodies might affect the degree of agglutination. Positive samples should be reassayed using the semi quantitative procedure.
6. Plasma samples should not be used due to the possibility of nonspecific results.
7. Reaction time longer than specified might cause false positive results due to drying affect.
8. Elevated serum levels of beta-lipoprotein and cholestrol might suppress a rise in ASO titre.
9. Penicillin or other antibiotic therapy might suppress a rise in ASO titre.

QUALITY CONTROL

1. It is recommended that the performance of the reagent be verified by the positive and negative controls provided in the kit as the reagents can be damaged due to microbial contamination or on exposure to extreme temperatures.
2. Positive and Negative Controls are not always required when the reagents are in continuous use as the variety of specimen being tested will ensure both agglutinated and nonagglutinated patterns frequently. However, such controls are provided in the kit for performing an occasional check. It is therefore, not necessary to run Positive and Negative Controls with every test. Controls provided are sufficient for 4 tests.

PRESENTATION

Kit containing the reagents :

Reagents	REF 41LA300-20 / 20	41LA300-50 / 50
Reagent 1 : ASO Latex Reagent	1 X 1.0 mL	1 X 2.5 mL
Reagent 2 : Positive Control Serum	1 X 0.25 mL	1 X 0.25 mL
Reagent 3 : Negative Control Serum	1 X 0.25 mL	1 X 0.25 mL
ACCESSORIES (*QS-Quantity Sufficient)		
Glass Slide	1 No.	1 No.
Disposable Applicator Sticks	25 Nos.	50 Nos.
Disposable Plastic Droppers	25 Nos.	50 Nos.
Rubber Teats	2 Nos.	2 Nos.

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SYMBOL LEGENDS

Symbol	Explanation of Symbol	Symbol	Explanation of Symbol
	Consult instructions for use	LOT	Batch code No.
IVD	*In vitro* diagnostic device		Manufacturer
+2 °C +8 °C	Store at +2 °C to +8 °C		Date of manufacture
	Keep away from sunlight		Use by (date or month of expiry)
X	Contains sufficient for < n > tests		Do not use if package is damaged
STERILE A	Sterile A (sterilized using aseptic processing techniques)	EC REP	Authorized Representative
REF	Catalogue number	CE	The product meets all the legal requirements for CE marking as per directive 98/79/EC

LIMITED EXPRESSED WARRANTY DISCLOSURE

ARKRAY Healthcare Pvt. Ltd. (ARKRAY) limits the warranty to the test kit in as much as the said test kit will function only within the limitations and specifications as described and illustrated in the product insert. Any deviation there from by the purchaser or the end user shall not be the liability and/or responsibility of ARKRAY. ARKRAY shall not be liable and/or responsible for any misuse of the said test kit after the date of expiry. If any defect is proved in the manufacture of the test kit, ARKRAY shall be liable only to the extent of the replacement of the said test kit or the refund of its purchase price thereof and shall not be liable for any consequential loss arising there from.

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The Manufacturing site's QMS is Certified for ISO 13485:2016, ISO 9001:2015

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3020 / ver 2.2

HAEMOGLOBINOPATHIES AND SEROLOGY LAB

Code: MMLT 395C

1. Preparing a Peripheral Blood Film

A. DROP PRESENTATION

The first and obvious way to study the cells of the blood is to examine fresh blood under the microscope in the form of a drop preparation and in a thin blood film or smear made on a glass slide. In this experiment, the students will examine some features of blood cells and record their observations. They will also practice making (preparing) blood films and examine them without staining. In later experiments, they will prepare and stain blood smears and identify and count various blood cells, reticulocytes, and platelets.

• Anticoagulated blood obtained from a student volunteer, or spare blood obtained from the clinical laboratory may be provided to the students to avoid skin pricks at this time (a drop of blood can be put on the slide without touching it). Students may also use their own blood from skin pricks.

APPARATUS AND MATERIALS

1. Disposable, sterile blood lancet/pricking needle.
    - Sterile cotton/gauze swabs
    - 70% alcohol/methylated spirit.
2. 8–10 thin, absolutely transparent, grease-free standard glass slides (75 mm × 25 mm).
    - Vaseline • Toothpicks.

PROCEDURES

While you prepare the drop preparation, your work-partner can make blood films from the same finger-prick blood.

1. Get a finger-prick under aseptic conditions. Discard the first 2 drops and allow a good drop to form. Holding a coverslip by its edges between your thumb and finger, touch its center to the blood drop, thus forming a bead.
2. Invert and carefully drop the coverslip (along with the blood drop under it) in the center of a glass slide. Do not press. The blood drop will spread into a thick film by the weight of the coverslip.
3. Using a toothpick, apply a little vaseline all around the edges of the coverslip to seal the capillary space under it. This will prevent evaporation of water and drying up of the preparation.
4. Examine the preparation under low and high magnifications and record your observations.

OBSERVATIONS

Note the degree of separation of cells. Do they lie in a single layer or in 2, 3 or more layers? The red cells are non-nucleated, flat biconcave disks, round, oval or pear-shaped, thinner in the center and appear as colorless, or pale pink structures. (When stained with Leishman's stain, they appear dull orange-pink). Note if there is any rouleaux formation (cells lying on top of each other like a pile of coins) and the number of cells in a rouleaux. Observe if any leukocytes are seen, and their types if possible.

B. PREPARATION OF A BLOOD FILM (BLOOD SMEAR)

Blood films can be made from anticoagulated, or finger-prick blood.

PROCEDURES

1. Place 3 or 4 slides on a white sheet of paper on your work-table, the surface of which should be even and smooth.
2. Allow a medium-sized drop of blood to form on the finger-tip.
3. Steady the pricked finger of your partner with your left hand. Lift a slide from the table, holding it along its long edges. Then touch its center, about 1 cm from the narrow end, to the blood drop. (If anticoagulated blood is being used place a drop of blood in a similar position with a dropper). Do not apply the blood drop at the finger to the slide placed on the table. One cannot see the amount of blood placed on the slide.
4. Place the slide flat on the table, with the blood drop to the right side (neither your fingers, nor the skin of the subject's finger should touch the surface of the slide).
5. Support the left end of the slide with your thumb and fingers of your left hand. Now grasp the long edges of a second slide, the "spreader", between thumb and fingers of your right hand, so that its free left end extends downwards and to the left at an angle of about 40° to the horizontal.
6. Place the narrow edge of the spreader on the first slide, at an angle of 40°, just in front of the blood drop (step 1, **Figure**). Pull the spreader back gently so that it touches the front of the blood drop. Hold it there, (or move it a little from side to side) till the blood, moving along the junction of the two slides by capillarity, almost reaches the ends of the spreader, except the last 2 mm on each side, thus distributing the blood evenly across its width. (If the blood drop is too big, you

may start to spread the smear before the whole of the blood spreads along the slide).

7. Steady the first slide with your left hand, and maintaining a light but even pressure and 40° angle (step 2, **Figure**), move the spreader forwards to the left in a single, smooth, fairly fast gliding motion, pulling the blood behind it in the form of a thin smear. The smear should be spread in about half a second. Any hesitation will result in striations in the film.
8. Make as many trials as possible to get acceptable films, keeping in mind the features of an ideal blood smear, as described below. Dry the film by waving the slide in the air (Do not try to blot-dry the film).

OBSERVATIONS

Examine the slides against diffuse light, with naked eye. What is the color of the smear? Does it appear thick, thin, or granular? Are there any striations— longitudinal or transverse? Are there any vacant places in the film? Is it uniformly distributed in the middle two-thirds of the slide? Is its head—the starting point, straight and about 1 cm from the end? Are its edges about 2 mm from the long sides of the slide? Is there a tail? Try to answer all these questions.
Examine the slides under low and high magnification. Describe what you see about the various cells and compare with what you saw in the drop preparation.

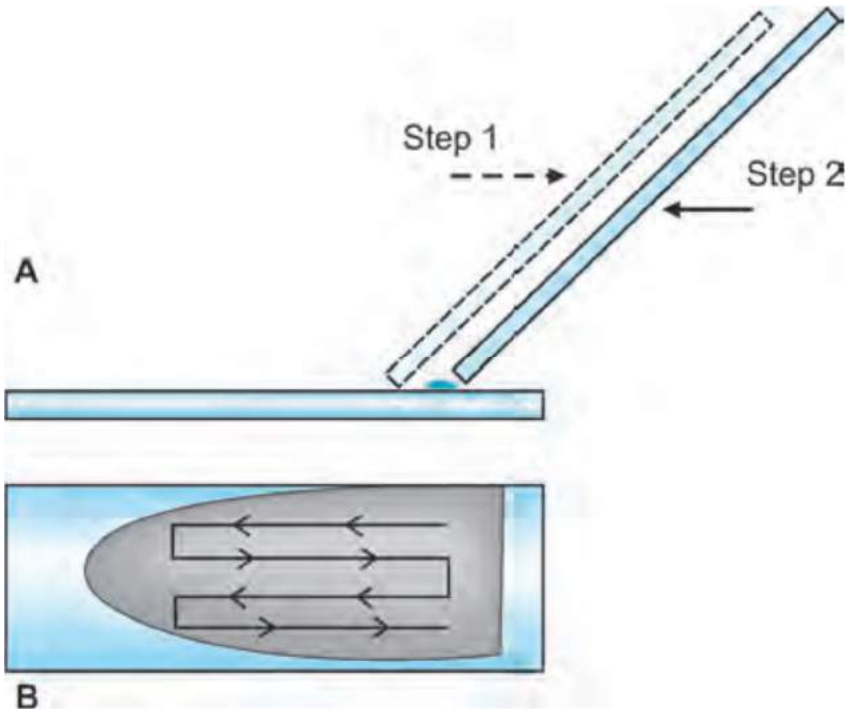


Figure: (A) Method of spreading a blood film. Step 1: The spreader is placed in front of the blood drop and pulled back till it touches the blood. Step 2: Spreader is pushed forwards to spread the film. (B) The appearance of a well-prepared film, showing the movement of the

objective over it may start to spread the smear before the whole of the blood spreads along the slide).

PRECAUTIONS

1. The slides should be absolutely free from dust and grease, because blood will not stick to areas where oils from your fingers have been left. New slides should be preferred. But if old ones are to be used, they should be properly cleaned, as described below.
2. The edge of the spreader should be smooth and not chipped, otherwise the slide would leave striations along or across the smear. Leukocytes may also be caught in chipped places and be carried towards the tail.
3. When applying the slide to the blood drop from a finger-prick, do not touch the skin with the slide, but only the periphery (top) of the blood drop. This is to avoid taking up epidermal squames or sweat.
4. The blood should be spread immediately after taking it on the slide. Any delay will cause clumping of cells due to partial coagulation. This will give a 'granular' appearance to the blood film, which is visible to the naked eye.
5. The angle of the spreader should be 35° to 40°. The more the angle of the spreader approaches the vertical, the thinner the film, and the lesser the angle, the thicker the film.
6. The pressure of the spreader on the slide should be slight and even and the pushing should be fairly quick while maintaining a uniform pressure throughout.
7. The film should be dried by waving it in the air immediately after spreading it. A delay can cause not only clumping, but also crenation and distortion of red cells in a damp atmosphere (if water is allowed to slowly evaporate from the blood plasma on the slide, crenation occurs due to gradual increase in the concentration of salts).
8. By turning the spreader over, you can use it to make 4 blood films.

Cleaning the Slides

Prepare acid-dichromate solution by mixing 1part of concentrated sulfuric or nitric acid with 9 parts of 2–3% potassium dichromate solution.

1. Wash the slides and coverslips with soap and water and rinse in running water. Then soak them overnight in the acid-dichromate solution. Follow this with a wash in running water, then in distilled water.

2. Dip the slides in 90–95% alcohol and dry with a clean, lint-free cloth.

• Another method is to use a good detergent for overnight soak in place of acid-dichromate solution.

• The acid-dichromate solution is best kept in a 1–2 litre, wide-mouth jar. After washing the used slides, they are put in this jar. The slides can then be treated as described above.

2. Study of Morphology of Red Blood Cells

Alterations in the morphology of red cells (their size, shape structure, staining characteristics, etc.) are commonly seen in various types of anemia and other diseases. A careful examination of the peripheral blood film can, therefore, provide important information in the diagnosis of these conditions.

PROCEDURES

Prepare and stain a 'thin' blood film. If a suitable film is available from the previous experiments, it may be used. Study the cells in the area between the tail and the thicker head of the smear, away from the edges, where the RBCs are spread out. Note the following features of the red cells:

Size and shape. Note that there is a moderate variation in the size around the diameter of about 7.5 μm. Most cells are round, though a few may be slightly oval.

Staining. Note the size of the central pallor (it normally occupies the central third) and compare the depth of color of different cells. Note if there are any granules. Though reticulocytes are present (0.5 to 2%), their basophilic network does not take up Leishman's stain, and a special process called 'supravital' staining is required (see next Expt). The nucleated red cells are not normally present in the peripheral blood.

Demonstration slides. Stained slides showing reticulocytes and abnormal morphology of red cells (obtained from pathology department) will be set up on the demonstration table. Examine and compare these smears with your own blood film. Note the descriptions listed on the cards beside the microscopes and enter these in your workbook.

Abnormal red cells. The following terms express some abnormal morphological states and the conditions with which they are associated:

1. Anisocytosis: Abnormal variation in size; seen in iron deficiency and megaloblastic anemias.

2. Basophilic stippling (punctate basophilia): Bluish granules, seen in lead poisoning, thalassemia.

3. Burr cells: Irregularly shaped red cells, seen in uremia.

4. Cabot's rings and Howell-Jolly bodies: Bluish remnants of nuclei that persist in iron-deficiency and megaloblastic anemias; rarely leukemias.

5. Leptocytes (target cells, also called Mexican hat cells): Central staining, a ring of pallor, and an outer rim of staining, seen in liver disease, thalassemias, sickle cell disease.

6. Hypochromia: Less dense staining, wider central pallor; seen in iron deficiency anemia.

7. Hyperchromia: More dense staining red cells; seen in pernicious anemia.

8. Microcytes and macrocytes: Small or large cells; seen in iron deficiency and pernicious anemia.

9. Normoblasts: Immature, nucleated red cells; seen in hypoxia, hemolysis.

10. Pappenheimer bodies: Visible in lead poisoning, carcinomatosis, after splenectomy.

11. Poikilocytosis: Variable shaped cells, seen in iron deficiency anemia.

12. Polychromasia: RBCs of different ages stain unevenly, younger cells being bluer. This is a response to bleeding, hemolysis, hematinics (e.g. ferrous sulphate, B12).

13. Rouleaux formation: Stacking of red cells on each other; the "visual analog" of high ESR.

14. Schistocytes: Fragmented RBCs, sliced by fibrin bands; seen in intravascular hemolysis.

15. Spherocytes: Smaller, spherical cells, appearing more dense, seen in congenital hemolytic anemia or, rarely, in hereditary spherocytosis.

16. Sickle cells: The RBCs are shaped like a sickle; it is due to the HbS which alters the shape of red cells.

RED BLOOD CELL MORPHOLOGY

Size variation	Hemoglobin distribution	Shape variation		Inclusions	Red cell distribution
Normal	Hypochromia	Target cell	Acanthocyte	Pappenheimer bodies (siderotic granules)	Agglutination
Microcyte	1+	Spherocyte	Helmet cell (fragmented cell)	Cabot's ring	
Macrocyte	2+	Ovalocyte	Schistocyte (fragmented cell)	Basophilic stippling (coarse)	Rouleaux
Oval macrocyte	3+	Stomatocyte	Tear drop	Howell-Jolly	
Hypochromic macrocyte	4+	Sickle cell	Burr cell	**Crystal formation**	
	Polychromasia (Reticulocyte)			HbSC HbC	

3.Serum iron Assay by Kit method

8th Edition, revised in February, 2018

Serum iron Assay Kit (Serum samples)

Catalog No: MBS2540518
Method: Colorimetric method
Specification: 100 Assays

This manual must be read attentively and completely before using this product.

If you have any problem, please contact with our Technical Service Center for help.

Application

This kit can be used to measure the concentration of iron in serum samples. This kit (100 Assays) can detect 96 samples.

Detection significance

Serum iron is an essential element in human body. Iron with physiological activity mainly exists in the form of ferroheme and transferrin in plasma. 65% of the iron in the body is bound up in hemoglobin molecules in red blood cells. About 4% is bound up in myoglobin molecules. Around 30% of the iron in the body is stored as ferritin or hemosiderin in the spleen, the bone marrow and the liver. Small amounts of iron can be found in other molecules in cells throughout the body. None of this iron is directly accessible by testing the serum. However, some iron is circulating in the serum. Transferrin is a molecule produced by the liver that binds one or two iron (III) ions, i.e. ferric iron, Fe^{3+}. Transferrin is essential if stored iron is to be moved and used.

Detection principle

Under the action of acidic solution and reductant, ferric ions can be separated from transferrin in serum, and reduced into ferrous ions (Fe^{2+}). The latter then bind to bipyridine and form pink complexes. The concentration of iron can be calculated by measuring the OD value at 520 nm indirectly.

Kit components

Item	Component	Specification	Storage
Reagent 1	100 mg/L Iron Standard stock solution	2 mL × 1 vial	4℃, 6 months
Preparation of 2 mg/L Iron Standard working solution: Take 0.2 mL of **Reagent 1** and add double-distilled water to a final volume of 10 mL. The prepared solution can be stored at 4℃ for 3 days.			
Reagent 2	Chromogenic agent A	Powder × 4 vials	4℃, 6 months, shading light
Reagent 3	Chromogenic agent B	Powder × 4 vials	4℃, 6 months, shading light
Reagent 4	Chromogenic agent C	50 mL × 4 vials	4℃, 6 months
Preparation of Iron Chromogenic agent: Dissolve 1 vial of **Reagent 2** and 1 vial of **Reagent 3** with 50 mL of **Reagent 4**. The prepared solution can be stored at 4℃ for 1 month with shading light.			

Note: During the experiment, the experimental vessel must be clean to avoid iron contamination which may affect the result of the experiment.

Experimental instruments

Test tubes, Vortex Mixer, Centrifuge, Water bath, Spectrophotometer (520 nm）

Preparation of sample

It is recommended to take 2~3 samples which expected large difference to do pre-experiment before formal experiment:

Serum: Detect the sample directly. (The serum must be centrifuged before test if it is turbid.)

Operation steps

(1) Blank tube: Add 0.5 mL of **Double-distilled water** into a 5 mL centrifuge tube.
Standard tube: Add 0.5 mL of **2 mg/mL Iron Standard working solution** into a 5 mL centrifuge tube.
Sample tube: Add 0.5 mL of **Sample** into a 5 mL centrifuge tube.

(2) Add 1.5 mL of **Iron Chromogenic agent**, mix fully with vortex mixer, then incubate in 100℃ water bath for 5 min. (Blank tube and standard tube can be treated without water bath.)

(3) Cool the tubes with running water, centrifuge the tubes at 3500 rpm for 10 min. (If the supernatant is still turbid, take the turbid supernatant into another centrifuge tube and centrifuge again.)

(4) Take 1.0 mL supernatant. Set to zero with double-distilled water, and measure the OD value of each tube with spectrophotometer at 520 nm with 0.5 cm optical path quartz cuvette.

Note: It can be refer to the following operating table

	Blank Tube	Standard Tube	Sample tube
Double-distilled water (mL)	0.5		
2 mg/L Iron standard working solution (mL)		0.5	
Sample (mL)			0.5
Iron Chromogenic agent (mL)	1.5	1.5	1.5

Mix fully with vortex mixer, then incubate in 100℃ water bath for 5 min. Cool the tubes with running water, centrifuge the tubes at 3500 rpm for 10 min. Take 1.0 mL supernatant. Set to zero with double-distilled water, and measure the OD value of each tube with spectrophotometer at 520 nm with 0.5 cm optical path quartz cuvette.

[Note]: When taking the supernatant for colorimetry measurement, it is suggested to take the supernatant carefully with the pipette to avoid adding sediment to optical path quartz cuvette and affect the OD value.

Calculation of results

Serum Iron content (mg/L)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{ the concentration of Iron standard (2 mg/L)}$$

Or

Serum Iron content (μmol/L)

$$= \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times \text{ the concentration of Iron standard (35.81 μmol/L)}$$

Note:

2 mg/L Iron standard = 2000 μg/L ÷ Molecular weight of Iron (55.847) = 35.81 μmol/L

Technical parameters

1. The sensitivity of the kit is 0.08 mg/L.
2. The intra-assay CV is 2.98% and the inter-assay CV is 3.19%.
3. The recovery of the kit is 98.1 %.
4. The linear range of the kit is 0.08-50 mg/L.

Notes

1. The kit is for scientific research only.
2. Instructions should be followed strictly, changes of operation may result in unreliable results.
3. The validity of kit is 6 months.
4. Do not use components from different batches of kit.

Appendix: Preparation of Iron Standard Curve

(This is for reference only)

Pretreatment

Dilute 100 mg/L Iron Standard stock solution with double-distilled water to a serial concentration. The recommended dilution gradient is as follows: 50, 40, 30, 20, 10, 5, 0 μmol/L.

Operation procedure

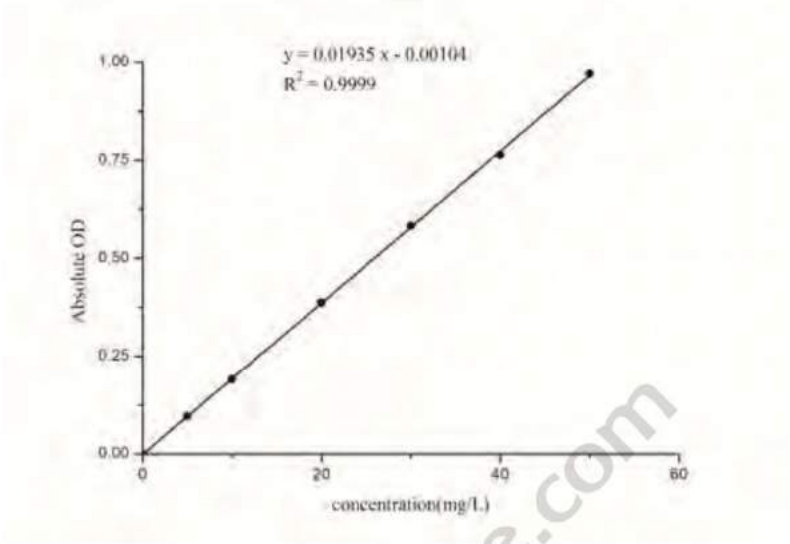
	Standard Tube
Standard working solution with different concentrations (mL)	0.5
Iron Chromogenic agent (mL)	1.5

Mix fully with vortex mixer, then incubate in 100℃ water bath for 5 min. Cool the tubes with running water, centrifuge the tubes at 3500 rpm for 10 min. Take 1.0 mL supernatant. Set to zero with double-distilled water, and measure the OD value of each tube with spectrophotometer at 520 nm with 0.5 cm optical path quartz cuvette.

Detection results

Concentration of Standard(mg/mL)	OD Values		Average absolute OD
0	0.000	0.000	0.000
5	0.095	0.097	0.096
10	0.187	0.194	0.191
20	0.385	0.389	0.387
30	0.584	0.582	0.583
40	0.742	0.786	0.764
50	0.974	0.967	0.971

Standard curve



4. Serum Ferritin Assay Kit

FOR RESEARCH USE ONLY!

BioVision
BioVision Incorporated

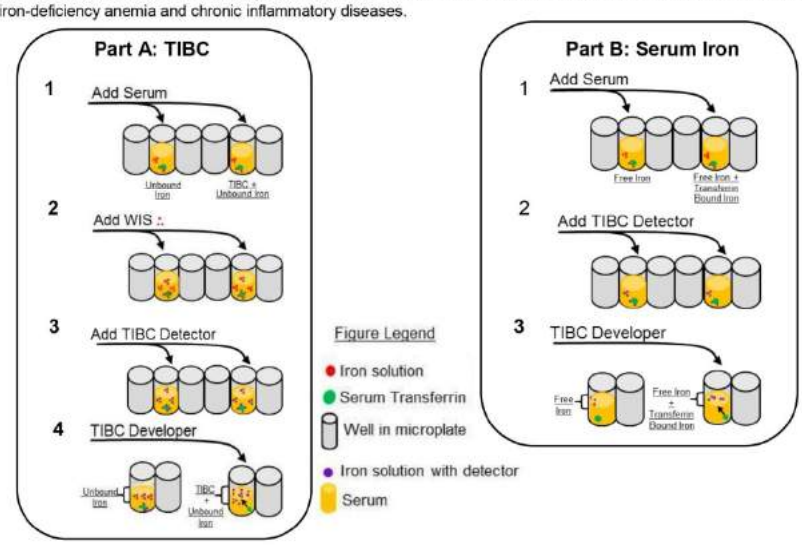
Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit (Colorimetric)

rev 08/19

(Catalog # K392-100; 100 assays; Store at -20°C)

I. Introduction:

BioVision's TIBC and Serum Iron Assay Kit measures both Total iron-binding capacity (TIBC) and Serum iron. Those values indicate the requisite iron for transferrin saturation and Serum Iron respectively. In humans, Transferrin is a blood protein that binds and transports iron throughout the body. Iron bound to transferrin and not bound are reflected in the following: 1) Total Iron Binding Capacity, 2) Unbound Iron, 3) Transferrin Saturation Bound Iron, and 4) Free Iron. Those measurements can be used for to detect and monito transferrin saturation and also iron-deficiency anemia and chronic inflammatory diseases.



II. Application:

- Determination of TIBC, Unbound Iron, Transferrin Saturation, Serum Iron

III. Sample Type:

- Serum or plasma. Serum-off-the clot is preferable to normal serum.

IV. Kit Contents:

Components	K392-100	Cap Code	Part Number
TIBC Assay Buffer	25 ml	WM	K392-100-1
Iron Solution	100 µl	Blue	K392-100-2
TIBC Detector	2 x 1.5 ml	Brown	K392-100-3
TIBC Developer	5 ml	NM	K392-100-4
Iron Standard (100 mM)	100 µl	Yellow	K392-100-5

V. User Supplied Reagents and Equipment:

- 96-well plate clear plate with flat bottom
- Microplate reader capable of absorbance reading

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **TIBC Assay Buffer:** Bring to 37°C before use. Store at -20°C or 4°C.
- **Iron Solution:** Store at -20°C. Immediately before use, prepare the Working Iron Solution (WIS) by adding 4 µl iron solution to 996 µl TIBC Assay Buffer. Make fresh solution as needed.
- **TIBC Developer and Iron Standard:** Store at -20°C or 4°C.
- **TIBC Detector:** Store at -20°C. Keep protected from light.

VII. Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Protocol:

1. **Sample Preparation:** For each sample, prepare duplicates for each (if needed): Unbound Iron, TIBC + Unbound Iron, Free Iron and Free iron + Transferrin Bound Iron. **For TIBC Assay:** Wells 1-4 include two parallel wells for each sample dilution (Unbound Iron and TIBC +

Unbound Iron). Add 10-50 µl serum/well. **For Serum Iron:** prepare two parallel wells for each sample dilution. Wells 5-8 include (Free Iron and Free Iron + Transferrin Bound Iron). Bring the final volume of each well to 50 µl with TIBC Assay Buffer.

Notes:

a) Use serum stored at -80°C. Avoid repeated freeze/thaw.

b) Bilirubin concentrations up to 210 mg/L do not interfere with the assay.

2. **Iron Standard Curve:** Prepare 1 mM Standard: Add 10 µl of 100 mM Iron Standard + 990 µl dH_2O. Next, add 0, 2, 4, 6, 8, 10 µl of 1 mM Iron Standard to each well to generate 0, 2, 4, 6, 8 and 10 nmol/well Iron Standard. Bring to 225 µl final volume with TIBC Assay Buffer. Then, add 25 µl TIBC Detector to each well. Discard diluted Standard after use. *The Standards can be prepared and added to the plate immediately prior to the final 10 minutes incubation.*

3. **TIBC & Serum Iron Assay:** Add reagents as specified in the tables below:

TIBC Assay		
	Unbound Iron (A)	**TIBC + Unbound Iron (B)**
WIS	125 µl	125 µl
Incubate @ 37°C for 10 minutes		
TIBC Detector	25 µl	25 µl
Incubate @ 37°C for 10 minutes		
TIBC Assay Buffer	50 µl	–
TIBC Developer	–	50 µl
Incubate @ 37°C for 10 minutes		

Serum Iron		
	Free Iron (C)	**Free Iron + Transferrin Bound Iron (D)**
TIBC Assay Buffer	175 µl	125 µl
Incubate @ 37°C for 10 minutes		
TIBC Detector	25 µl	25 µl
Incubate @ 37°C for 10 minutes		
TIBC Developer	–	50 µl
Incubate @ 37°C for 10 minutes		

4. **Measurement:** Measure absorbance at OD 570 nm for Standards and Samples. The OD at the end of the final incubation is the value to be used in calculations. The plate may be measured between 24°C-37°C. However, each incubation should be performed at 37°C.

5. **Calculations:** Subtract 0 Standard reading from all Standards and plot the Iron Standard Curve. For each Sample, determine the $TIBC_{(570\ nm)}$ by using the following equation: **$TIBC_{(570\ nm)}$= B – A** or $OD_{(TIBC+Unbound\ iron)} - OD_{(Unbound\ Iron)}$ **(See Step 3)**. Determine the Serum $Iron_{(570\ nm)}$ by using the following equation: **Serum $Iron_{(570\ nm)}$= D – C** or $OD_{(Free\ iron\ +\ transferrin\ bound\ iron)} - OD_{(Free\ iron)}$ **(See Step 3)** Apply the OD values from $TIBC_{(570\ nm)}$ and Serum $Iron_{(570\ nm)}$ to the Standard Curve to get X and Y nmol respectively, of iron in each Sample. TIBC and Serum Iron are represented as µmol iron/L of serum. Calculate the TIBC and Serum Iron as shown below:

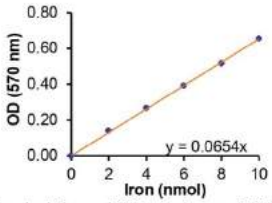
I) $TIBC = \frac{X}{V\ serum}$ **x dilution factor x 10^3 = µmol/L**

II) $Serum\ Iron = \frac{Y}{V\ serum}$ **x dilution factor x 10^3 = µmol/L**

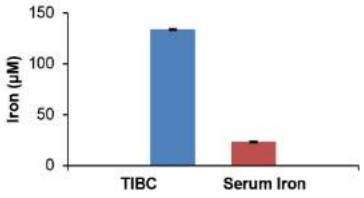
III) $\%\ Transferrin\ Saturation = \frac{Serum\ Iron}{TIBC}$ **x 100**

Where: **X** is the TIBC iron amount from Standard Curve (nmol),
Y is the Serum iron amount from the Standard Curve (nmol),
10^3 is conversion factor mL → L,
V is the volume of Serum Sample (µl)

A)



B)



Figures: (A) Iron Standard Curve, **(B)** Serum Iron and TIBC determination of Serum. Assays were performed following the kit protocol.

VIII. Related Products:

Iron Colorimetric Assay Kit (K390)
Heme Colorimetric Assay Kit (K672)
Ferrostatin-1 (2230)
Transferrin, Rat Plasma (7543)
Apotransferrin, Mouse Plasma (7540)
Human CellExp™ Transferrin, human recombinant (7467)
Ferritin (human) ELISA Kit (K7420)
Deferoxamine Mesylate (1883)
Transferrin (HOLO), Human Plasma (7542)
Apotransferrin, Human Plasma (4707)
Apotransferrin, Rat Plasma (7541)
Lactoferrin, Human Milk (7550)

FOR RESEARCH USE ONLY! Not to be used on humans.

5. Ferritin ELISA assay

Intended Use:

The Eagle Biosciences Ferritin ELISA assay kit is intended for the quantitative determination of ferritin in serum or plasma by enzyme linked immunoassay (ELISA). The Ferritin ELISA assay kit is for research use only and not to be used in diagnostic procedures.

Assay Background:

The serum ferritin concentration is proportional to the amount of iron in stores in the human body. The measurement of serum ferritin can help differentiate between the anemia caused by iron deficiency and other forms of anemia. Studies have demonstrated that serum ferritin concentration is a useful, noninvasive screening test for iron overload, which may allow the detection of idiopathic hemochromatosis in the precirrhotic stages4.

Principle of Procedure:

The Ferritin ELISA assay kit for the quantitative determination of serum ferritin is basically a two stage reaction:

STAGE 1: The binding of human serum ferritin to a solid phase antihuman ferritin, and the simultaneous binding of the purified antihuman ferritin conjugated with alkaline phosphatase to the insoluble immune-complex.

STAGE 2: Reaction of alkaline phosphatase with a substrate solution consisting of phenylphosphate disodium and 4-amino-antipyrine. Following the addition of potassium ferricyanide a color develops, the optical density (490 - 510nm) of which is directly proportional to the ferritin concentration in the sample.

The intensity of the color developed during the 2nd Stage incubation is directly proportional to both time and temperature; therefore, an increase in the 2nd Stage incubation time will increase the intensity of the color, while a decrease in the 2nd Stage incubation time will decrease the intensity of the color. The same applies for an increase or decrease in the incubation temperature.

Materials Provided:

The expiration date for the Ferritin ELISA assay kit and each component is stated on the label(s). Store all components at 2-8oC.

Prediluted Ferritin Calibrator Solutions: 6 vials containing 0.3ml human spleen ferritin calibrated to concentrations of 6, 20, 60, 200, 600, and 2000 ng/ml, against WHO reference material (94/572), in phosphate buffered saline with rabbit serum and sodium azide as a preservative. Store at 2 - 8C. Do Not Use After Expiration Date on Vial.

Solid Phase Antihuman Ferritin: 96 microwells ("wells"), in the form of eight 1 X 12 strips, coated with rabbit antihuman spleen ferritin. Stored in a bottle containing borate buffer with bovine serum albumin, rabbit serum, and sodium chloride with sodium azide as a preservative. Store at 2 - 8C. Do Not Use After Expiration Date on Bottle.

Sample Diluting Buffer: 1 bottle containing 20 ml of phosphate buffered saline with rabbit serum and sodium azide as a preservative. Store at 2 - 8C. Do Not Use After Expiration Date on Bottle.

Conjugated Antihuman Ferritin: 1 bottle containing 23 ml of alkaline phosphatase conjugated rabbit antihuman spleen ferritin dissolved in 0.15 M phosphate buffered saline with 5% normal rabbit serum, and sodium azide as a preservative. Store at 2 - 8C. Do Not Use After Expiration Date on Bottle.

Substrate Solution: 1 bottle containing 23 ml of phenylphosphate disodium, 4-amino-antipyrine in 10% diethanolamine with sodium azide as a preservative. Store at 2 - 8C. Do Not Use After Expiration Date on Bottle.

Potassium Ferricyanide: 1 bottle containing 15 ml of potassium ferricyanide (0.24%) in water. Store at 2 - 8C. Do Not Use After Expiration Date on Bottle.

Well Holder: 1 micro well holder.

Sample Preparation:

Collect 5 ml of venous blood aseptically. Allow the blood to coagulate and separate the serum from the clot by centrifugation. Plasma may also be used for ferritin analysis. Moderate hemolysis will not interfere with the assay. If the assay will be performed within 7 days, store the serum refrigerated. If more than 7 days will elapse before the test is performed, the serum specimen should be frozen. Serum specimens may be stored frozen for 4 months without change in the ferritin content.

Assay Procedure:

Determine the number of Solid Phase Antihuman Ferritin micro wells needed for the assay (each strip contains 12 wells) and remove any unnecessary wells before proceeding with step 1. Replace unused wells in the bottle of buffer and store at 2-8°C. Allow all reagents, sera, and samples to reach room temperature before performing assay.

1. Remove the appropriate number of micro wells from the Solid Phase Antihuman Ferritin bottle, place in the Well Holder, and shake dry.

2. Beginning with micro well C1 (skip wells A1 and B1), pipette 10ul of each Prediluted Ferritin Calibrator Solution and sample, in duplicate, into separate wells. Micro wells A1 and B1 measure non-specific binding (NSB) and will contain only the Conjugated Antihuman Ferritin.

3. Pipette 200 µl of Conjugated Antihuman Ferritin into all micro wells.

4. Incubate on a vibrator or clinical rotator table, set at 180 -200 rpm, for 2 hours at room temperature.

5. Wash with deionized water by filling each micro well with water and shaking to decant. Repeat 3 times. After the final wash, tap the tops of the micro wells on absorbent material for about 30 seconds to drain.

6. Pipette 200 µl of the Substrate Solution into each well.

7. Incubate for 30 minutes at room temperature.

8. Develop the color by adding 100µl of the 0.24% Potassium Ferricyanide to each micro well and mix thoroughly (eg 1 minute at 180 - 200 rpm).

9. Zero the Microplate Reader with a blank prepared with 200 µl of the Substrate Solution and 100 µl of the Potassium Ferricyanide.

10. Read the absorbance of all samples at 500 ± 10nm. If possible, use a correction wavelength of 600 -630nm and record the net values. Readings should be taken within one hour of the completion of the assay.

11. If automatic background subtraction is not available, it is suggested that the plate be read a second time at 600 - 630nm and these values be manually subtracted from the initial 500nm readings. Failure to
compensate for the background absorbance may increase the variability of the assay and result in potentially erroneous values.

12. Calculate the results.

Calculations:

The serum concentration of serum ferritin can be calculated using a logit log data reduction program. Construct a calibration curve by plotting the net absorbance values obtained for each Prediluted Ferritin Calibrator Solution (mean O.D. of Calibrator minus NSB) on the vertical axis (Y) and the corresponding ferritin concentration in ng/ml on the horizontal axis (X).

Calculate the average absorbance value for each control and sample. Determine the location of the average absorbance value on the Y-axis. Follow this point horizontally until it intersects the calibration curve. Follow this point of intersection with the curve vertically until it intersects the X-axis. This X-axis value will be the serum ferritin concentration of the control or sample. For maximum precision, samples in excess of 1000 ng/ml should be diluted as follows:

1. Dilute the sample 1:10 with Sample Diluting Buffer.
2. Further dilutions may be made if necessary.
3. Re-assay these dilutions using the standard assay procedure and multiply the results by the dilution factor.

6. Bone Marrow Examination

Red blood cells, most white blood cells, and platelets are produced in the bone marrow, the soft fatty tissue inside bone cavities. Sometimes a sample of bone marrow must be examined to determine why blood cells are abnormal or why there are too few or too many of a specific kind of blood cell. A doctor can take two different types of bone marrow samples:

- Bone marrow aspirate: Removes fluid and cells by inserting a needle into the bone marrow and sucking out (aspirating) fluid and cells

- Bone marrow core biopsy: Removes an intact piece of bone marrow using a coring device (similar to a large diameter needle)

The bone marrow aspirate shows what cells, normal and abnormal, are present in the bone marrow and provides information about their size, volume, and other characteristics. Special tests, such as cultures for bacteria, fungi, or viruses, chromosomal analysis, and analysis of cell surface proteins can be done on the sample.

The core biopsy removes an entire piece of bone marrow and shows not only what cells are present but also how full the bone marrow is with cells and where the cells are located within the marrow.

Although the aspirate often provides enough information for a diagnosis to be made, the process of drawing the marrow into the syringe breaks up the fragile bone marrow. As a result, determining the original arrangement of the cells is difficult.

When the exact anatomic relationships of cells must be determined and the structure of the tissues evaluated, the doctor also does a core biopsy. A small core of intact bone marrow is removed with a special bone marrow biopsy needle and sliced into thin sections that are examined under a microscope.

Both types of samples are usually taken from the hipbone (iliac crest), often during a single procedure. Aspirates are rarely taken from the breastbone (sternum). In very young children, bone marrow samples are occasionally taken from one of the bones in the lower leg (tibia).

A bone marrow sampling begins with cleaning, sterilizing, and anesthetizing the skin over the bone. The procedure generally involves a slight jolt of pain, followed by minimal discomfort. The procedure takes a few minutes and causes no lasting damage to the bone.

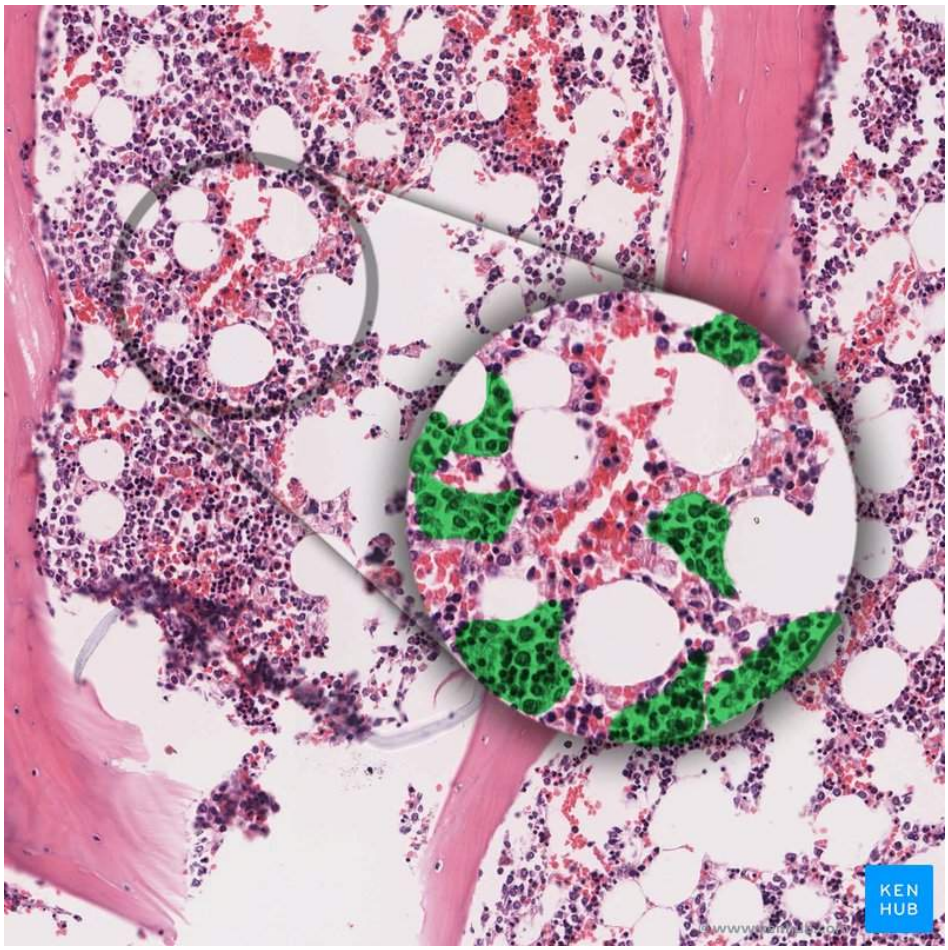
Clinical Significance

Bone marrow aspirations and biopsies remain a mainstay for the workup of hematologic malignancies and their definitive diagnosis. These methods avoid more invasive procedures, can yield an accurate diagnosis in a relatively short amount of time, and are relatively uncomplicated to perform. The quick nature of the procedure, the potential for avoidance of sedation or general anesthesia, and the ability to perform the procedure on an outpatient basis facilitate an efficient path towards diagnosis and treatment as well as a nontraumatic experience for the patient.

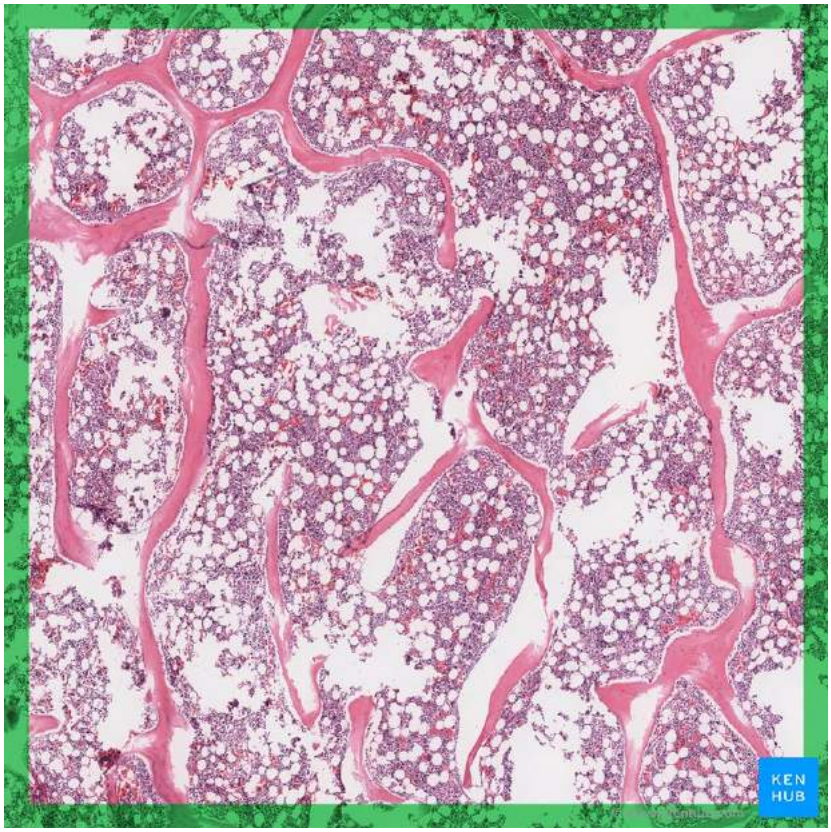
Red bone marrow

Clusters of haematopoietic cells known as haematopoietic islands are widely distributed throughout the loose connective tissue network observed in red marrow. These islands are found

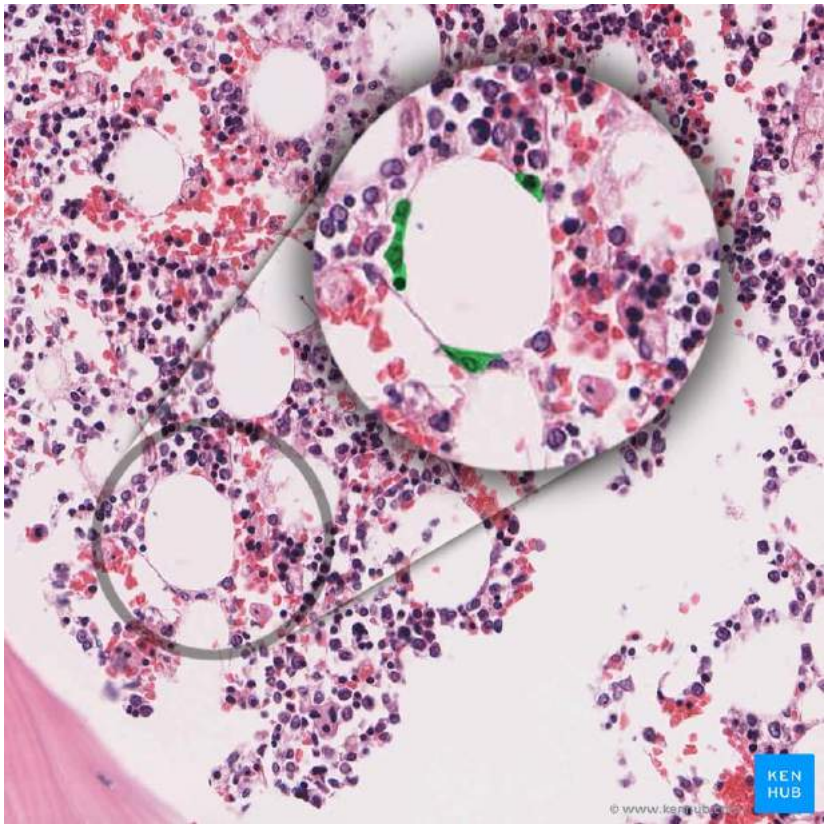
next to relatively large, yet thin walled, sinusoids that also communicate with nutrient vessels of the bone. The sinusoids are situated at a central part of a roundabout circulation such that the nutrient arteries that leave the nutrient canals to supply the bones anastomose in the bone marrow and subsequently terminate in arterioles that coalesce to form the sinusoids. The sinusoids then drain to significantly larger veins that form nutrient veins, which then leave the bone via the same nutrient canals that the arteries enter by.



Red marrow is most abundant in all skeletal structures from intrauterine life up until around the 5th year of life. As time progresses, red marrow is restricted to the central flat bones (i.e. cranial bones, clavicle, sternum, ribs, scapula, vertebrae, and pelvis) and the proximal ends of the proximal long bones of the upper and lower limbs.

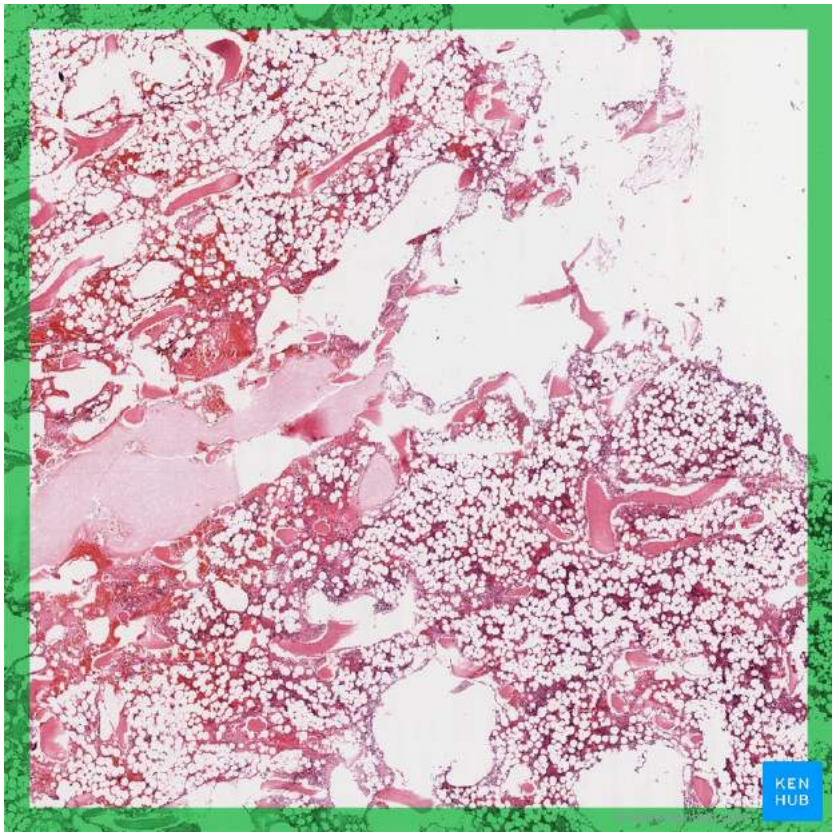


The supporting substance that supports the haematopoietic and adipocyte cells in the marrow is made up of reticulin. This is a fine type III collagen that is produced by mesenchyme derived reticular cells (fibroblast-like cells). Other housekeeping cells like macrophages exist in the stroma and facilitate haematopoiesis by phagocytosing cellular debris generated from the process.



Yellow bone marrow

Depending on the age and haematological demand of an individual, the reticular cells become swollen as a result of increased lipid uptake. Subsequently, yellow marrow is formed. It contains mainly supportive connective tissue that provides scaffolding for the neurovascular structures that traverse the cavitation. There are also numerous adipocytes in addition to very few dormant haematopoietic clusters. These latent haematopoietic centres can be reactivated in the event of an increase demand for red blood cells.



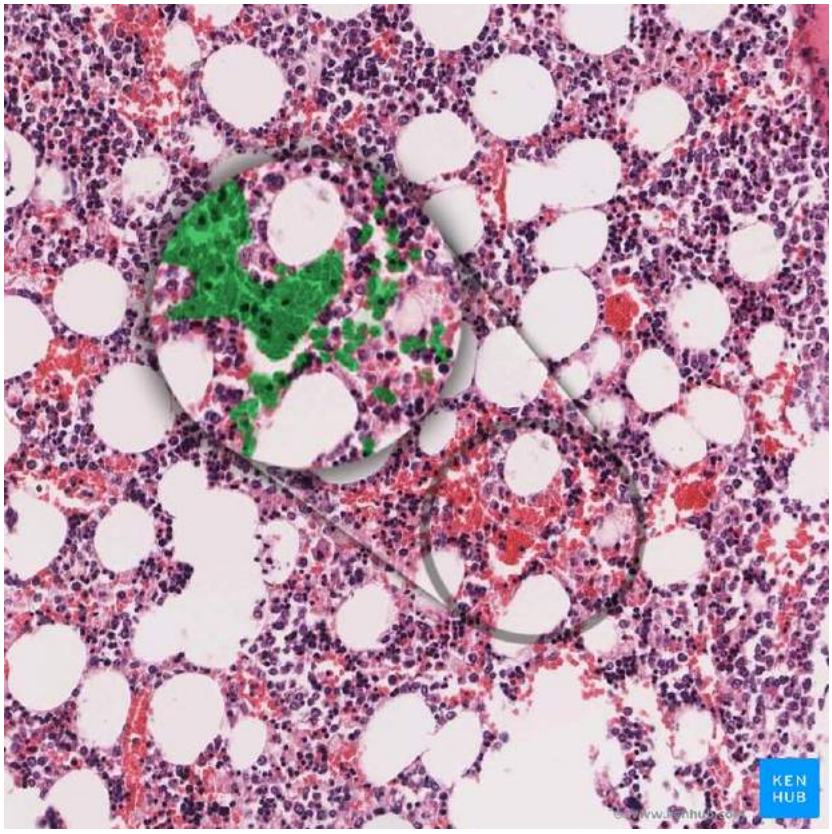
Cell types

Histological analysis of the bone marrow will reveal an abundance of progenitor cells and their derivatives at different stages of development. Typically, the progenitor cells are larger than their end products. The suffix "-blast" is often used to denote that the cell line being referenced are the stem cells for that series (i.e. erythroblasts are the precursor cells for red blood cells [erythrocytes]). The following is a list of the cell lines found in the bone marrow:

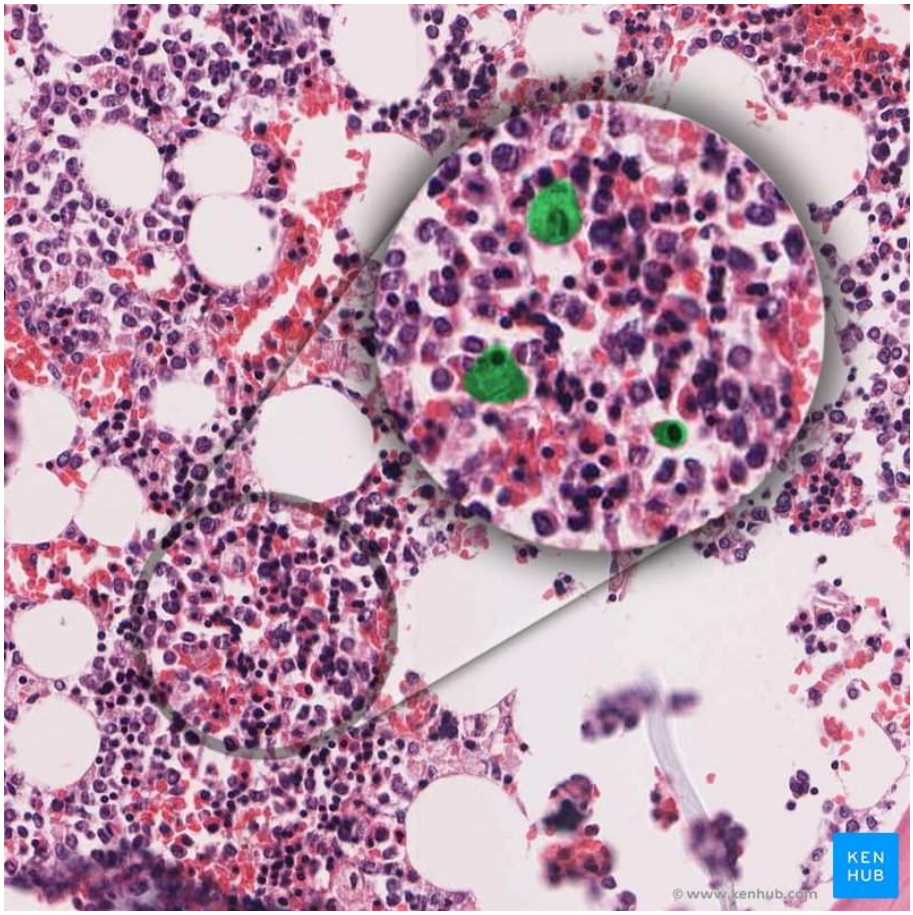
- Granulocytes – are a special line of white blood cells that possess secretory granules in their cytoplasm. There are three granulocytes; these are eosinophils, basophils and neutrophils.

- Monocytes – are leukocytes that differentiate into macrophages. Recall that there are different subtypes of macrophages depending on the region of the body that they are found in (i.e. Kupffer cells of the liver).

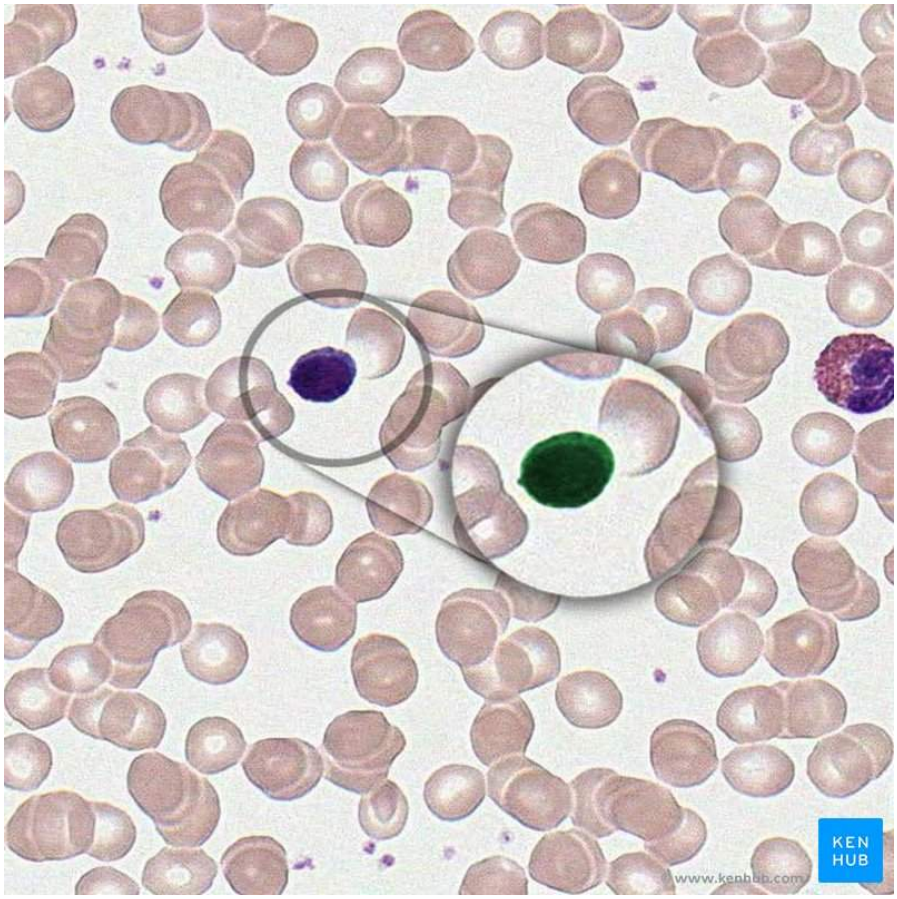
- Erythrocytes – are the anucleate, biconcave, oxygen-carrying species.



- Megakaryocytes – is another large species that is responsible for thrombocytogenesis (i.e. platelet production).



Lymphocytes – are all produced in the bone marrow. However, education and maturation of one subset of lymphocytes occurs in the thymus (i.e. T-lymphocytes)



The stroma also contains a myriad of stem cells of mesenchymal origin. These include multipotent cell lines that are capable of differentiating into cartilaginous cell lines (chondrocytes), bone cells (osteoblasts and osteoclasts) in addition to adipocytes, myocytes (muscle) and endothelial cells.

7. **Vitamin B12 (Vit-B12) ELISA**

FOR RESEARCH USE ONLY!

BioVision
BioVision Incorporated

Vitamin B12 (Vit-B12) ELISA Kit

09/18

(Catalog # E4638-100, 100 assays, Store at 4°C)

I. Introduction:

Vitamin B12 (Vit-B12) is a water-soluble vitamin that is involved in the metabolism of every cell of the human body: it is a cofactor in DNA synthesis, and in both fatty acid and amino acid metabolism. It plays an important role in the synthesis of myelin, and in the maturation of developing red blood cells in the bone marrow. BioVision's Vitamin B12 ELISA kit is a competitive ELISA assay for the quantitative measurement of Vit-B12 in serum, plasma and cell culture supernatants. The density of color is proportional to the amount of Vit-B12 captured from the samples.

II. Application:

This ELISA kit is used for *in vitro* quantitative determination of Vitamin B12 in samples.
Detection Range: 0.781 - 50 ng/ml
Sensitivity: < 0.469 ng/ml
Assay Precision: Intra-Assay: CV < 8%; Inter-Assay: CV < 10% (CV (%) = SD/mean X 100)
No significant cross-reactivity or interference between Vit-B12 and analogues was observed.

III. Sample Type:

Serum, plasma, tissue homogenates and other biological fluids.

IV. Kit Contents:

Components	E4638-100	Part No.
Micro ELISA Plate	8 X 12 strips	E4638-100-1
Lyophilized Standard	2 vials	E4638-100-2
Sample / Standard dilution buffer	20 ml	E4638-100-3
Biotin- detection antibody (Concentrated)	60 µl	E4638-100-4
Antibody dilution buffer	10 ml	E4638-100-5
HRP-Streptavidin Conjugate (SABC) (Avoid light)	120 µl	E4638-100-6
SABC dilution buffer	10 ml	E4638-100-7
TMB substrate (Avoid light)	10 ml	E4638-100-8
Stop Solution	10 ml	E4638-100-9
Wash buffer (25X)	30 ml	E4638-100-10
Plate sealers	5	E4638-100-11

V. User Supplied Reagents and Equipment:

- Microplate reader capable of measuring absorbance at 450 nm
- 37°C incubator

VI. Storage and Handling:

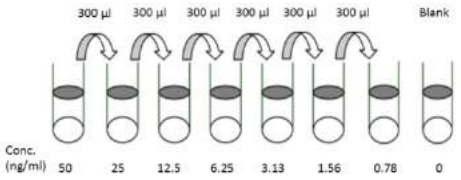
The entire kit may be stored at 4°C for up to 6 months from the date of shipment.

VII. Reagent and Sample Preparation:

Note: Prepare reagents within 30 minutes before the experiment. Before using the kit, spin tubes and bring down all components to the bottom of tubes.

1. **Biotin- detection antibody working solution**: Calculate the total volume of the working solution: 0.05 ml / well × quantity of wells with additional 0.1 - 0.2 ml of the total volume. Dilute the Biotin- detection antibody with Antibody dilution buffer at 1:100 and mix thoroughly.
2. **HRP-Streptavidin Conjugate (SABC)**: Calculate the total volume of the working solution: 0.1 ml / well × quantity of wells with additional 0.1 - 0.2 ml of the total volume. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly.
3. **Wash Buffer**: Dilute 30 mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4°C. If crystals have formed in the concentrate, warm it with 40°C water bath and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use.
4. **Standard Preparation:**

- Reconstitute the lyophilized Vit-B12 standard by adding 1 ml of Standard/Sample Dilution Buffer to make the 50 ng/ml standard stock solution. Use within 2 hours after reconstituting.
- Allow solution to sit at room temperature for 10 minutes, then gently vortex to mix completely.
- Prepare 0.6 ml of 25 ng/ml top standard by adding 0.3 ml of the above stock solution in 0.3 ml of Standard/Sample Dilution Buffer. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay.
- Suggested standard points are: 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0 ng/ml



FOR RESEARCH USE ONLY! Not to be used on humans.

155 S. Milpitas Blvd., Milpitas, CA 95035 USA | T: (408)493-1800 F: (408)493-1801 | www.biovision.com | tech@biovision.com

5. Sample Preparation:

Note: Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles.

- **Serum**: Coagulate the serum for 2 hour at room temperature or overnight at 4°C. Centrifuge at approximately 1000×g for 20 min. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
- **Plasma**: Collect plasma using EDTA-Na_2 as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
- **Tissue homogenates**: Rinse the tissues with ice-cold PBS (0.01M, pH=7.4) to remove excess hemolysis blood thoroughly. Tissue pieces should be weighed and then minced to small pieces which will be homogenized in PBS (the volume depends on the weight of the tissue. 9 mL PBS would be appropriate for 1 g of tissue. Some protease inhibitor is recommended to add into the PBS.) with a glass homogenizer on ice. To further break the cells, sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to retrieve the supernatant.
- **Cell culture supernatant**: Centrifuge supernatant for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8°C. Collect the clear supernatant and carry out the assay immediately or aliquot and store at -20°C.
- **Other biological fluids:** Centrifuge samples for 20 min at 1000×g at 4°C. Collect the supernatant and carry out the assay immediately.
- End user should estimate the concentration of the target protein in the test sample first, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit.

VIII. Assay Protocol:

Note: Bring all reagents and samples to room temperature 30 minutes prior to the assay. It is recommended that all standards and samples be run at least in duplicate. A standard curve must be run with each assay.

1. Prepare all reagents, samples and standards as instructed in section VII.
2. Wash plate 2 times with **1X Wash Solution** before adding standard, sample and control wells.
3. Add 50 µl of each **standards** or **samples** into appropriate wells. Immediately add 50 µl **Biotin-labeled Antibody Working Solution** into each well. Cover with the Plate sealer we provided. Gently tap the plate to ensure thorough mixing. Incubate for 45minutes at 37°C.
4. Discard the solution and wash 3 times with **1X Wash Solution**. Wash by filling each well with Wash Buffer (350 µl) using a multi-channel pipette or autowasher. Let it soak for 1-2 minutes, and then remove all residual wash-liquid from the wells by aspiration. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers or other absorbent materials.
5. Add 0.1 ml of **SABC working solution** into each well, cover the plate and incubate at 37°C for 30 min.
6. Discard the solution and wash 5 times with **1X Wash Solution** as step 4.
7. Add 90 µl of **TMB substrate** into each well, cover the plate and incubate at 37 °C in dark within 15-30 min. The shades of blue should be seen in the first 3-4 wells by the end of incubation.
8. Add 50 µl of **Stop Solution** to each well. Read result at 450 nm within 20 minutes.

IX. Calculation:

For calculation, **(the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well).** The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Vit-B12 concentration of the samples can be interpolated from the standard curve. If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

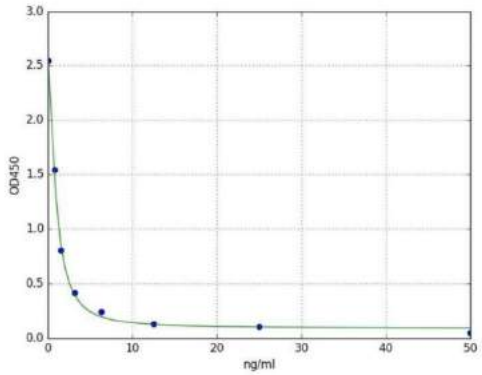


Figure: Typical Standard Curve: These standard curves are for demonstration only. A standard curve must be run with each assay.

FOR RESEARCH USE ONLY! Not to be used on humans.

X. RECOVERY:

Matrix	Recovery range (%)	Average(%)
serum(n=5)	86-105	94
EDTA plasma(n=5)	92-105	98
heparin plasma(n=5)	85-99	93

XI. LINEARITY:

Sample	1:2	1:4	1:8	1:16
serum(n=5)	90-105%	88-102%	88-105%	85-103%
EDTA plasma(n=5)	85-101%	87-100%	92-100%	86-95%
heparin plasma(n=5)	86-95%	82-100%	83-95%	85-99%

XII. RELATED PRODUCTS:

- Folic Acid ELISA Kit (E4523)
- Vitamin D3 (human) ELISA kit (K4806)
- Ascorbic Acid Colorimetric/Fluorometric Assay Kit (K661)
- Ascorbic Acid Colorimetric Assay Kit II (FRASC) (K671)
- Homocysteine ELISA Kit (E4543)

8. Sickling test

Atlas Medical CE

SICKLE-TEST
RAPID SCREEN FOR HbS

IVD *For In-Vitro and professional use only*

Store at 2-8°C

INTENDED USE

Atlas sickle kit is a qualitative solubility test for Sickle Haemoglobin The test can be performed in two ways:
1. A screening test to detect sickle haemoglobin (HbS)
2. A centrifugation test to differentiate the sickle cell trait (AS) from sickle cell anaemia (SS).

INTRODUCTION

Sickle cell disease (also called sickle cell anemia) is an inherited blood disorder that affects red blood cells. The sickle cell gene causes the body to produce abnormal hemoglobin. In sickle cell disease, the hemoglobin clumps together, causing red blood cells to become stiff and develop a C-shaped ("sickle") form. These sickled red blood cells can block blood vessels, reducing blood flow in many parts of the body. This process results in tissue and organ damage.

Each red blood cell contains about 280 million hemoglobin molecules. Hemoglobin is the most important component of red blood cells. It is composed of protein (globulin) and a molecule (heme), which binds to iron.

In the lungs, the heme component takes up oxygen and releases carbon dioxide. The red blood cells carry the oxygen to the body's tissues, where the hemoglobin releases the oxygen in exchange for carbon dioxide, and the cycle repeats. The oxygen is essential for all cells in the body to function.

Sickle cell disease reduces or denies adequate oxygen to many parts of the body. This contributes to the severe pain experienced as a sickle cell crisis and both short- and long-term organ damage.

MATERIALS

MATERIALS PROVIDED

- Reagent A (Buffered Saponin)
- Reagent B(Reducing agent, Sodium Dithionite).

PREPARATION OF THE WORKING SOLUTION

Bring 1 bottle of Reagent 'A' and 1 vial Reagent 'B' up to Room Temperature. Add Reagent 'B' to Reagent 'A' and mix well for 5 minutes. ***record date on bottle.***

PROCERURE

1. Using the solution prepared as above, place 2 ml quantities into the required number of 75 x 12mm tubes.
2. Using whole anti coagulated blood (EDTA), add (20 µl) to each tube. Mix well and **stand for 3 – 5 minutes**. Hold against viewer or 1cm away for best results.
3. Always use known positive and negative controls.

RESULTS

- Negative : Clear haemolysed solution.
- Positive:Turbid red solution, partially or completely obscuring the lines on the viewer.
- Centrifugation : Positive results should be centrifuged for 5 minutes at 1000 RCF.

INTERPRETATION

- HETEROZYGOUS: red-pink supernatant with a dark red band at the top.
- HOMOZYGOUS: Yellowish supernatant with a dark red band at the top.
- NEGATIVE: Slight greyish matter on top of deep red haemolysate.

NOTES

1. The working solution should be kept refrigerated and will remain stable for up to 2 weeks. Allow the reagent to reach to room temperature before use.
2. ANAEMIC SAMPLES Adjust haematocrit to approximately 50% by removal of plasma. Do not add double volume of sample.
3. False Positives may be caused by abnormal plasma protein or when patients are receiving parental nutrition.
4. False Negatives may be found if old or outdated reagents are used, or the blood of small children under the age of 6 months if the proportion of HbS is less than 20%, or following Blood transfusion in severe anaemia.

STORAGE AND STAIBILITY

- The reagents should be stored refrigerated between 2 - 8°C.
- Never Freeze or expose to elevated temperature.
- The reagent is stable until the expiry date stated on the product label. Do not use the reagents past the expiry date

PRECAUTIONS AND LIMITATIONS

- The reagents are intended for in vitro diagnostic use only.

- Do not use reagents if it is turbid or contain particles as this may indicate reagent deterioration or contamination.
- Protective clothing should be worn when handling the reagents.
- Don't use these reagents if the label is not available or damaged.
- Don't use the kit if damaged or the glass vials are broken or leaking and discard the contents immediately.
- Test materials and samples should be discarded properly in a biohazard container.
- Wash hands and the test table top with water and soap once the testing is done.
- Heamolysed blood sample should not be used for testing.
- The test should be performed at room temperature in a well let area with very good visibility.
- Failure to follow the procedure in this package insert may give false results or safety hazard.
- Close the vial tightly after each test.
- The reagent is considered toxic, so don't drink or eat beside it.

REF	Product Reference No.	IVD	For in-vitro diagnostic use.
	Caution.		Store at
	Read product insert before use.		Number of tests in the pack.
LOT	Lot (batch) number.		Manufacturer.
	Expiry date.		Manufacturer telephone number.
	Manufacturer fax number.		

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PPI011A01
Rev D (03.11.2015)

9. Kleihauer acid elution test

Material and method

The test makes use of the fact that F hemoglobin (HbF) is resistant to acid elution while A hemoglobin (HbA) is not. If a fixated thin layer blood smear is immersed in an acid buffer solution, adult erythrocytes will lose their hemoglobin in the buffer and keep only their stroma, while fetal erythrocytes will remain intact. The percent of fetal red blood cells in maternal blood is used to calculate the volume of the FMH.

Sampling

Samples will be harvested on EDTA or ACD through the usual phlebotomy technique and following the laboratory's standard operating procedure. Test tubes are visually inspected on arrival at the laboratory. Samples with blood clots or hemolysis are not accepted for processing since they yield false positive or negative results. Similarly, if the test tubes are faulty new samples will be needed. It is recommended that samples reach the laboratory in less than 2 hours since harvesting. Test tubes will be labeled according to the standard operating procedures of the laboratory. The time allotted to results has to be adequate in order to allow the administration of supplementary anti-D IgG in case this should prove necessary (less than 72 hours after birth or any risk event).

1. Test samples: 3 ml of whole blood sampled from Rh D negative or positive pregnant women or women lately confined, at no more than 2 hours after giving birth or after an event that could induce FMH
2. Control samples

- 1-2 ml of cord blood from a Rh D positive newborn
- 3 ml of blood from an adult male ABO identical with the cord blood, Rh negative.

Materials and reagents

Distilled water, isotonic saline solution, dibasic sodium phosphate, 0.1 M citric acid, 80% ethyl alcohol, DiaQuick Panoptic reagents (Reagent Ltd., Budapest, Hungary), laboratory glassware and consumables.

Apparatus

Microscope with cell counting device, centrifuge, automated pipettes with adjustable volume, analytic scales, 37º C thermostat.

Phosphate buffer preparation

36 ml of 0.1 M citric acid and 14 ml of dibasic sodium phosphate are mixed in a volumetric flask. pH is measured and adjusted to the critical value of 3,2-3,3 by adding citric acid, if it is too elevated, or dibasic sodium phosphate if it is too low. The buffer will be kept at 4° C.

Blood smear preparation

Positive (artificial mixes of adult male blood and cord blood of the same type) and negative (blood from repeat donors) control smears are prepared. All smears will follow identical processing.

Elution and staining

- smears are fixated by immersion in an Erlenmeyer flask which contains 80% ethyl alcohol for 5 minutes and are subsequently air-dried
- fixated smears are immersed in another Erlenmeyer flask containing phosphate buffer at 37º C for 5 minutes
- smears are washed with distilled water and are left to dry
- the staining procedure is as follows:

○ smears are immersed in an Erlenmeyer flask containing DiaQuick Panoptic red solution for one second; this step is repeated 10 times
○ smears are immersed in an Erlenmeyer flask containing DiaQuick Panoptic blue solution for one second; this step is repeated 5 times
○ smears are washed with distilled water and left to dry for further examination. If the smears have been properly processed, they should meet the following criteria:
● complete elution of HbA makes adult erythrocytes easy to identify ● fetal erythrocytes are intensely stained ● leucocytes are easy to differentiate and cannot be mistaken for fetal erythrocytes ● presence of artifacts is minimal.

Screening test

· 25 fields are examined at 400x magnification; · if no fetal cells are found the result is negative; · if a single fetal erythrocyte is encountered quantitative assessment will follow.

Quantitative assessment

In order to enhance the precision of the calculations, at least 2000 cells will be counted using 200x or 400x magnifications.

Calculation

Before calculating the absolute count of fetal erythrocytes the following must be taken into account (15):
● fetal red blood cells are 22% larger than adult ones, thus the volume will be higher than indicated by the erythrocyte count;
● only 92% of fetal erythrocytes stain;
● some adult erythrocytes have a certain degree of HbF preservation;
● a total volume of 5000 ml of maternal blood is arbitrarily considered;
● maternal total erythrocyte mass (in a 5000 ml volume) is 1800 ml.
The fetal bleed (VFEM) should be calculated as fallows:
● Uncorrected volume of bleed =
= 1800 x FE / AE
● Corrected for fetal volume =
= (1800 x FE/AE) x 1,22 = J
● Corrected for staining efficiency =
= J x 1,09 = fetal bleed,
where VFEM = Volume of fetal erythrocytes in maternal circulation (ml), AE = Adult erythrocytes count, FE = Fetal erythrocytes count.

10. Alkali denaturation test

Principle

Fetal cells are not denatured in an alkaline solution. Adult cells will be denatured and demonstrated by a change in colour.

Scope and Related Policies

The alkaline denaturation test should be used as a screening test to identify Maternal-Cord samples which have been incorrectly labeled.

Any confirmed errors in labeling should be reported to the Charge Technologist or designate

Specimens

EDTA anticoagulated whole blood or clotted sample

Materials

Equipment: Block for test tubes

Supplies: Test tubes – 10 x 75 mm

Reagents: 0.1N NaOH

Saline

Quality Control

Known Adult and Cord samples, one of each.

Procedures

1.1	From 1N NaOH stock solution: take 25 mL 1N NaOH + 225 mL 0.9% saline (total 250 mL).	
1.2	Label 10 x 75 mm test tubes.	1.2.1 Positive control (adult cell).
		1.2.2 Negative control (cord cells).
		1.2.3 Patient.

1.3	Add 5 drops red cells from bottom of the specimen to appropriately labeled tube.
1.4	Add 10 drops 0.1N NaOH to each tube.
1.5	Mix and observe color change (10-15 seconds).

Reporting

Interpretation

1.6 Fetal cells – No change in color.

1.7 Adult cells – Red to brown.

1.8 If the sample you are testing consists of Fetal cells report as fetal cells present. If the sample consists of Adult cells report as adult cells present.

11. Laboratory Tests Relevant to Paroxysmal nocturnal hemoglobinuria (*PNH*)

For many years, Ham's test has been the standard way to identify the PNH clone among RBCs. Only one other disease is associated with a positive Ham's test: hereditary erythroid multinuclearity with positive acidified serum (HEMPAS), or congenital dyserythropoietic anemia (CDA) type II, which can be readily differentiated from PNH by the medical history, bone marrow aspirate morphology, and a negative sucrose hemolysis test. Hence, Ham's test is highly specific for PNH.

The Ham's test is based on the tenet that complement will attach to RBCs at somewhat acid pH and that PNH RBCs are sensitive to complement fixation. Whole defibrinated blood collected in heparin is utilized. Cells from the patient and controls are tested for hemolysis with (a) unmodified serum, (b) acidified serum (pH 6.8), (c) heat-inactivated serum (55°C for 3 min.), and (d) heated serum with guinea pig complement. Positivity should be encountered with: (1) PNH cells only and (2) acidified serum; (3) positivity is undone by heating (destroying complement), and (4) is not reestablished with guinea pig complement. This test can be modified to determine complement lysis sensitivity, to distinguish PNH type II from PNH type III RBCs [9].

The sucrose lysis test has been the standard screening test for PNH. Low ionic strength isotonic sucrose causes serum globulin aggregates to fix complement on the RBC surface. Consequently, a scant amount of serum added to this solution will lyse PNH RBCs preferentially to normal RBCs. Heparinized, defibrinated whole blood is employed. A small amount of type-specific serum is added to a 10% (w/v) sucrose solution with washed patient or control erythrocytes and the mixture is incubated (1 hr, 25°C). Greater than 5% hemolysis is considered positive for PNH. The sucrose hemolysis test can also be used to determine complement lysis sensitivity.

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Beratung und Vertrieb: dianova GmbH
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Warburgstr. 45 • 20354 Hamburg • www.dianova.de

Product: PNH KIT
Cat. Ref: PNH-25T
50 Tests per vial with the next monoclonal antibodies combination: CD157/CD45/CD64
Reagent provided: 25 test (20µl / test)
Description: PNH Kit is a screening Kit for Paroxysmal Nocturnal Hemoglobinuria (PNH) whit three-color direct immunofluorescence reagent for use in flow cytometry to evaluate the possible loss of expression of the glycosylphosphatidylinositol (GPI) anchor (GPI-anchored proteins) molecule on neutrophils and on monocytes.
The monoclonal conjugates are provided in aqueous buffered solution containing protein stabilizer, and ≤0.09% sodium Azide.
The vial contain:

- PE Anti-Human CD157, clone SY11B5, isotype IgG1
- PerCP Anti-Human CD45, clone HI30, isotype IgG1, 6th International Workshops on Human Leucocyte Differentiation, WS Code N-L103
- APC Anti-Human CD64, clone 10.1, isotype IgG1, 6th International Workshops on Human Leucocyte Differentiation, WS Code MA36

CD157 is expressed by macrophages, neutrophils, bone marrow stromal cells, endothelial cells, follicular dendritic cells, and T and B cell progenitors prior to the rearrangement of the antigen receptors. Besides the ectoenzyme activity, it also plays a role in neutrophil migration and adhesion.

CD45 recognizes a 180- to 220-kilodalton (kd) human leucocyte antigen that is a member of the leucocyte common antigen (LCA) family. The CD45 antigen is present on all human leucocytes and is weakly expressed on hematopoietic progenitor cells.

The CD64 molecule is a single chain, heavily N-glycosylated type I transmembrane protein, with a molecular weight of 72 kDa. After
removal of the N-linked carbohydrates, a core protein of 55 kDa is found. CD64 is also known as the high-affinity receptor for IgG (FcγRI). It is one, with CD32 (FcγRII) and CD16 (FcγRIII), of three distinct receptors for IgG found on human leukocytes. Clone 10.1 recognizes the EC3 epitope of CD64

PNH is an acquired clonal hematopoietic stem-cell disorder related to the occurrence of a somatic mutation in the PIG-A gen, located in the human X chromosome. This genetic alteration results in a partial or total deficiency of all proteins normally linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (GPI-anchored proteins) (2-3). Typical clinical features of PNH are bone marrow failure of variable severity, thrombosis in unusual sites, chronic intravascular hemolytic anemia that leads to hemoglobinuria, iron deficiency anemia, and increased incidence of acute myeloid leukemia.

Storage: Store in the dark at 2-8 °C. Do not use after expiration date stamped on vial. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the product is suspected, contact our Technical Services. (tech@immunostep.com).

Application: It is recommended for use in flow cytometry. This reagent is effective for direct immunofluorescence staining of human tissue for flow cytometric analysis using the amounts indicated in each vial.
Although a rare disease, the PNH assay is frequently requested since the screening of PNH should be performed in patients with hemoglobinuria, patients with Coombs-negative intravascular hemolysis, especially patients with concurrent iron deficiency, patients with venous thrombosis involving unusual sites, patients with aplastic anemia, patients with refractory anemia-MDS and patients with episodic dysphagia or abdominal pain with evidence of intravascular hemolysis (1)

Precautions:

1. The device is not intended for clinical use including diagnosis, prognosis, and monitoring of a disease state, and it must not be used in conjunction with patient records or treatment.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.

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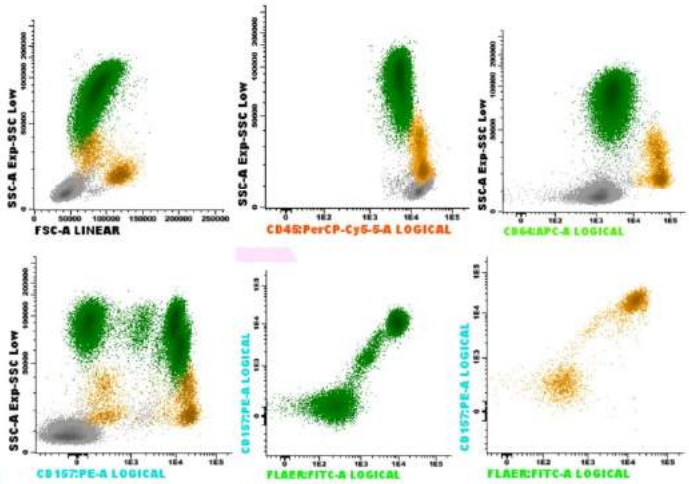
Preparation[6]:

Whole blood sample must be taken aseptically by means of a venepuncture in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). Perform a white blood cell (WBC) count on all samples to be evaluated. If the WBC count is greater than 10 x 10^3 WBC/mm3 , dilute the sample with 1X PBS (calcium- and magnesium-free) with 0,5% fetal calf serum (FCS) and 0.1% sodium azide. The analysis requires 100 µl of the whole blood sample per tube, assuming a normal range of approximately 4 to 10 x 10^3 leucocytes per µl.

1. Pipette 20 µL of reagent PNH kit into the tube.
2. Pipette 100 µL of well-mixed sample into each tube. If necessary, use PBS with 0,5% FCS and 0,1% NaN_3 to reach a final volume of 100 µl per tube.
3. Cap tubes and vortex gently to mix. Incubate for 15 minutes at room temperature in the dark.
4. Add lysing solution to each tube (volume according to the manufacturer).
5. Cap tubes and vortex gently. Incubate for 10 minutes at room temperature in the dark.
6. Centrifuge for 5 minutes at 540 g.
7. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet (must remain 50 µl of residual volume)
8. Add 2 ml of PBS with 0,5% FCS and 0,1% NaN_3
9. Cap tubes and vortex gently to mix.
10. Centrifuge for 5 minutes at 540 g.
11. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet (must remain 50 µl of residual volume)
12. Re-suspend the cell pellet in 200 µl of PBS with 0,5% FCS and 0,1% NaN_3
13. Run samples within 1 hours of staining or store at 4° C (maximally for 3 h until measured in the flow cytometer. If samples are not analyzed immediately, vortex thoroughly just before acquisition.

FOR MORE INFORMATION, PLEASE VISIT OUR WEBSITE: www.immunostep.com

Example:



The histograms are biparametric representations (Side Scatter versus Fluorescence Intensity) of a lysate stabilized whole-blood PNH sample gated on Leukocytes. Human peripheral blood was stained with PNH kit and Flaer FITC. Monocytes are represented by the yellow color and Neutrophils by green color. Cells were analyzed on a FACSCanto II (Becton Dickinson, San Jose, CA) flow cytometer.

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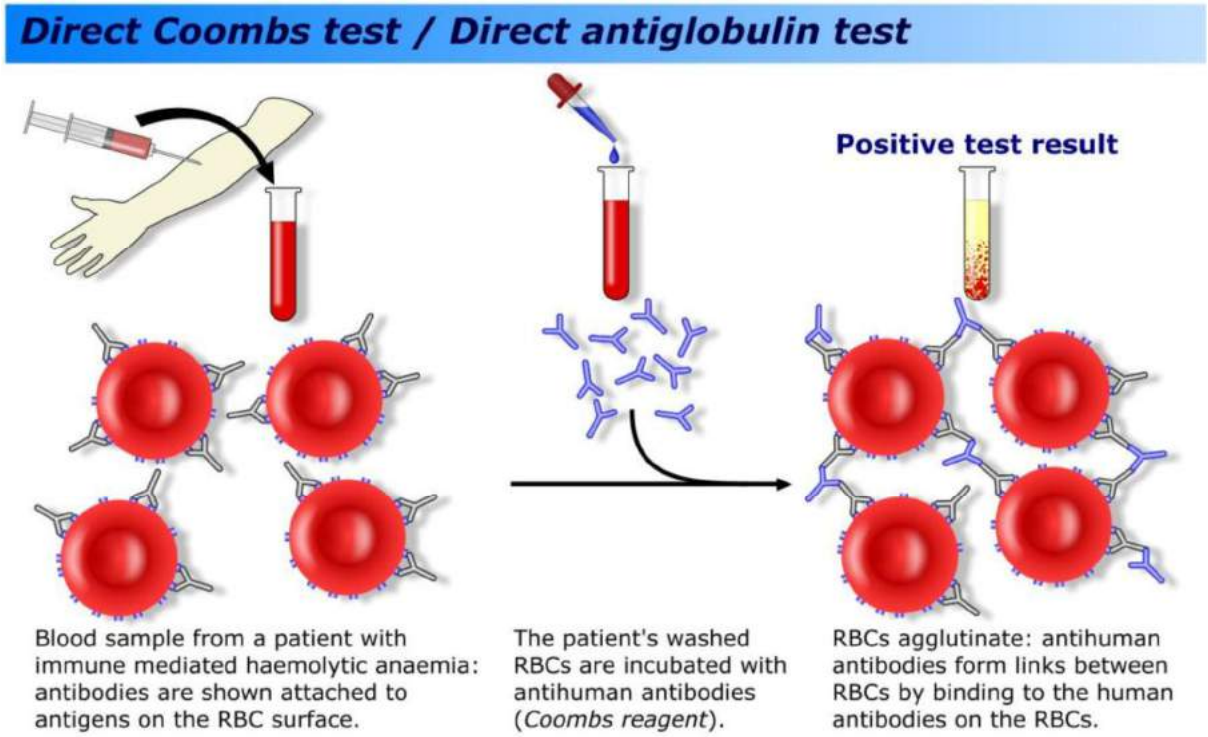
12. **Erythrocyte Hemolysis Assessments and Direct Coomb's Test**

Coomb's Test (The Antiglobulin Test) It refers to clinical blood tests that used in immunohematology to find certain antibodies which cause autoimmune hemolysis of RBCs.

Types of Coomb's Test 1. Direct Coomb's Test -The patient's cells, after careful washing are tested for sensitization that has occurred in vivo (inside the body). -This test is done on the newborn's blood sample, usually in the setting of a newborn with jaundice. -The test is looking for foreign antibodies that are already adhered to the infant's RBCs, a potential cause of hemolysis.
-A positive DAT may also be caused by the presence of allo-antibodies (e.g. owing to a delayed hemolytic transfusion reaction), so details of any transfusion in the past months must be checked for.



2. Indirect Coomb's Test
Normal RBCs are incubated with a serum suspected of containing an antibody and subsequently tested, after washing for in vitro-bound antibody.

Aims of the Test -To detect incomplete Rh antibodies. -IgG antibodies sensitizing RBCs but cannot agglutinate RBCs suspended in saline, while IgM antibodies agglutinate saline-suspended RBCs completely.

Principle of the Test -In certain diseases or conditions, an individual's blood may contain IgG antibodies that can specifically bind to antigens on the RBC surface membrane. -RBCs coated

with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement. -In order for agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the C3b or C3d component of complement, must be added to the system. -Because antibodies are gamma globulins, they can form bridges between RBCs sensitized with antibodies and cause them to agglutinate.

Materials Phosphate Buffered Saline (PBS): NaCl 0.9%, pH 7.0 ± 0.2 at 22ºC ± 1ºC. IgG sensitized red cells Inert antibody serum Weak anti-D Water bath or dry heat incubator equilibrated to 37ºC ± 2ºC. Coomb's cell washer Low Ionic Strength Solution (LISS)

Procedure

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

13. Hemoglobin Electrophoresis

Electrophoresis is a process by which molecules can be separated according to their molecular weight and electrical charge by applying electric current to them. Each molecule travels through the medium at a different rate depending on its size and charge, and on the type of medium used. There are different types of media for electrophoresis e.g. agarose gel, acrylamide gel, cellulose acetate and paper.

Hemoglobin Electrophoresis

Hemoglobin electrophoresis at pH 8.4-8.6 using cellulose acetate membrane is a rapid, simple, reliable confirmatory test. It is satisfactory for the detection of the most common clinically important hemoglobin variants.

Principle

At alkaline pH, hemoglobin is a negatively charged protein and when subjected to electrophoresis will migrate toward the anode (+). Structural variants have a change in the charge on the surface of their molecule. This will cause them to separate from Hb A at alkaline pH. Migration of molecules depends on their molecular weight and electrical charge. The higher the negative charge the faster the movement.

Notes:

- Hb A has the highest amount of negative charges, and thus it is the fastest in migration.
- Hb A is soluble, has a low molecular weight and has a high negative charge.
- Hb S is insoluble, has a low molecular weight and a lower negative charge.

Equipment

- Electrophoresis tank and power source
- Wicks of filter
- Blotting paper
- Applicators
- Cellulose acetate membrane.

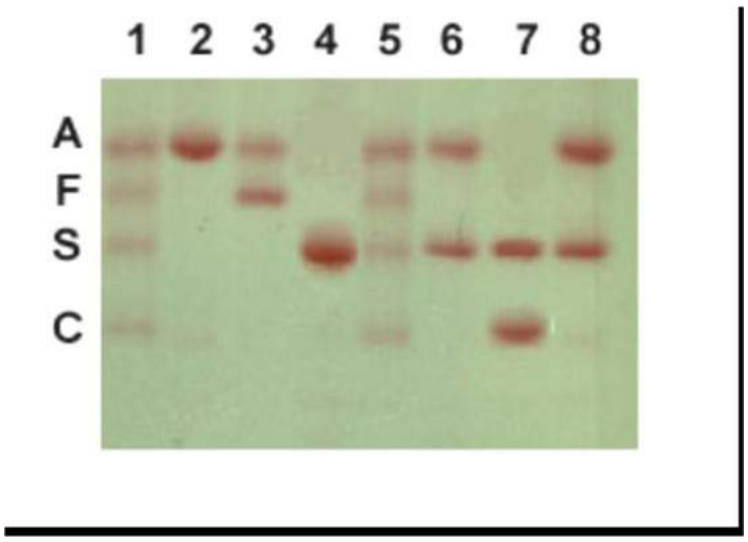
Reagents

- Electrophoresis buffer: tris EDTA/ borate (TEB) pH 8.5
- Wetting agent
- Ponceau stain
- Destaining solution: 5% acetic acid
- Hemolysing agent

Method

1. Centrifuge samples at 1200g for 5 min. Dilute 20 µl of packed cells with 150 µl of the hemolyzing agent. Mix gently and leave for 5 min. if purified hemolysates are used, 40 µl of 10 g/dl hemolysate with the 150 µl of lysing agent.
2. With the power supply disconnected, prepare the electrophoresis tank by placing equal amounts of TEB buffer in each of the outer buffer compartments. Wet 2 chamber wicks in the buffer, and place one along each divider/bridge support ensuring that they make good contact with the buffer.

3. Soak the cellulose acetate by lowering it slowly into a reservoir of buffer; leave the cellulose acetate to soak for 5 min before use.
4. Fill the sample well plate with 5 µl of each diluted sample/control and cover with coverslip or a short glad slide to prevent evaporation. Load a second sample well plate with the wetting agent.
5. Clean the applicator tips immediately prior to use.
6. Remove the cellulose strip from the buffer and blot twice between two layers of blotting paper.
7. Load the applicator by depressing the tips into the sample wells twice, and dispense this first load. Reload the applicator and apply samples into cellulose acetate.
8. Place the cellulose acetate plates across the bridges, with the plastic side uppermost. Place two glass slides across the strip to maintain good contact. Electrophorese at 350 V for 25 min.
9. After 25 min electrophoresis, immediately transfer the cellulose acetate to ponceau S and fix and stain for 5 min.
10. Remove excess stain by washing for 5 min in the 1st acetic acid reservoir and for 10 min in each of the remaining two. Blot once, using blotting paper and leave to dry.
11. Label the membranes and store in protective plastic envelope.



Automated Hb Electrophoresis

In today's modern laboratories, there are automated electrophoresis machines which offer a new level of continuous processing electrophoresis automation. With true walk-away convenience, the system requires technologist intervention only to load samples, reagents, and slides, and to start the run. The technologist reviews and prints results when processing is complete. No instrument monitoring is necessary and the technologist does not have to physically move gels around. These instruments perform all electrophoretic phases, including sample application, migration, staining, destaining, clearing, drying, and scanning in a fully automated process.

Atlas Medical CE

G6PD QUALITATIVE KIT

IVD *For In-Vitro diagnostic and professional use only*

2℃ 8℃ Store at 2-8 °C.

INTENDED USE

The G-6-PD assay is an enzymatic method for the qualitative determination of G-6-PD activity in dried blood spots and whole blood specimen. The test is intended for use as a screening method for red cell glucose-6-phosphate dehydrogenase deficiency in newborns and adults.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD or G6PDH) is a cytosolic enzyme that catalyzes the chemical reaction. This enzyme participates in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells (such as erythrocytes) by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione in these cells that helps protect the red blood cells against oxidative damage from compounds like hydrogen peroxide. Of greater quantitative importance is the production of NADPH for tissues involved in biosynthesis of fatty acids or isoprenoids, such as the liver, mammary glands, adipose tissue, and the adrenal glands. G6PD reduces NADP+ to NADPH while oxidizing glucose-6-phosphate. Clinically, an X-linked genetic deficiency of G6PD predisposes a person to non-immune hemolytic anemia.

TEST PRINCIPLE

The G-6-PD Assay utilizes glucose-6-phosphate-dehydrogenase, which in the presence of NADP, catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate. The NADPH produced is directly proportional to the concentration of Glucose-6-phosphate dehydrogenase present in the sample. The NADPH produced fluoresces under long wave UV lamps, the intention of fluorescence being proportional to the activity of the enzyme in the sample.

The assay procedure is according to reaction described by Beutler.

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{G-6-PD}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

The NADPH produced in the reaction fluoresces under long-wave UV-light. If there is a marked deficiency of this enzyme, or if G-6-PDH is lacking entirely, no fluorescence will be observed.

MATERIALS

MATERIALS PROVIDED

- **Reagent lyophilized:**

Contents of solution	Concentration in the test
Glucose-6-phosphate	1 mmol/l
NADP	0,75 mmol/l
GSSG (oxidized glutathione)	0,8 mmol/l
Saponin	0,2%
Tris(hydroxymethyl)-aminomethane	225 mmol/l, pH 7.8

- **Dilution buffer** is ready to use.
- **Filter paper.**(Optional)

MATERIALS NOT PROVIDED

- Timer.
- G-6-PDH Controls.
- Single or multichannel automatic pipettes.

PRECAUTIONS

- Wear protective clothing and disposable gloves when dealing with samples and reagents. Wash hands after operations.
- Do not use reagents beyond the labeled expiry date.
- Do not mix or use components from kits with different batch codes.
- The used tests, specimens and potentially contaminated materials should be discarded according to the local regulations.
- Dispose of all specimens and materials used to perform the test as bio hazardous waste.
- Follow the instructions for use carefully before testing.
- All reagents contain (0.1%) sodium azide which is toxic and can be absorbed through the skin. When drained, the drains should be thoroughly flushed with water.
- Don't use the kit if damaged or the glass vials are broken or leaking and discard the contents immediately.
- Don't use these reagents if the label is not available or damaged.
- Do not use any solutions that have become turbid or discolored.

PREPARATION AND STABILITY OF REAGENT SOLUTION

1. Dissolve the contents of reagent lyophilized bottle (one bottle) with 25 mL of Dilution buffer (one bottle).
2. Reagents stable for four weeks at 2-8°C or two months at -20 °C after reconstitution. Stable for 24 months lyophilized in unopened vial at 2-8°C (See Expiry Date on label).
3. Dilution buffer stable for 24 months at 2-8°C (See Expiry date on the label).

SAMPLE COLLECTION HANDLING AND STORAGE

A. DRIED BLOOD SPOTS (DBS):

1. Collect dried blood spots sample from the infant's heel or whole blood.
2. Apply a drop of blood on filter paper to absorbent and let dry completely.
3. After the sample is taken and the blood has dried, the cards must be stored at 2-8°C.
   NOTE: Spots not stored under these conditions gradually lose the enzyme activity due to heat inactivation, causing potential risk of misclassifying samples as screen-positive.

B. WHOLE BLOOD SPECIMEN:

Whole blood may be used instead of dried blood. Heparin, citrate, oxalate and EDTA are suitable anticoagulants. Do not freeze blood. It is recommended to store blood specimen in the refrigerator and use them within 3-4 days after collection. Use 0,005 ml (5 microliters) for the assay.

PROCEDURE

1. Punch out a disk of blood-stained paper (5 mm in diameter) (3 mm can also be used) and introduce into a vial (volume 1-3 ml). In case of using whole blood sample, transfer 5 µl of the blood sample to the vial.
2. Transfer 5 µl of each normal and deficient Control (not provided in the kit) to separate vials.
3. Transfer 0.1 ml of reagent solution to each vial.
4. Mix well then incubate for 10 minutes at 25°C.
   NOTE: Test Solution (One blood disk or whole blood sample with reagent solution).
5. Transfer 0.01 ml of the test solution to a new filter paper.
6. When the filter paper is dry (approximately after 1 hour), view under a long-wave UV-lamp in a darkened room.

INTERFERING OF THE RESULTS-EXPECTED VALUES

- Normal Control will show strong fluorescence after 10 min incubation. Deficient control will show very weak or no fluorescence after 10 min incubation.
- Samples obtained from normal or slightly reduced G-6-PDH activity will show strong fluorescence. Failure to fluoresce after 10 minute incubation suggests a total or marked deficiency of G-6-PDH.
  Each sample is classified according to the obtained result as follows:
  Totally Deficient: Very weak or no fluorescence after 10 min incubation
  Partially Deficient: Weak to moderate fluorescence after 10 min incubation
  Normal: Strong fluorescence after 10 min incubation.

- It is highly recommended to repeat the examination of all the classified Deficient neonates after a period of 6 months.
- It is recommended that samples which have been determined as deficient or intermediate by this procedure be assayed by a quantitative G-6-PDH.

PERFORMANCE CHARACTERISTICS

1. Accuracy:

This kit was compared against the commercial kit and showed an excellent correlation of the results obtained (R -squared > 0.99).

2. Sensitivity:

In a study performed in our laboratory, we evaluated the G6PD residual enzymatic activity in two hundred and thirty three samples concomitantly using a quantitative kit and our qualitative kit .The values of the samples, as measured by the quantitative kit ranged from 0.1 U/g Hb to 13.8 U/g Hb. The fluorescence of the samples was compared to the values obtained and it was shown that the sensitivity of the kit is 2.5 U/g Hb when the assay is performed according to the instructions. Samples with lower activity would show the same fluorescence as a 2.5 U/g Hb sample.

3. Specificity:

The oxidation of glucose-6-phosphate by G6PDH is specific. Any nonspecific formation of NADPH due to oxidation of other substrates due to endogenous enzymes occurs during the elution time while the other substrates are exhausted.

4. QC Requirements:

It is not recommended to run the test at temperatures lower than 24°C.

5. Reproducibility

Normal, deficient, and intermediate samples were assayed three times over a period of several days. Results obtained for each of the samples were identical for the replicate assays.

6. Correlation

Two hundred and thirty three (233) samples including normal, intermediate and deficient enzyme levels were assayed simultaneously by G-P-PD Qualitative kit and Quantitative kit. All samples were identified similarly by the two test kits.

7. Interfering Substances:

The effects of bilirubin, triglyceride and protein, at concentrations which mimic severely icteric, lipemic, and abnormal protein specimens, were determined by spiking whole blood and preparing dried blood spot specimens.

Substance	Amount (mg/dL)	G6PD (U/gHb)	Recovery (%)
Unconjugated Bilirubin	0	17.77	
	40	16.15	90.87
Conjugated Bilirubin	0	17.77	
	40	17.18	96.66
Triglycerides (Liposyn II)	0	18.32	
	1000	17.49	95.46
gG	0	18.20	
	2500	17.73	97.43

The effect of these substances on the assay's results is marginal (2.57 – 9.13%) and will never lead to the misclassification of a positive (Deficient) sample.

LIMITATIONS OF THE EXAMINATION PREOCEDURE

Low results are not diagnostic per se of G-6-PD deficiency but indicate the need for further study of the newborn from which a presumptive screen positive sample was received.

1. In some forms of G-6-PDH deficiency, young erythrocytes manifest normal enzyme activity. Blood from patients who have just experienced a hemolytic crisis must first be treated by the procedure described by Herz et.al (4) to separate the older erythrocytes from the prevailing population of young ones. Use 0,005 ml of the suspension so obtained for the assay.
2. If the patient has received a blood transfusion this test is clinically significant only after 30 days have elapsed because the donor's erythrocytes generally manifest a normal G-6-PDH activity and can thus bias the result before the expiration of this time.

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PPI1897A01
Rev A (02.09.2019)

REF	Catalogue Number		Temperature limit
IVD	*In Vitro* diagnostic medical device		Caution
	Contains sufficient for <n> tests and Relative size		Consult instructions for use (IFU)
LOT	Batch code		Manufacturer
	Fragile, handle with care		Use-by date
	Manufacturer fax number		Do not use if the package is damaged
	Manufacturer telephone number		Date of Manufacture
	Keep away from sunlight		Keep dry

15. RADIAL IMMUNODIFFUSION

This protocol is based on the EDVOTEK® protocol "Radial Immunodiffusion". 10 groups of students

1. EXPERIMENT OBJECTIVE

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

2. EXPERIMENT COMPONENTS for 10 groups of students

COMPONENTS	Store
A. Antibody solution	4-8ºC
B. Standard antigen solution	4-8ºC
C. UltraSpec-Agarose™	4-8ºC
D. Buffer powder	4-8ºC
E. Unknown concentration of antigen	4-8ºC
1 Sleeve Petri dishes	
2 10 ml pipettes	
10 Well cutters	
80 Transfer pipettes	
70 Microtest tubes	
1 Graph paper templates	
1 Practice loading solution	

NOTE: Store all components at the indicated temperatures upon receipt.

NOTE: No human material is used in this practice.

NOTE: All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

2.1 Requirements

- Automatic Micropipets and Tips (5-50 µl)
- Pipet Pumps (for 10 ml pipets)
- Ruler
- Plastic Box or Dish
- Plastic Wrap
- Foil
- Paper Towels
- Distilled Water
- Heat plate, Bunsen burner, or microwave
- 400 to 600 ml beaker or Erlenmeyer flask

- 150 ml beaker or flask
- Water bath
- 250 ml Graduated Cylinder
- 37°C Incubation Oven

NOTE: Make sure glassware is clean, dry, and free of soap residue. For convenience, you can buy additional disposable transfer pipettes to the steps of extraction and washing liquids.

3. BACKGROUND INFORMATION

Radial immunodiffusion

The fundamental reaction of immunology involves the interaction of **antibodies (Ab)** and **antigens (Ag)**. These interactions are useful in the defense of the body against bacterial and viral infections and toxins. The defense capabilities are dependent upon the recognition of antigens by humoral components of the immune system. Specific antibodies are then produced in response to exposure to the antigen.

The formation of **antigen-antibody complexes** is the first step in removing infectious agents from the body. Because each antibody can bind more than one antigen and each antigen can be bound by more than one antibody molecule, very large macromolecular complexes can form. These complexes form precipitates which can be cleared from the body through various means. These precipitates are also useful for laboratory and diagnostic tests.

When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate at the interface of their diffusion fronts. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing the various antibody-antigen reactions in a system.

Double diffusion in two dimensions is a simple procedure invented by the Swedish scientist, Ouchterlony. Antigen and antibody solutions are placed in separate wells cut in an agarose plate. The reactants diffuse from the wells toward each other and precipitate where they meet at equivalent proportions. A single antigen will combine with its homologous antibody to form a single precipitation line.

Radial immunodiffusion (RID) is a technique that can quantitatively deter- mine the concentration of an antigen. Unlike many gel and liquid precipitation techniques which qualitatively detect antigen, RID is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins.

Antibody is incorporated into molten agarose which is poured into a Petri dish and allowed to solidify. Small wells are cut into the agarose and are filled with known concentrations of antigen which corresponds to the antibody in the agarose. Samples of unknown concentrations are placed in similar wells. The antigens in solution then diffuse outwards from the well in a circular pattern surrounding the well. Antibody is present in excess and diffusion of the antigen will continue until a stable ring of antigen-antibody precipitate forms. There are antigen-antibody complexes throughout the zone surrounding the well within the precipitin line. At the precipitin line is where the greatest number of complexes can be found because the antigen and antibody are present in roughly equal proportions. This is known as the **equivalence zone** or **equivalence point**. Generally, it takes 24 to 48 hours for optimal diffusion to occur and precipitation to become apparent.

For each antigen, an endpoint precipitation ring of a certain diameter will form. From the known standard concentrations, a standard curve can be drawn by plotting antigen concentration versus the diameter squared measurements of the rings. From this linear calibration curve the concentration of the unknown antigen samples may be determined.

4. EXPERIMENTAL PROCEDURES

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

4.1 Laboratory safety

No human material is used in this experiment.

1. Gloves and safety goggles should be worn at all times as good laboratory practice.

2. NOT PIPETTE WITH THE MOUTH, use appropriate devices.

3. Exercise caution when working with equipment using together heat and mix of reagents.

4. Wash hands with soap and water after working in the laboratory or after using biological reagents and materials.

5. Be careful when using any electrical equipment in the laboratory.

6. Dispose of RID plates using appropriate laboratory waste disposal procedures.

If you are unsure of something, ASK YOUR INSTRUCTOR

4.2 Approximate time requirements for pre-lab and experimental procedures

Your individual schedule and time requirements will determine when the RID plates should be prepared. It takes approximately 30 minutes to prepare the plates (generally 10 minutes of this time is required for solidification).

Students can prepare the plates, if time allows.

4.3 PreLab Preparations

Notes preparations teacher practice

The class size, length of classes of practices and equipment availability are factors that must be considered in the planning and implementation of this practice with their students. These guidelines can be adapted to fit your specific circumstances.

Laboratory notebooks:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Registration laboratory activities

Students must register in their book practices the activities listed below.

Before starting the experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

General instructions

PREPARATIONS BEFORE PRACTICE PREPARE AGAROSE IN BUFFER

1. In a 400 to 600 ml beaker or Erlenmeyer flask, add entire contents of buffer powder package (component D) to 200 ml distilled water. Swirl the flask until the powder is in solution. Remove 50 ml for use as dilution buffer to a separate beaker.
2. Add the entire contents of agarose package (component C) to the flask or beaker containing 150 ml of buffer. Swirl to disperse large clumps. With a marking pen, indicate the level of solution volume on the outside of the flask or beaker.
3. The solution must be boiled to dissolve the agarose. This can be accomplished with a hot plate or microwave. Cover the beaker with foil and heat the mixture to boiling over the burner with occasional swirling. Wear safety goggles and use hot gloves. Boil until all the agarose is dissolved. Check to make sure that there are no small, clear particles of agarose. The final solution should be clear.

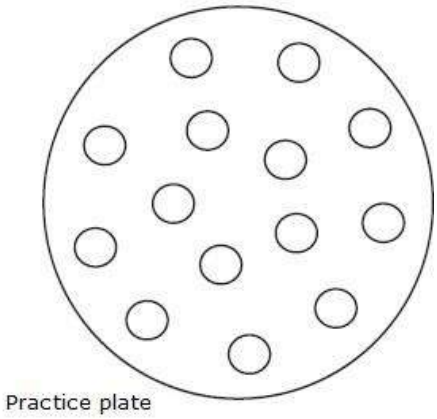
   Heat the mixture to dissolve the agarose powder. The final solution should be clear (like water) without any undissolved particles.

   A. Microwave method:
      - Cover flask with plastic wrap to minimize evaporation.
      - Heat the mixture on High for 1 minute.
      - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

   B. Hot plate method:
      - Cover the flask with foil to prevent excess evaporation.
      - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.
4. If detectable evaporation has occurred, add hot distilled water to adjust the volume of solution up to the original level as marked on the flask or beaker in step 2. Do not use cool water, or the agarose solution may cool too quickly and prematurely solidify.
5. Cool the agarose solution to 55°C in a waterbath. Swirl occasionally while cooling.

PREPARATION OF PRACTICE PLATES

1. If practice plates are to be made, pipette 2.5 ml of molten agarose into each of 10 petri dishes with a 10 ml pipette. Gently spread the agarose with the pipette on the bottom of the plate to cover the entire surface. Return the remaining agarose to waterbath.
2. Allow agarose plates to set up and cool. Refrigerate if plates are not to be used within a few hours.



Practice plate

PREPARATION OF ANTIBODY PLATES

1. Pour 26 ml of molten agarose solution to a large tube or flask.
2. Add entire contents of Antibody Solution (Component A) to the 26 ml warm agarose solution. With a pipet, stir the solution to mix. Keep the solution warm (such as in the 55°C waterbath) so it does not prematurely set up. The antibody concentration will be 1 mg/ml.

NOTE: Ensure that the temperature of the agarose solution is at 55°C by adding the Antibody Solution to prevent degradation of the antibodies.

3. With a 10 ml pipette, dispense 2.5 ml into the bottom of each Petri dish, gently spread the agarose with the pipette to cover the bottom. Allow the agarose to solidify. This will take approximately 10 minutes. If the plates are not to be used the day of the preparation, they can be wrapped in plastic wrap and stored in the refrigerator for no longer than one week.
4. Each group requires 1 antibody plate and 1 practice plate.

PREPARATIONS ON THE PRACTICE DAY PREPARATION OF ANTIGENS

Students will prepare serial dilutions of the Standard Antigen Solution (Component B) to determine the standard curve.

6. Label 10 microtest tubes with “Standard”.
7. Label 10 microtest tubes with “Unknown”.
8. Label 10 microtest tubes with “Buffer”.
9. Aliquot 75 µl of Standard Antigen Solution (Component B) into each tube labeled “Standard”.
10. Aliquot 10 µl of Unknown Antigen Solution (Component E) into each tube labeled “Unknown”.
11. Aliquot 1 ml of Buffer (retained from plate preparation step) into each tube labeled “Buffer”.
12. Each group requires one tube each of Standard, Unknown, and Buffer.

NOTE: Solutions can be aliquoted before the day of practice, in which case they should be stored at 4-8°C until the day of practice.

PREPARATION OF INCUBATION CHAMBER

1. Obtain plastic container or dish with lid. If a lid is not available, the container may be covered with plastic wrap.
2. Line the bottom of the container with several paper towels. Add distilled water to the towels to saturate. There should not be any liquid above the paper towels. All the liquid should be absorbed into the towels. Cover the chamber with the lid or plastic wrap.

4.4 Material that should receive each group

Distribute the following to each student group, or set up a work station for students to share materials.

- 1 tube Buffer
- 1 tube Standard
- 1 tube Unknown
- 4 microtest tubes
- 1 practice plate
- 1 experimental RID plate
- 1 well cutter
- 1 template
- Micropipetting device and tips (or 8 transfer pipets)
- Graph paper
- Ruler
- Marking pen

4.5 Avoiding common pitfalls

1. Follow instructions carefully when preparing gels. Make sure the agarose is completely dissolved.
2. Make neat, clean wells with the well cutters. Take measures to ensure that the wells are properly spaced according to the template on page 5.
3. Add samples to the wells carefully and precisely. Avoid overfilling the wells.
4. Do not tip or invert plates when transferring to the humidity chamber.
5. Placing the humidity chamber in a 37˚C incubation oven will expedite the formation of precipitin arcs.

5. STUDENT EXPERIMENTAL PROCEDURES

A. PREPARATION OF AGAROSE PLATES

1. Place the template under the plate so the pattern is centered.
2. Cut the wells using the well cutter (provided in the kit) in a gentle punching motion. Remove the agarose plugs with a flat-edged toothpick or spatula.

B. PREPARATING THE STANDARDS (SERIAL DILUTION)

1. Label four microtest tubes: 1:2, 1:4, 1:8, and 1:16.
2. Using a micropipet, add 50 microliters of Buffer to each tube.
3. With a fresh pipet tip, add 50 microliters of "Standard" to the tube labeled 1:2. Mix.
4. With a fresh pipet tip, transfer 50 microliters of the 1:2 dilution to the tube labeled 1:4. Mix.
5. With a fresh pipet tip, transfer 50 microliters of the 1:4 dilution to the tube labeled 1:8. Mix.
6. With a fresh pipet tip, transfer 50 microliters of the 1:8 dilution to the tube labeled 1:16. Mix.
7. There are now five antigen samples for the standard curve (see chart).

DILUTION	CONCENTRATION
Undiluted	2 mg/ml
1:2	1 mg/ml
1:4	0,5 mg/ml
1:8	0,25 mg/ml
1:16	0,125 mg/ml

C. PRACTICE WELL LOADING (OPTIONAL)

This experiment contains practice loading solution. This solution is included to allow instructors and students to practice loading the sample wells before performing the actual experiment. Use a micropipetting device or one of the plastic transfer pipettes included in the experiment to practice loading the sample wells with the practice loading solution. Make enough copies of the template for each lab group.

1. One practice plate should be prepared for every two groups. Enough reagents have been provided for this purpose.
2. Using the well cutters provided, cut several wells in the agarose as shown in the template below. Refer to Student Instructions for preparation of sample wells.
3. Practice loading the sample wells with the Practice Loading Solution using a micropipetting device. Load 5 µl per well and make sure the sample covers the entire surface of the well. If a micropipetting device is not available, use the transfer pipets provided, taking care not to overfill the wells. If using transfer pipets, put in just enough sample to cover the bottom of the well.

D. LOADING THE SAMPLES

1. On the bottom of the plate, number the wells on the perimeter of the plate 1 through 5. Leave the center well unlabeled.
2. Load wells 1 through 5 using the same pipette tip or transfer pipette. In well #5, load 5 µl of the 1:16 antigen dilution. Make sure the sample covers the entire surface of the well by carefully spreading it with the pipette tip.
3. In well #4, load 5 µl of the 1:8 antigen dilution.

4. In well #3, load 5 µl of the 1:4 antigen dilution.
5. In well #2, load 5 µl of the 1:2 antigen dilution.
6. In well #1, load 5 µl of the undiluted antigen.

NOTE: You may use the same pipette tip or transfer pipette to load wells #1 through #5, starting with the most dilute antigen dilution and ending with the most concentrated. Use a fresh tip for the unknown.

7. With a fresh pipette tip or microtipped transfer pipette, load 5 µl of your unknown in the center well.
8. Label the cover of the Petri dish with your lab group number or your initials. Place the cover on the dish, place the dish right side up (do not invert) inside the incubation chamber on the paper toweling. Cover the incubation chamber and place in a 37°C incubation oven or at room temperature for 24 to 48 hours.

E. READING THE RESULTS

The precipitin rings will be visible in 24 to 48 hours. Carefully hold a plate up so that the overhead room lights shine through it. You should be able to see opaque circles around each well where antigen and antibody have precipitated.

With a ruler, measure the diameter (through the centers of the wells) of the precipitin ring in millimeters. To plot the standard curve, square the diameter value and plot antigen concentration on the X-axis and the diameter squared on the Y-axis. Draw the best fit line through these points. Calculate the value of the unknown antigen concentration from this graph.

6. EXPERIMENTAL RESULTS

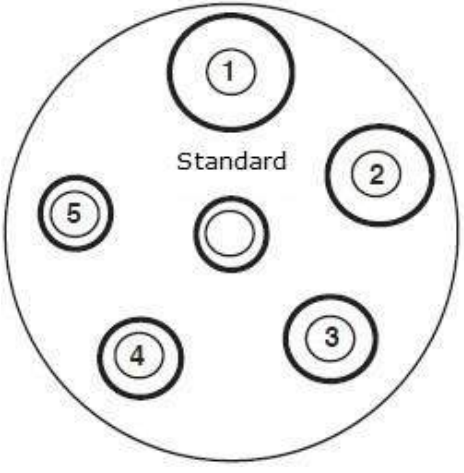
6.1 Experimental Results and Analysis

On regular graph paper, a linear standard curve should be obtained. If the curve is not linear,

the unknown concentration cannot be accurately determined. Precipitin rings will vary based on the concentration of the antigens, antibody, agarose and the time and temperature of incubation.

From the standard curve, the unknown concentration can be determined by finding the diameter squared value on the Y-axis, finding the intersecting point on the standard curve line, and obtaining the value on the X-axis. The value on the X-axis is the concentration of antigen in the solution.

The concentration provided was **0.40 mg/ml**.



6.2 Study Questions

Answer the following study questions in your laboratory notebook or on a separate

worksheet.

1. What do the circular precipitin rings represent?
2. Why do the ring sizes change until equilibrium is reached?
3. Predict the results if a very low concentration of antigen were loaded into a well. What would happen if not enough antibody was incorporated into the agarose?
4. Compare and contrast Radial Immunodiffusion with its close relative, the Ouchterlony plate technique.

16. Precipitin reaction (The Ring Test):

Precipitation ring test

Principle:

- The **ring** or **interfacial test** is a simple serological method that exemplifies the precipitin reaction in solution. This antigen-antibody reaction can be indicated by the formation of a visible precipitate, a flocculent or granular turbidity, in the test fluid. Antiserum is injected into a small-diameter test tube, and the antigen is then carefully added to form a discrete upper layer. After a period of incubation of up to 4 hours, a ring of precipitate is visible at the point of contact (interface) in the presence of the antigen-antibody reaction. The rate at which the visible ring forms relies on the concentration of antibodies in the serum and the concentration of the antigen.
- To determine the precipitation reaction, a series of dilutions of the antigen is used because both insufficient and excessive amounts of antigen will prohibit the formation of a visible precipitate. The insufficient amount of antigen gives rise to

zone of antibody excess and excessive amounts of antigen will give rise to zone of antigen excess. The visible precipitate ring is formed at zone of equivalence.

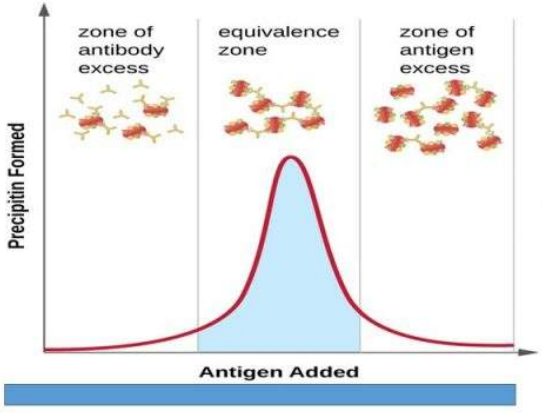


Fig. Antigen-antibody reaction on varying concentration

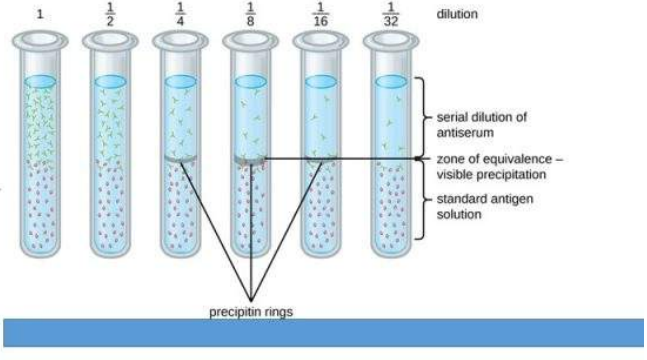


Fig: Ring test: Precipitin reactions at various dilutions

Requirements:

1. Saline (0.85%) NaCl
2. Bovine globulin antiserum
3. Normal bovine serum diluted to 1:25, 1:50, and 1:75 with physiological saline.
4. 0.5ml pipettes
5. Serological test tubes (8* 75mm)
6. Incubator
7. Serological test tube rack

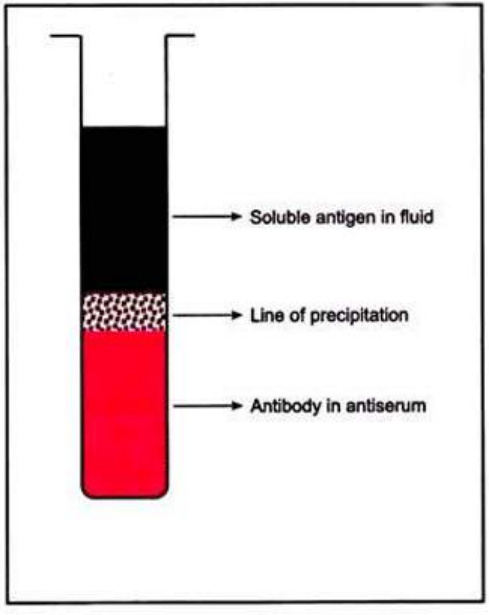
Procedure:

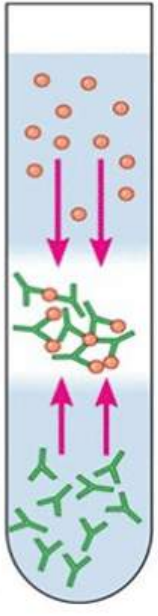
- Label three serological test tubes according to the antigen dilution to be used (1:25, 1:50, and 1:75) and the fourth test tube as a saline control.
- Transfer 0.3 ml of each of the normal bovine serum dilutions into its appropriately labelled test tube by using a different 0.5-ml pipette.
- Transfer 0.3 ml of saline into the test tube labelled as control by using a clean 0.5-ml pipette.

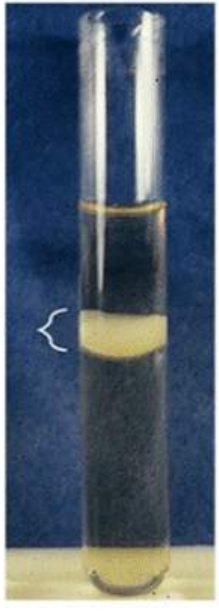
- Carefully overlay all four test tubes with 0.3 ml of bovine globulin antiserum.
- Tilt the test tube to prevent mixing of the sera, and allow the antiserum to run down the side of the test tube.
- Incubate all test tubes for 30 minutes at 37°C.
- Examine all test tubes for the development of a ring of precipitation at the interface. Indicate the presence or absence of a ring in the Lab Report.
- Determine and record the antigen dilution that produced the greatest degree of precipitation; this is indicative of the optimal antibody: antigen ratio.

Observations and Results interpretation:

- **Positive ring test:** Observation of white ring at the junction of antigen and antibody reaction indicates positive result
- **Negative test:** absence of white ring formation


Soluble antigen in fluid
Line of precipitation
Antibody in antiserum


Antigens
(soluble)
Zone of
equivalence:
visible precipitate
Antibodies



17. Quantitative of Immunoglobins in plasma (IgG and IgM) by ELISA

There are many instances within the life sciences where detection and quantification of antigens or antibodies within a sample in a timely and cost-effective manner is important. From identifying immune responses in vaccinated or infected individuals, detecting expression of a protein you wish to express on the surface of a cell, to performing quality control testing. Having a tool capable of making such assessments is key.

The enzyme-linked immunosorbent assay (ELISA) is one such test that has proven invaluable as both a research and diagnostic tool.

What is an ELISA test?

An ELISA is an immunological assay commonly used to measure antibodies or antigens, including proteins or glycoproteins, in biological samples. Like other immunoassays, they rely on binding of antibodies to their targets to facilitate detection.

Typically, ELISA assays are performed in 96-well plates, a format that makes them amenable to screening many samples at once. Serum, plasma, cell culture supernatants, cell lysates, saliva, tissue lysates and urine are all common sample types used for these assays, but most liquid sample types could be used in theory. It is, however, important to consider that some sample types may include inhibitory factors, such as buffer components that share similar antigenic epitopes[1] or factors like proteases[2] that may damage the target or detection components, that may interfere with the assay's performance.

There are several different assay formats, but all rely on binding of either the target itself or an antibody/antigen able to capture the target to the surface of the plate. A detection step involving a conjugated antigen, or more often antibody, is then employed to enable successful binding to be detected and quantified, most often by colorimetric detection.

Types of ELISA and ELISA test diagram

The ELISA was originally conceptualized, independently, in 1971 by Eva Engvall and Peter Perlman[3] at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen[4] in the Netherlands. They sought an immunoassay method able to detect the presence of antigens or antibodies to replace the radioimmunoassay, which employed potentially hazardous radioactively labeled antigens or antibodies, and thus devised an enzyme-based alternative.

There are now four main types of ELISA, direct, indirect, sandwich and competitive. The images below (Figure 1) illustrate detection of antigens; however, the same principle applies for antibody detection essentially with the roles of the antigen and primary antibody reversed.

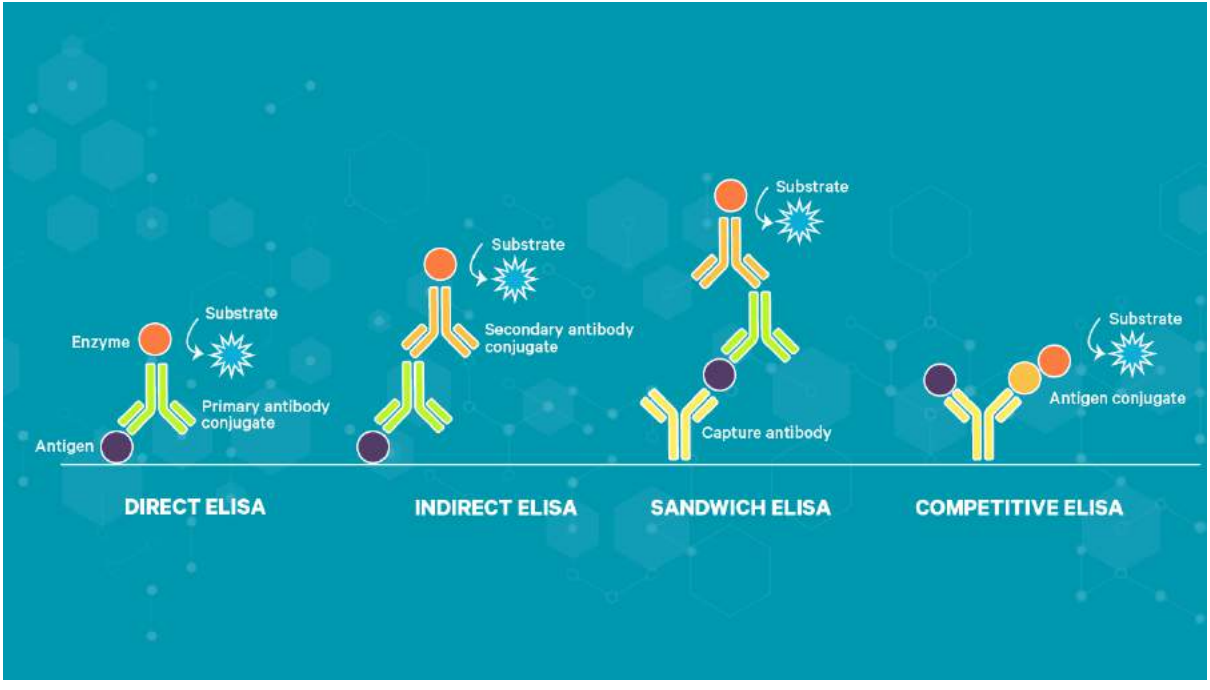

Substrate
Enzyme
Primary antibody conjugate
Antigen
DIRECT ELISA
Substrate
Secondary antibody conjugate
INDIRECT ELISA
Substrate
Capture antibody
SANDWICH ELISA
Substrate
Antigen conjugate
COMPETITIVE ELISA

Figure 1: Types of ELISA.

Direct ELISA test

With a direct ELISA, the antigens or antibodies in a sample are adsorbed directly to the test plate in a non-specific manner. A conjugated detection antibody or antigen specific for the target is then applied to the wells. Following this, a detection substrate is used to produce a measurable color change that can be quantified in a plate reader.

As this assay has few steps, it is quicker and offers less opportunity for the introduction of errors than the other ELISA methods. However, as the adsorption step is non-specific, background noise may be high. The absence of a secondary antibody step means there is no signal amplification, reducing assay sensitivity. It also requires conjugated detection antibodies/antigens to be created for each target required.

Indirect ELISA test

Originally developed in 1978[5] for the detection of human serum albumin, the indirect ELISA, or iELISA, works in a very similar way to the direct ELISA except for the addition of a secondary antibody step. This enables amplification of the test signal, overcoming the limitation of the direct ELISA. It also negates the need for target-specific conjugated detection antibodies/antigens as the conjugated secondary antibody need only be species specific for the primary antibody. Where total sample antigens are bound to the plates, like the direct ELISA, background noise remains an issue. However, if the assay is used for the detection of sample antibodies, purified target antigen is coated onto the plates, with the primary antibody coming from the sample. This greatly reduces background noise and consequently these assays are most popular for determining antibody titers in samples.

Disadvantages of the indirect ELISA include a longer protocol with more opportunities for errors and potential of cross-reactivity with the secondary antibody.

Sandwich ELISA

Developed in 1977[6], as its name suggests, the sandwich ELISA sandwiches the antigen between antibodies. The technique can employ the direct or indirect ELISA format (the sandwich ELISA depicted above is based on the indirect ELISA) except that rather than non-specific binding of antigens to the assay plate, the capture antibody makes this a specific process. This combination further improves assay sensitivity and specificity.

They do, however, require the determination of compatible capture and detection antibody pairs to function effectively with which cross-reactivity can be problematic. This assay typically has the most steps too, offering greater opportunity for error. Due to the selective nature of the antigen

binding step, sandwich ELISAs are particularly useful where the antigen is in a complex mix as antigen purification is not required.

Competitive ELISA

A competitive ELISA, also known as an inhibition ELISA or blocking ELISA, is possibly the most complex of the ELISA techniques. Originally developed in 1976[7] for the detection of human choriogonadotropin, the assay works by detecting interference to an expected output signal level, producing an inverse relationship. The more of the target there is in the sample applied, the lower the assay output signal will be. Multiple formats are possible for which the other ELISA types can be adapted into a competitive format. However, there are two general principles; the sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. Alternatively, sample antigen or antibody may compete with a labeled reference for binding to a limited amount of antibody or antigen, respectively.

The competitive ELISA will have some of the same advantages and limitations as the format from which it has been adapted. However, it can be helpful when the antigen is small, limiting the ability of two antibodies to bind concurrently, as required for the sandwich ELISA, or when only one antibody is available.

ELISA steps

While there are variations in the protocols for the different types of ELISA, there are a number of stages to the assay common to most that should be considered.

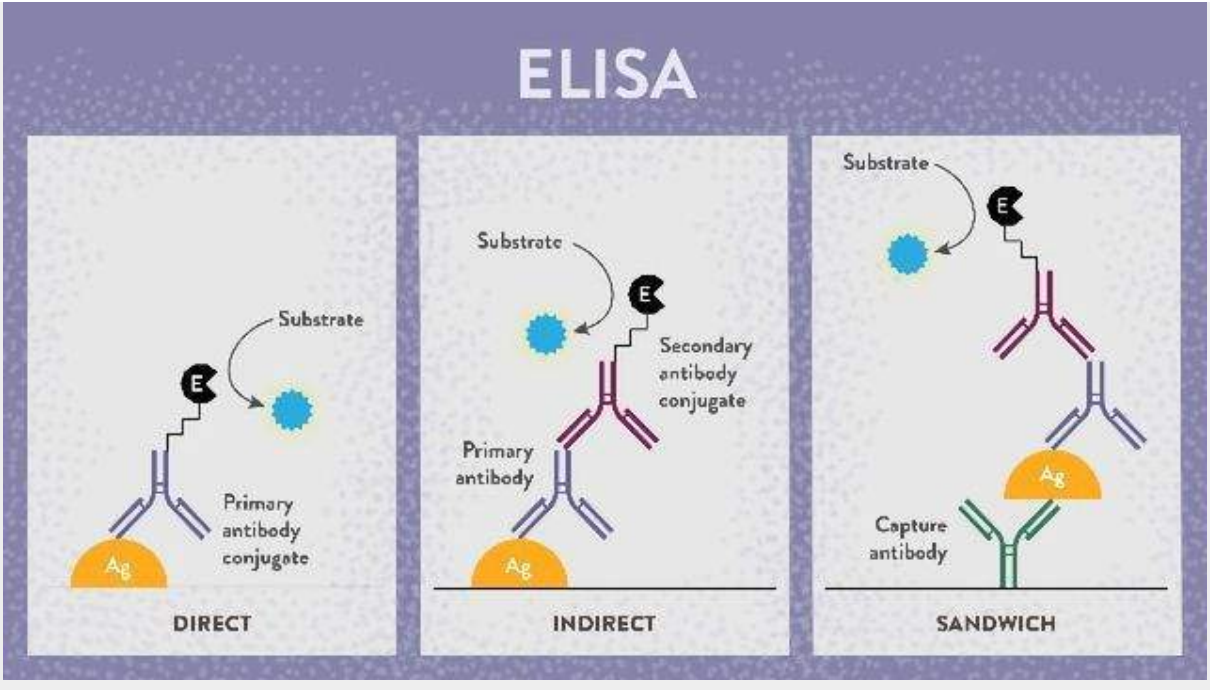

ELISA
Substrate
E
Primary antibody conjugate
Ag
DIRECT
Substrate
E
Secondary antibody conjugate
Primary antibody
Ag
INDIRECT
Substrate
E
Ag
Capture antibody
SANDWICH

Plate coating

The first step in most ELISAs is the binding of the first component of the assay to the test plate. This is often done through passive adsorption, which is a non-specific process, so the result will depend on what is applied to the plate. If antigen-containing sample is applied, then the result is a plate for which selective steps are still required as a whole variety of antigens will bind, not just those of interest. If, however, purified antigen — as in the case of an indirect ELISA for antibody detection — or capture antibody — as in the case of the sandwich ELISA — is applied, then the result is a plate that already has selective properties.

Multiple plate types, most of which are polystyrene or polystyrene derivatives, with differing binding properties are available depending on the target and nature of the assay. Some targets,

including heavily glycosylated proteins, carbohydrates, DNA, lipids, short peptides and proteins in the presence of detergents, do not adsorb well. In these cases, it is advisable to use plates that have been treated to permit covalent linkage to the surface instead.

Incubations

Following the addition of reagents to the ELISA plates, they must be incubated to allow time for binding or reactions to take place. The temperature and duration of each incubation will depend upon the assay step and the action being performed. Incubation for 1 hour at 37 °C is commonly used, for example following sample application. However, steps like blocking may be performed overnight in a fridge and incubation during detection is often performed at room temperature for much shorter times.

Washing

Plate washing is a vital step between the application of each component of the assay right up until detection. After solutions are emptied from the wells, the wells are washed with a buffer — often phosphate-buffered saline-Tween 20 (PBST) — to remove any residual unbound antigens, antibodies or reagents that remain. This may be done by hand with a multichannel pipette or using an automated plate washer. Failure to wash sufficiently may result in high background signal, whereas too much washing can result in low sample signals. Inconsistent washing is likely to introduce inconsistencies across the plate, resulting in unreliable results.

Blocking

Following the coating of ELISA plates with proteins, blocking is often necessary to prevent any non-specific binding of detection antibodies in the following protocol steps (Figure 2). Mixed proteins unrelated to the assay are added to and incubated in the plate, occupying any available non-specific binding sites. Common protein blocking buffer choices include skimmed dried milk,

bovine serum albumin (BSA) and casein. Alternatively, nonionic detergents, such as Tween 20 or Triton X-100, may be used as blocking agents but do not provide permanent blocking like proteins. Ineffective plate blocking can lead to increased background noise and reduce assay sensitivity and specificity.

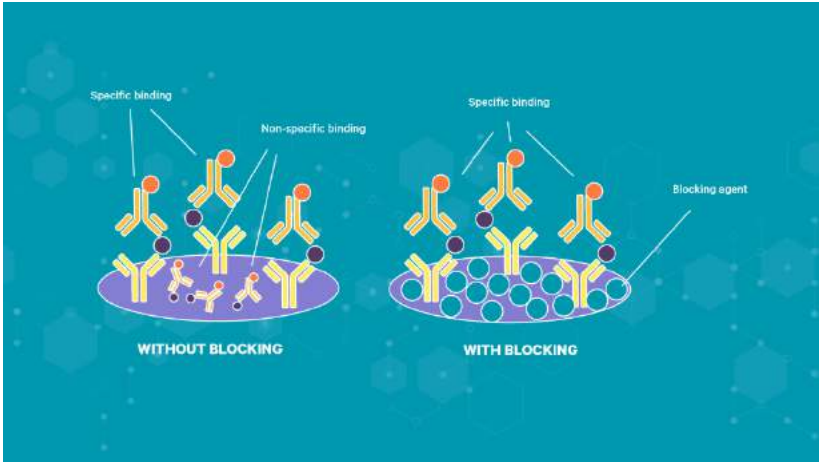


Figure 2: How the blocking process prevents non-specific binding.

Antibodies

Experimental antibodies are the cornerstone of most ELISAs and it is of paramount importance to choose the right ones, especially where multiple antibodies are used. Both monoclonal and polyclonal antibodies can be used, each with their own advantages and limitations. Monoclonal antibodies offer high specificity but are more costly. Polyclonal antibodies on the other hand can bind a target at multiple binding sites, amplifying the signal and improving sensitivity.

The use of a methods employing a secondary antibody adds extra steps, prolonging the assay time, increasing the opportunity for mistakes and requiring more optimization to find an appropriate compatible antibody pair. However, sensitivity gains from the use of a polyclonal secondary antibody may make this worthwhile or essential to the development of an effective assay.

Detection

Irrespective of the ELISA type used, all end in a detection step, most often utilizing enzyme-mediated visible color change chemistry which can then be measured using UV-Vis spectrophotometry. An enzyme-conjugated antigen or antibody is applied to test wells where it will bind if its target is present. When an appropriate substrate for the enzyme is added to the plate, it causes a color change that is proportional to the amount of target bound inside the well. Horseradish peroxidase (HRP) is a common conjugate used in partnership with the substrate 3,3',5,5'-tetramethylbenzidine (TMB), which turns blue in response to HRP and then yellow on the addition of a sulfuric acid solution that stops the reaction.

The absorbance values for each well can then be determined using a microplate reader (at 450 nm in the case of TMB following the addition of stop solution) — a type of UV-Vis spectrophotometer — and corrections and calculations, such as subtraction of average blank well values, averaging of technical replicates or ratio calculation to standards, made according to the assay design.

An example of an ELISA workflow are depicted in Figure 3.

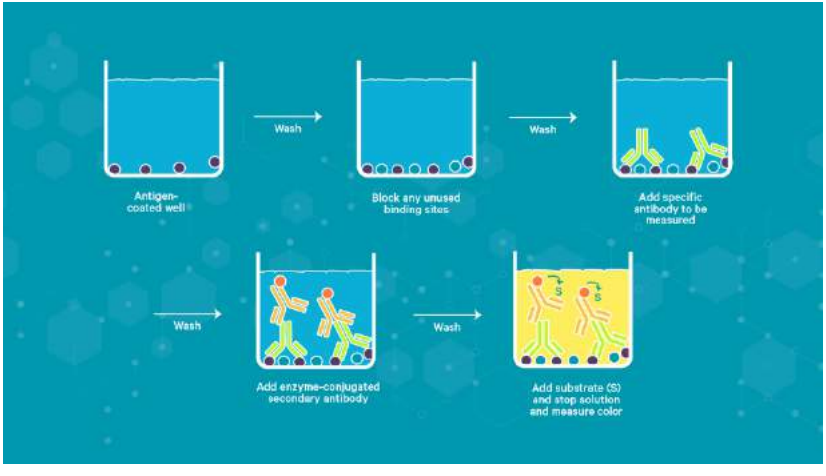


Figure 3: Example of an iELISA workflow.

ELISA test results,

ELISA results may be interpreted **quantitatively, qualitatively** or **semi-quantitatively**. In a quantitative assay, a serial dilution of a known standard is used to enable the generation of a standard curve, normally of optical density (OD) versus concentration. From this, the precise quantities of target in the unknown samples can be calculated (Figure 4).

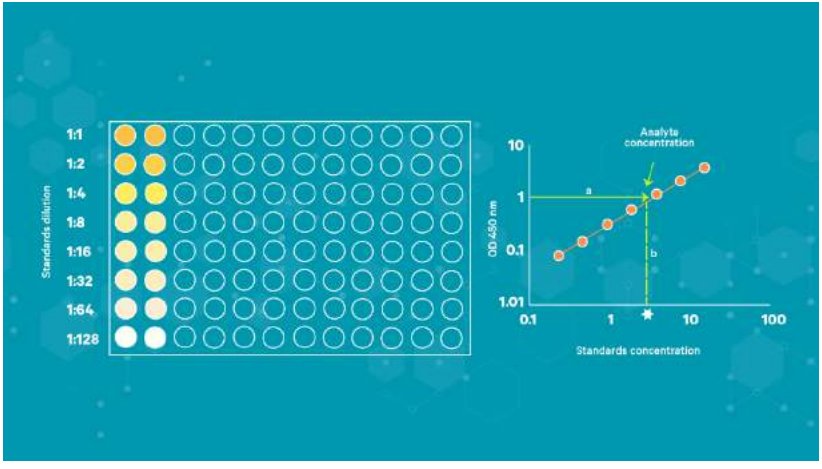


Figure 4: Example of standard curve calculation and target concentration determination of an unknown for an ELISA experiment.

In a qualitative assay, data will still normally be collected numerically from the use of a microplate reader, but results will be interpreted as positive or negative by comparison to blank and/or negative wells with no standard curve.

Semi-quantitative assays also collect numerical data without the use of a serial dilution or standard curve. However, the inclusion of both negative and positive standards, often high and low positives, facilitates the relative comparison of levels between the wells of unknown samples to those of known status. With the use of these standards to monitor assay reproducibility, a cut-off value may be set with results over the threshold determined to be positive and those below negative. Some assays may also incorporate an “amber zone”. For samples whose values fall in this region, retesting or further investigation is recommended.

While a quantitative assay may be desirable in some settings, the inclusion of a full standard curve

on each plate takes up valuable wells. Therefore, a trade-off exists depending on the information the user hopes to obtain from the assay result.

Like many assays, the results obtained from an ELISA are rarely likely to be 100% accurate, with both false positive and false negative results a possibility. Consequently, most tests will be associated with measures of sensitivity and specificity; the closer they are to 100%, the better the test.

There are many factors that can impact these values such as:

- High background from non-specific binding or cross-reactivity
- Poor affinity of antibodies for their targets
- Suboptimal assay conditions
- Sample condition and complexity

Calculating sensitivity and specificity values is therefore an important step in the development of many ELISA assays, particularly in a diagnostic setting, to determine how informative and how reliable any results obtained are.

Rat IgG/IgE ELISA Kit (Sandwich ELISA) User manual

Assay Specifications

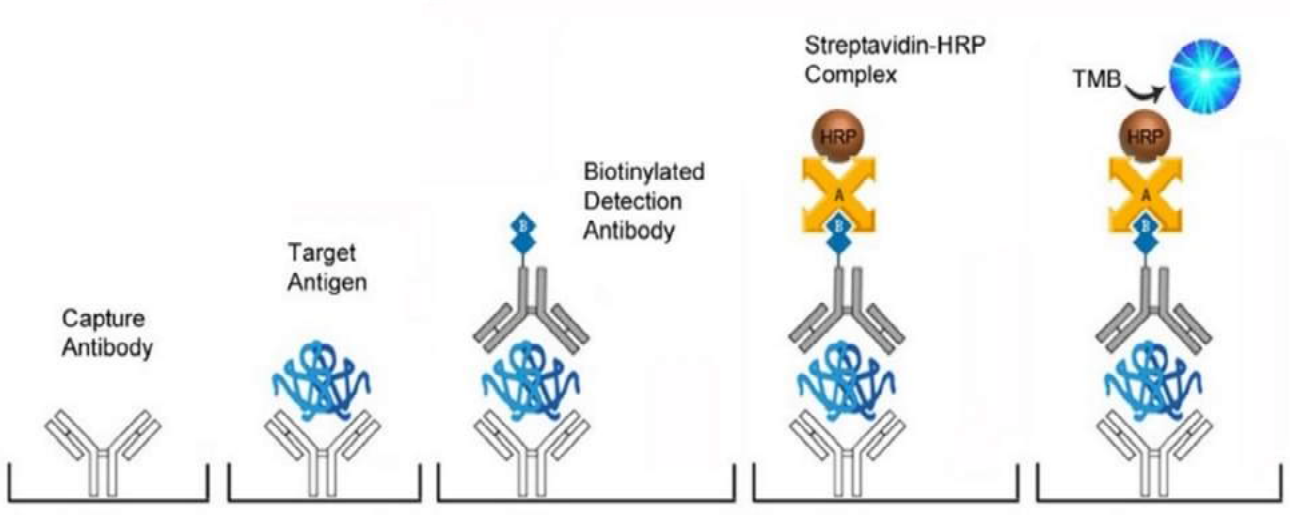
Target:	IgG
Synonyms:	IgG
Specificity:	This kit is for the detection of Rat IgG. No significant cross-reactivity or interference between IgG and analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with

	untested analogs.
Sample Types:	This kit is intended for use with samples such as Plasma and Serum. It has been empirically tested using the standard supplied with the kit (t ypically a recombinant protein).
Detection:	Colorimetric - 450nm(TMB)
Detection Range:	3.13–200 ng/ml
Sensitivity:	Typically less than 1.88 ng/ml
Performance:	Intra-Assay CV (<5.84%); Inter-Assay CV (<6.31%)
Limitations:	This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

Assay Principle

This assay is based on the sandwich ELISA principle. Each well of the supplied microtiter plate has been pre-coated with a target specific capture antibody. Standards or samples are added to the wells and the target antigen binds to the capture antibody. Unbound Standard or sample is washed away. A biotin-conjugated detection antibody is then added which binds to the captured antigen. Unbound biotinylated detection antibody is washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate is then added which binds to the biotin. Unbound Avidin-HRP conjugate is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm. An OD standard curve is generated using known antigen concentrations; the OD of an unknown sample can then be compared to the standard curve in order to determine its antigen concentration.

ASSAY PRINCIPLE IMAGE



Kit Components

Component	Quantity
Coated 96-well Strip Plate	1
Standard (Lyophilized)	2 vials
Sample Diluent	3 vial x 20 ml
Biotinylated Detection Antibody (100x)	1 vial x 120µl
Biotinylated Detection Antibody Diluent	1 vial x 14 ml
HRP Conjugate (100x)	1 vial x 120µl
HRP Conjugate Diluent	1 vial x 14 ml
Wash Buffer (25x)	1 vial x 30 ml
TMB Substrate	1 vial x 10 ml
Stop Solution	1 vial x 10 ml
Adhesive Plate Sealers	4
Instruction Manual	1

Kit Storage

Store all kit components at 4°C. Store Coated 96-well Strip Plate, Standard, Biotinylated Detection Antibody and HRP Conjugate at -20°C if the kit will not be used within 1 month. The Substrate should never be frozen. Once individual reagents are opened it is recommended that the kit be used within 1 month. Unused Strip Plate wells should be stored at -20°C in a sealed bag containing desiccant in order to minimize exposure to moisture. Do not use the kit beyond its expiration date .

Other Required Supplies

- Microplate reader with 450nm wavelength filter
- High-precision pipette and sterile pipette tips
- Eppendorf tubes
- 37°C incubator
- Deionized or distilled water
- Absorbent paper

Assay Planning

Before using this kit, researchers should consider the following:

1. Read this manual in its entirety in order to minimize the chance of error.
2. Confirm that you have the appropriate non-supplied equipment available.
3. Confirm that the species, target antigen, and sensitivity of this kit are appropriate for your intended application.
4. Confirm that your samples have been prepared appropriately based upon recommendations (see Sample Preparation) and that you have sufficient sample volume for use in the assay.
5. When first using a kit, appropriate validation steps should be taken before using valuable samples. Confirm that the kit adequately detects the target antigen in your intended sample type(s) by running control samples.

6. If the concentration of target antigen within your samples is unknown, a preliminary experiment should be run using a control sample to determine the optimal sample dilution (see Experimental Layout and Sample Preparation).
7. Ensure that the kit is properly stored and do not use it beyond its expiration date.
8. When using multiple lots of the same kit do not substitute reagents from one kit to another. Review each manual carefully as changes can occur between lots. To control for inter-assay variability include a carry-over control sample.

Experimental Layout

The following is an example of how to layout a study. A dilution series of the positive control Standard should be run in duplicate or triplicate, with the last well in each series being the negative control blank.

Samples should also be run in duplicate or triplicate. Unknown samples should be run as a dilution series in order to identify the optimal dilution that produces an OD reading within the OD range of the positive control Standard dilution series.

Example 1: Standard Curve and dilution series of an unknown sample.

	1	2	3	4
A	Standard Dilution 1	Standard Dilution 1	Sample (1:1)	Sample (1:1)
B	Standard Dilution 2	Standard Dilution 2	Sample (1:10)	Sample (1:10)
C	Standard Dilution 3	Standard Dilution 3	Sample (1:100)	Sample (1:100)

D	Standard Dilution 4	Standard Dilution 4	Sample (1:1k)	Sample (1:1k)
E	Standard Dilution 5	Standard Dilution 5	Sample (1:10k)	Sample (1:10k)
F	Standard Dilution 6	Standard Dilution 6	Sample (1:100k)	Sample (1:100k)
G	Standard Dilution 7	Standard Dilution 7	Sample (1:1,000k)	Sample (1:1,000k)
H	Negative Control	Negative Control	Sample (1:10,000k)	Sample (1:10,000k)

Example 2: Standard Curve and samples run in duplicate.

	1	2	3	4
A	Standard Dilution 1	Standard Dilution 1	Sample A	Sample E
B	Standard Dilution 2	Standard Dilution 2	Sample A	Sample E
C	Standard Dilution 3	Standard Dilution 3	Sample B	Sample F
D	Standard Dilution 4	Standard Dilution 4	Sample B	Sample F

E	Standard Dilution 5	Standard Dilution 5	Sample C	Sample G
F	Standard Dilution 6	Standard Dilution 6	Sample C	Sample G
G	Standard Dilution 7	Standard Dilution 7	Sample D	Sample H
H	Negative Control	Negative Control	Sample D	Sample H

Sample Collection

This assay is intended for use with samples such as Plasma and Serum. The sample collection protocols below have been provided for your reference.

Breast Milk - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cell Lysates - Collect and pellet the cells by centrifugation and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication 4 times. Alternatively freeze the cells to -20°C and thaw to room temperature 3 times. Centrifuge at 1500×g for 10 minutes at 2 - 8°C to remove cellular debris. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Erythrocyte Lysates - Centrifuge whole blood for 20 minutes at 1000×g to pellet the cells and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Freeze (-20°C)/thaw (room temperature) the cells 3 times. Centrifuge at 5,000×g for 10 minutes at 2-8°C

to remove cellular debris. Collect the supernatant for assaying.
Erythrocyte lysates must be diluted with Sample Dilu ent before running. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Platelet-Poor Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. It is recommended that samples should be centrifuged for 10 minutes at 10,000×g for complete platelet removal. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below.
Avoid repeated freeze-thaw cycles.

Sperm and Seminal Plasma – Allow semen to liquefy at room temperature or 37°C. After liquefaction, centrifuge at 2,000×g for 10-15 minutes. Collect seminal plasma supernatant for assaying. Wash the precipitated protein 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication then centrifuge at 2,000×g for 10-15 minutes. Collect the supernatant for

assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Tissue Homogenates–Because preparation methods for tissue homogenates vary depending upon tissue type, users should research tissue specific conditions independently. The following is one example only. Rinse tissues in PBS* to remove excess blood and weigh before homogenization. Finely mince tissues and homogenize them in 5-10mL of PBS*with a glass homogenizer on ice. Lyse the cells by ultrasonication or freeze (-20°C)/thaw (room temperature) 3 times.
Centrifuge homogenate at 5000×g for 5 minutes. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter and collect the supernatant for assaying. Store un-diluted samples at

-20°C or below. Avoid repeated freeze-thaw cycles.

Cell culture supernatants, cerebrospinal, follicular, and lung lavage fluids, saliva, sweat, tears, and other biological fluids - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

* 1xPBS (0.02mol/L pH7.0-7.2)

Sample Collection Notes

1. LifeSpan recommends that samples are used immediately upon preparation.
2. Avoid repeated freeze/thaw cycles for all samples .
3. In the event that a sample type not listed above is intended to be used with the kit, it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Due to factors including cell viability, cell number, or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18-25°C) before performing the assay without the use of extra heating.
7. Sample concentrations should be predicted before being used in the assay. If the sample

concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

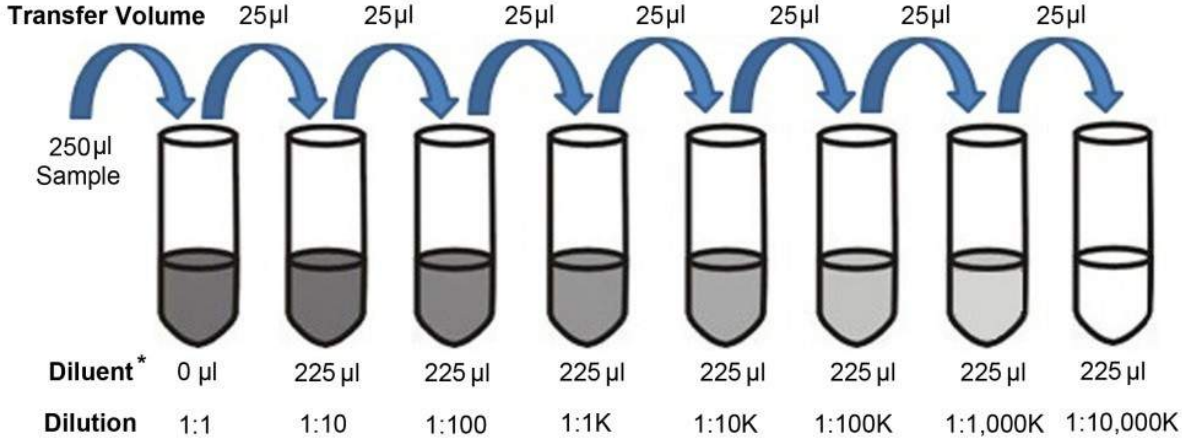
8. LifeSpan is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer-supplied samples used with the kit.

Sample Preparation

The resulting Optical Density (OD) values of your sample must fall within the OD values of the standard curve in order for the calculated antigen concentration to be accurate. In many cases samples will need to be diluted in order to lower the antigen concentration to sufficient levels. Information about antigen concentrations within various sample types may be available from the published literature; however, it is often necessary to run a dilution series of each sample type. The following will prepare sufficient volumes to run the Sample dilution series in triplicate. In the case of small volume samples, a preliminary step dilution, such as 1:5 or 1:10, can be made using PBS (0.02mol/L pH7.0-7.2) as the diluent.

* **The final dilution should always be made using the same buffer that is used to dilute the Standards, and/or generate the Standard Curve.**

Running duplicate or triplicate wells for each sample is recommended.



Standard Preparation

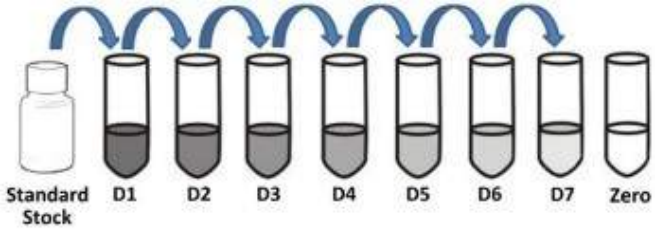
The following are instructions for the preparation of a Standard dilution series which will be used to generate the standard curve. The standard curve is then used to determine the concentration of target antigen in unknown samples (see the **Calculation of Results** section). The following will prepare sufficient volumes to run the Standard dilution series in duplicate. Reconstituted Standard and prepared standard dilutions should be used immediately and not stored for future use.

Standard Stock Solution (200 ng/ml): Briefly centrifuge the vial to ensure that all lyophilisate is collected at the bottom of the vial.
Reconstitute 1 tube of lyophilized Standard with 1.0 ml of Sample Diluent. Incubate at room temperature for 10 minutes with gentle agitation (avoid foaming).

D1 (200 ng/ml): Pipette 500µl of Stock Standard into 0µl of Sample Diluent
D2 (100 ng/ml): Pipette 250µl of D1 into 250µl of Sample Diluent **D3** (50 ng/ml): Pipette

250µl of D2 into 250µl of Sample Diluent **D4** (25 ng/ml): Pipette 250µl of D3 into 250µl of Sample Diluent **D5** (12.5 ng/ml): Pipette 250µl of D4 into 250µl of Sample Diluent **D6** (6.25 ng/ml): Pipette 250µl of D5 into 250µl of Sample Diluent **D7** (3.125 ng/ml): Pipette 250µl of D6 into 250µl of Sample Diluent

Zero Standard (0 ng/ml): Use Sample Diluent alone



Reagent Preparation

During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Bring all reagents to room temperature (18-25°C) before use.

Wash Buffer: If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved. Prepare 750 ml of Working Wash Buffer by diluting the supplied 30 ml of 25x Wash Buffer Concentrate with 720 ml of deionized or distilled water. Wash Buffer can be stored at 4°C once prepared.

1x Biotinylated Detection Antibody: Calculate the required amount needed before beginning the experiment (100µl/well) and include a 200µl excess. Centrifuge the stock tube before use. Dilute the concentrated Biotinylated Detection Antibody to the working concentration using the Biotinylated Detection Antibody Diluent (1:100).

1x HRP Conjugate: Calculate the required amount needed before beginning the experiment

(100 µl/well) and include a 200µL excess. Dilute the HRP Conjugate to the working concentration using the HRP Conjugate Diluent (1:100).

TMB Substrate Solution: Using sterile techniques remove the needed volume of TMB Substrate solution for the number of wells you are planning to run. Dispose of unused TMB Substrate solution rather than returning it to the stock container.

Reagent Preparation Notes

1. It is highly recommended that standard curves and samples are run in duplicate within each experiment.
2. Once resuspended, standards should be used immediately, and used only once. Long-term storage of reconstituted standards is NOT recommended.
3. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
4. Do not prepare Standard dilutions directly in wells.
5. Prepared reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
6. All solutions should be gently mixed prior to use.
7. Reconstitute stock reagents in strict accordance with the instructions provided.
8. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated. Pipetting

volumes of less than 10µl is not recommended.

9. TMB Substrate solution is easily contaminated; sterility precautions should be taken. TMB Substrate solution should also be protected from light.
10. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
11. Due to the antigen specificity of the antibodies used in this assay, native or recombinant proteins from other manufacturers may not be detected by this kit.

Assay Procedure

Bring all reagents and samples to room temperature with out additional heating and mix thoroughly by gently swirling before pipetting (avoid foaming). Prepare all reagents, working standards, and samples as directed in the previous sections.

1. Add 100µl of **Standard, Blank, or Sample** per well, cover with a plate sealer, and incubate for 90 minutes at 37°C.
2. Aspirate the liquid of each well, don't wash.
3. Add 100µl of **1x Biotinylated Detection Antibody** to each well, cover with a plate sealer, and gently agitate to ensure thorough mixing. Incubate for 1 hour at 37°C.
4. Aspirate the liquid from each well and wash 3 times. Wash by adding approximately 350 µl of Wash Buffer using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Allow each wash to sit for 1-2 minutes before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.

5. Add 100µl of **1x HRP Conjugate** working solution to each well, cover with a new plate sealer, and incubate for 30 minutes at 37°C.
6. Aspirate the liquid from each well and wash 5 times as outlined in step 4.
7. Add 90µl of **TMB Substrate** solution to each well, cover with a new plate sealer, and incubate for 15 minutes at 37°C. Protect from light and monitor periodically until optimal color development has been achieved.
8. Add 50µl of **Stop Solution** to each well. The blue color will change to yellow immediately. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the TMB substrate solution.
9. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

Assay Procedure Notes

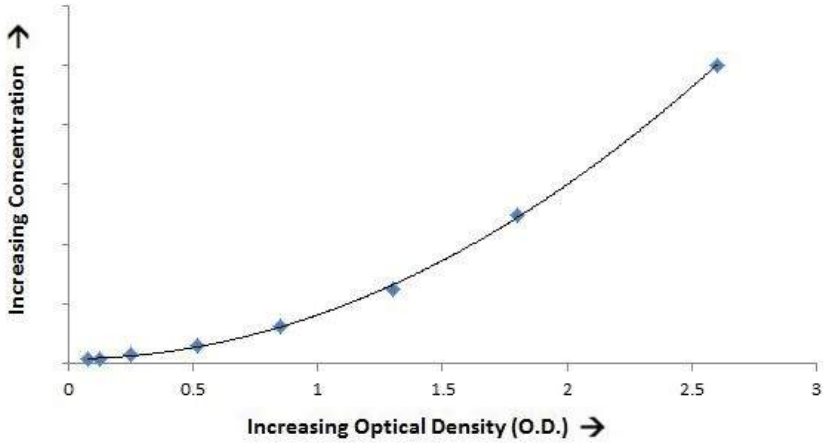
1. **ELISA Plate**: Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be placed in a sealed bag containing desiccant and stored at
   -20°C.
2. **Solutions**: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Applying Solutions:** All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
4. **Assay Timing**: The interval between adding sample to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.
5. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
6. **Washing**: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.
7. **Controlling Substrate Reaction Time**: After the addition of the TMB Substrate, periodically monitor the color development . Stop color development before the color becomes too deep by adding Stop Solution. Excessively strong color will result in inaccurate absorbance readings.
8. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings, remove any
   residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.
9. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.
10. **Stop Solution**: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
11. **Mixing**: During incubation times, the use of a micro-oscillator at low frequency is recommended. Sufficient and gentle mixing is particularly important in producing reliable results.
12. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is

performed by the same operator from the beginning to the end.

Calculation of Results

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

Typical Data: The following standard curve is an example only and should not be used to calculate results for tested samples. A new standard curve must be generated for each set of samples tested.



VIROLOGY AND PARASITOLOGY LAB

Code: MMLT 396B

Diagnostic tests in virology, Animal cell cultures, Media, sterilization, demonstration of cell line.

Animal Cell Culture: Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established.

Primary Culture: Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

Cell Line: After the first subculture, the primary culture becomes known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span (i.e., they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

Cell Strain: If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

Cell Culture Equipment

Basic Equipment

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
- Incubator (humid CO2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–20°C)
- Cell counter
- Inverted microscope
- Liquid nitrogen (N2) freezer or cryostorage container
- Sterilizer (i.e., autoclave)

Expanded Equipment

- Aspiration pump (peristaltic or vacuum)
- pH meter

- Confocal microscope
- Flow cytometer

Additional Supplies

- Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multi-well plates)
- Pipettes and pipettors
- Syringes and needles
- Waste containers
- Media, sera, and reagents
- Cells

Culture media: Today, with standardized media and sophisticated incubation conditions, culturing animal cells is considerably easier than it used to be. Since 1950s, tissue culture media were developed and conditions were worked out which closely simulate the situation in vivo. In particular, the environment is regulated with regard to the temperature, osmotic pressure, pH, essential metabolites (such as carbohydrates, amino acids, vitamins, proteins and peptides), inorganic ions, hormones and extracellular matrix. Among the biological fluids that proved successful for culturing cells, serum is the most significant. Five to twenty percents of serum is usually added to media for optimal cell growth. Serum is an extremely complicated mixture of compounds including undefined components, therefore much work has gone towards creating a chemically defined alternative to serum.

A wide variety of culture media is currently available. The choice of culture media is dependent on the requirements of cells. The components of suitable culture media include:

Basic media: The most basic media are balanced salt solutions (BSS), e.g., phosphate-buffered saline (PBS), which may be used for washing cells and for short incubations in suspension. More complex defined media are used for growth and maintenance. Defined media can also vary in complexity, by the addition of a number of constituents, e.g., from Eagle's minimum essential medium (MEM) which contains essential amino acids, vitamins and salts, to McCoy's medium, which contains a larger number of different amino acids, vitamins, minerals and other extra metabolites (such as nucleosides).

Buffering capacity: A number of supplements to the basic media are necessary to enable them to be used for culturing cells. Cell cultures have an optimum pH for growth, generally between pH 7.4- 7.7. The type of buffering that is used for the media depends on the growth conditions. When cells are incubated in a CO_2 atmosphere equilibrium is maintained between the medium and the gas phase. A bicarbonate−CO_2 buffering system is most often used due to its low toxicity towards the cells. HEPES, a much stronger buffer, may also be used, however, in this case much greater concentrations of HEPES than bicarbonate are required when used in a CO_2 atmosphere. Each type of media has a recommended bicarbonate concentration and CO_2 tension to achieve the correct pH and osmolarity. Nevertheless, this may vary slightly among laboratories, therefore, a sample of media should be left under the normal incubating conditions and monitored overnight, the buffering can then be adjusted accordingly. HEPES buffer should normally be used in conjunction with bicarbonate for which a relationship between the HEPES and bicarbonate exists for differing CO_2 levels, although, HEPES alone can maintain pH in the

absence of exogenous CO_2. The addition of 1-5 mM pyruvate to the medium increases the endogenous cellular production of CO_2 and limits the need for a CO_2 atmosphere. Some defined media have been devised for this purpose, e.g., Leibovitz L-15 medium. Cells which produce large amounts of endogenous CO_2 under certain incubation conditions may require HEPES to buffer this CO_2 product. The density of the culture may affect the CO_2 requirement, however, in general phenol red in the medium will indicate the state of the pH at any given time.

Glutamine and amino acids: In addition to buffering the medium, there are other growth requirements including amino acids, the requirement for which may vary with cell culture type. Commonly the necessary amino acids include cysteine and tyrosine, but some non-essential amino acids may be needed. Glutamine is also required by most cell lines and it has been suggested that cultured cells use glutamine as an energy and carbon source in preference to glucose, although glucose is present in most defined media. Glutamine is usually added at a final concentration of 2 mM, however, once added to the medium the glutamine is only stable for about 3 weeks at 4 °C.

Serum: Although there is much research aimed at attempting to reduce the requirement of cells for serum, by alternative supplementation of the media, it is apparent that most cell lines still require serum for adequate growth. Various sources of serum may be used such as calf, fetal calf, and horse. Many continuous cultures utilize calf serum, but often fetal calf serum provides the best growing conditions. The level of serum used depends on the particular cell line and should be determined empirically. Batches of serum may vary considerably in their ability to support cellular growth. It is therefore important to test batches of serum and have sufficient quantities of a batch that shows suitable growth supporting characteristics stored at -20 °C. To check these properties, cloning efficiency and growth characteristics (morphology, growth patterns) should be carried out over a concentration range of 2% to 20% serum. This wide concentration range will disclose if the alteration in the concentration of serum is possible to give optimal growth characteristics for a particular cell line.

Antibiotics and antimycotics: Unless good sterile conditions can be maintained (e.g., using laminar flow hoods) it is necessary to incorporate antibiotics and antimycotics into the media. A wide range of suitable preparations are available from relatively specific antibiotics, e.g., penicillin/streptomycin solutions, to broader spectrum antibacterial/antimycotic agents such as kanamycin or amphotericin B. The antibiotics chosen should clearly not to be toxic to the cells in culture and may depend on the type of contamination experienced in the individual laboratory.

Supply and preparation of culture media: The choice of culture media used will depend on the type of primary cell, cell line, and the incubation conditions. However, it is best to start with the medium recommended by the original supplier of the cells. Changing the medium necessitates the investigation on growth characteristics such as growth curves and cloning efficiency. To render a change in culture conditions from one medium to another it is advisable to ‘condition’ the cells, by increasing the ratio of new to old medium with successive passages.

Culture media have a limited storage life and the recommendations indicated by the supplier should be followed. Liquid defined media may have a storage life at 4 °C of up to one year, while glutamine lasts only 3 weeks at 4 °C. Serum lasts for about one year at - 20 °C.

Culture media can be supplied in powdered form which requires dissolving and filter sterilizing, as a 10x concentrate liquid which requires dilution prior to use, or as a 1x liquid media. Media preparation, in all cases, requires high quality water.

Bottles of media should be prepared in small batches, for instance two weeks supply at a time. This will ensure that constituents such as glutamine do not have time to deteriorate and also that if contamination should occur it is confined to a few bottles.

Culturing animal cells

Primary cultures or continuous cell lines: If you remove tissue from an embryo, dissociate it into a suspension of single cells, and plate them out onto a culture dish, a series of characteristic events occurs. Firstly, cells are in a lag phase, usually no more than 1-2 days in length, during which there is little or no increase in cell number. During this time, cells are "conditioning" the medium, undergoing internal cytoskeletal and enzyme changes and adjusting to the new medium.

Secondly, the cells undergo a period of rapid division, so-called log phase growth. Then, as they approach confluency and form contacts with one another, their rate of division slows and they begin to express a program of differentiation characteristic of their tissue of origin. Muscle cells fuse and acquire cross-striation, epithelial cells from the kidney or gut become linked by junctional complexes and transport ions from one surface to another, heart cells begin to beat spontaneously.

Cultures such as those just described are referred to as primary cultures, because they are prepared from cells taken directly from the animal. The cells divide or not (depending on what they are accustomed to), acquire differentiated characteristics, and ultimately die. For the next experiment, it's back to the animal again to obtain new tissue and prepare new culture.

Alternatively, in the case of cells that divide in culture, it is possible to 'passage' or 'subculture' them by inducing them to detach from the substrate, 'splitting' them (i.e., diluting them several-fold in medium and replating them into new dishes), and allowing them to reenter log phase growth. However, the properties of the cultured cells often change gradually with passaging, as more rapidly dividing cell populations come to predominate and more 'differentiated' cells, which divide more slowly, are lost.

When cells are repeatedly subcultured, most cease division after a finite number of generations, typically between 20 and 80. This is thought to reflect the same process of senescence that occurs in cells in situ. However, it is possible to develop populations of cells that can be passaged indefinitely and that express a reasonably stable phenotype. These are referred to as established or continuous cell lines. Some cell lines have arisen spontaneously in normal cells being passaged in culture, but the majority has been obtained by culturing tumor cells. In addition to their infinite life span (their 'immortality'), such cell lines frequently share several additional properties that distinguish them from 'normal' cells in culture. They divide more rapidly, they do not require attachment to the substratum for growth, and when reintroduced into animals, they form tumors. Cell lines with these properties are sometimes referred to as transformed cell lines.

Cultivation of Viruses in the Cell lines

PRINCIPLE

Viruses infect healthy cells grown in the laboratory. When susceptible cells are used for inoculation of viruses, they show pathological changes and the viruses can be harvested from the cells for further tests. The growth of viruses in the cell lines can be known by a) cytopathic effects, b) immunofluorescence, c) haemagglutination and haemadsorption, and d) interference.

Many viruses kill the infected viral cells in which they grow and bring about detectable changes in morphology of the cells. These changes are collectively known as cytopathic effects. Some viruses however do not produce any cytopathic effect (e.g., rubella virus). The most important precaution to be taken during maintenance of cell lines is sterility. Contamination of cell lines should be prevented and even cross contamination among cell lines should be avoided.

REQUIREMENTS

I Equipments

Inverted microscope, incubator, haemocytometer and biological safety cabinet.

II Reagents and lab wares

Sterile glassware, pre-sterilized tissue culture plasticware, Pasteur pipettes and measuring pipettes, membrane filter, syringes, vials, discard jar, Eagle's, minimum essential medium (MEM), sodium bicarbonate ($NaHCO_3$), EDTA trypsin mixture, foetal calf serum (FCS), sterile double distilled water, virus inoculum, spirit and sodium hypochlorite.

Monolayer of a cell culture in a culture flask is treated with trypsin or versene to disperse cells.

III Specimen

Suspected virus infected specimen like the cerebrospinal fluid (CSF), stool, rectal swab, and throat swab.

PROCEDURE

1 Discard the trypsin versene mixture and add a small amount of MEM with 10% FCS to the monolayer of cells.

2 Count the cells with the medium in a hemocytometer for appropriate splitting.

3 Inoculate the cells into sterile flasks or tubes for viral inoculation

4 Fill the new flasks with MEM and incubate in horizontal position.

5 Select a healthy monolayer, which is also confluent, for viral inoculation.

6 Inoculate the monolayer of cells with virus using sterile Pasture pipette, and incubate at 37°C.

7 Observe for the cytopathic effect (CPE) 7 days after inoculation.

OBSERVATIONS

After incubation, the flasks are observed for confluency and healthy monolayer of cells and virus infected cells are classified.

Viruses are known to produce cytopathic effects are identified by observing the same in the infected cell lines.

Non-cytopathogenic viruses are identified by other methods like immunofluorescence, haemagglutination and haemadsorption, and interference

RESULTS AND INTERPRETATION

The cell lines are observed for any cytological alterations that are diagnostic of viral infections

Detection of Measles Virus by ELISA

Principle: This ELISA kit adopts Indirect-ELISA method as its principle. The micro ELISA plate provided in the kit has been pre-coated with inactivated purified measles virus antigen. When samples are added into the micro ELISA plate wells, the anti- measles virus antibody in the sample will combine with the pre-coated mixed antigen to form antigen-antibody compound. Free components will be washed away.

HRP conjugated Mouse-anti-human IgG monoclonal antibody is added to each well and react with the compound to form “antigen-antibody-HRP conjugated antibody ” compound. Free components will be washed away. The TMB substrate is added to initiate the color developing reaction. The shade of developed color is proportional to the concentration of measles virus IgG antibody.

Experimental instrument

Micro-plate Reader with 450 nm wavelength filter

High-precision transferpettor, EP tubes and disposable pipette tips

37℃ incubator or water bath

Deionized or distilled water

Absorbent paper

Requirements of sample

1. Human serum/plasma or whole blood can be used as sample. Anticoagulants (heparin, EDTA, and sodium citrate) has no interference to the test results.

2. Serum/plasma samples can be kept stable for 4 days at 2~8℃, but it is recommended to be used within 72 hours. Whole blood sample should be used immediately after collecting. Serum/plasma samples can be kept stable for 1 year when stored at -20℃ immediately for long-term storage, while whole blood samples should be diluted with Sample Diluent and store the supernatant at -20℃ for 1 year. It is suggested that the freeze-thaw cycles should be no more than 3 times.

3. Avoid using samples with hemolysis or lipidemia. Polluted samples can't be detected.

Assay procedure

1. Sample preparation:

(1) Add 500 μL of Sample Diluent into 1.5 mL centrifuge tube. Label the tubes.

(2) Collect 10 μL of whole blood, or take 5 μL of serum/plasma. Then add the samples into corresponding centrifuge tube, gently shake the tubes to mix thoroughly.

(3) For whole blood sample, stand the centrifuge tube at room temperature (or 4℃). Take the supernatant for detection after all erythrocytes are settled to the bottom of the tube. For serum/plasma, sample can be used directly after mixed thoroughly.

2. Take the kit and sample out from cold storage and stand for 30 min to room temperature. Set the constant temperature box or water bath to 37±1℃.

3. Preparation of washing buffer: Dilute the 20×Concentrated Wash Buffer with distilled water, purified water or deionized water at the ratio of 1:19, mix thoroughly.

4. Add sample: Reserve 1 well for positive control, 1 well for negative control (100 μL of corresponding control solution for each well). Set 1 well for blank control and add 100 μL of Sample Diluent. Add 100 μL of diluted sample (take supernatant of whole blood sample for detection) to other wells. Cover the plate with the plate sealer. Incubate for 60 min at 37℃.

Carefully remove the plate sealer and discard the liquid in the wells. Add 300 μL of diluted washing buffer, gently shake for 10 sec and discard the liquid, tap the plate to make it dry. Repeat this washing process 5 times. Or operate this procedure with a plate-washer by setting the machine as the conditions above.

5. HRP conjugated: Add 100 μL of HRP Conjugate Working Solution to each well except blank control well. Cover the plate with sealer. Incubate for 30 min at 37℃. Carefully remove the plate sealer and discard the liquid in the wells. Add 300 μL of diluted washing buffer, gently shake for 10 sec and discard the liquid, tap the plate to make it dry. Repeat this washing process 5 times.

6. Add substrate: Add 50 μL of Substrate Reagent A and 50 μL of Substrate Reagent B to each well. Gently tap the plate to ensure thorough mixing. Cover with a new plate sealer. Incubate for 15 min at 37℃ in the dark.

7. Stop reaction: Add 50 μL of Stop Solution to each well, gently tap the plate to ensure thorough mixing.

8. OD Measurement: Set the Micro-plate Reader wavelength at 450 nm/630 nm to detect the A value of each well within 30 min. Or set the Micro-plate Reader to zero with the blank well to determine the A value of each well directly when using single wavelength 450 nm for detection.

Interpretation of results

1. Positive result: The titer of measles virus IgG antibody in sample was not less than 200 mIU/mL.

2. Negative result: The titer of measles virus IgG antibody in sample was less than 200 mIU/mL.

3. Suspicious result: The titer of measles virus IgG antibody in sample was at critical value, and it is recommended to do repeat detection or re-test after two weeks, and take dynamic observation of the change in antibody level.

Detection of Dengue Virus by ELISA.

DENGUE IgG MICROLISA

CE

GAC- ELISA Test for the Detection of Dengue IgG Antibodies in Human Serum/Plasma

1. INTRODUCTION

The mosquito-borne dengue viruses (serotype 1-4) cause dengue fever, a severe flu-like illness. The disease is prevalent in third world tropical regions and spreading to sub-tropical developed countries - including the United States. WHO estimates that 50-80 million cases of dengue fever occur worldwide each year, including a potentially deadly form of the disease called dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Primary infection with dengue virus results in a self-limiting disease characterized by mild to high fever lasting 3 to 7 days, severe headache with pain behind the eyes, muscle and joint pain, rash and vomiting. Secondary infection is the more common form of the disease in many parts of Southeast Asia and South America. This form of the disease is more serious and can result in DHF and DSS. The major clinical symptoms can include high fever, haemorrhagic events, and circulatory failure, and the fatality rate can be as high as 40%. Early diagnosis of DSS is particularly important, as patients may die within 12 to 24 hours if appropriate treatment is not administered.

Primary dengue virus infection is characterized by elevations in specific IgM antibody levels 3 to 5 days after the onset of symptoms; this generally persists for 30 to 60 days. IgG levels also become elevated after 10 to 14 days and remain detectable for life. During secondary infection, IgM levels generally rise more slowly and reach lower levels than in primary infection, while IgG levels rise rapidly from 1 to 2 days after the onset of symptoms.

2. INTENDED USE

Dengue IgG MICROLISA is designed for in-vitro qualitative detection of Dengue IgG Antibodies in human serum or plasma and is used as a screening test for testing of collected blood samples suspected for DENGUE. The kit detects all four subtypes; DEN1, DEN2, DEN3 & DEN4 of Dengue Virus.

3. PRINCIPLE

DENGUE IgG MICROLISA test is an enzyme immunoassay based on "GAC-Capture ELISA".

Anti-human IgG antibodies are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated.

Antibodies to Dengue if present in the specimen, will bind to the Anti-human IgG antibodies adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRPO) conjugated dengue antigen (DEN1-4) is added to each well. This dengue antigen conjugate will bind to Dengue specific IgG antibodies which is complexed with anti- human IgG antibodies. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of Dengue antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain Dengue IgG antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

4. DESCRIPTION OF SYMBOLS USED

The following are graphical symbols used in or found on J. Mitra diagnostic products and packing. These symbols are the most common ones appearing on medical devices and their packing. They are explained in more detail in the European Standard EN ISO 15223-1:2016.

	Manufactured By	IVD	*In vitro* diagnostic medical device
Σ	No. of tests		See Instruction for use
LOT	Lot Number Batch Number		Temperature Limitation
	Manufacturing Date		Caution See instruction for use
	Expiry Date	REF	Catalogue Number
	Do not use if package is damaged		Keep away from sunlight
	Keep Dry		

5. KIT PRESENTATION

- 96 Test Pack

6. KIT & ITS COMPONENTS

COMPONENT	DESCRIPTION	96 TESTS
Microwells	Breakaway microwells coated with Anti-human IgM antibodies packed in a pouch with dessicant.	1 Plate (96 wells)
Sample Diluent	Buffer containing protein stablizers and antimicrobial agents as preservative.	50 ml (2 bottle)
Enzyme Conjugate	Dengue antigen labelled with horseradish peroxidase with protein stablizers. Ready to use	13 ml
Wash Buffer Concentrate (25X)	PBS with surfactant. Dilute 1:25 with distilled water before use.	50 ml
TMB Diluent	Buffer solution containing H_2O_2 with preservative.	10 ml
TMB Substrate	To be diluted with TMB Diluent before use.	10 ml
Control —	Ready to use, Normal human serum negative for Dengue antibodies with preservative.	1.5 ml
Control +	Ready to use , positive for Dengue IgG antibodies antibodies with preservative.	1.5 ml
Calibrator	Ready to use, positive for Dengue IgG antibodies with preservative.	2 ml
Stop Solution	Ready to use, 2N sulfuric acid	15 ml
Plate Sealers	Adhesive packed sheets for sealing microtiter plate/strips	4 nos.

7. STORAGE AND STABILITY

Store the kit & its components at 2-8°C. Expiry date on the kit indcates the date beyond which kit should not be used.

8. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent dilution
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37°C
- Disposable gloves
- Disinfectant Solution

9. SPECIMEN COLLECTION & HANDLING

1. Human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum from the clot as soon as possible to avoid haemolysis. Fresh serum/ plasma samples are preferred.
2. Specimens should be free of microbial contamination and may be stored at 2-8°C for one week, or frozen at -20°C or lower. Avoid repeated freezing and thawing.
3. Do not use heat inactivated samples as their use may given false results. Haemolyzed and lcteric hyperlipemic samples may give erroneous results.

10. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

Dengue IgG MICROLISA test is best used with fresh samples that have not been frozen and thawed. However most frozen samples will perform well if the procedure suggested below is followed.

Allow the frozen sample to thaw in a vertical position in the rack. Do not shake the sample. This allows particles to settle to the bottom. If a centrifuge is available, the sample should be centrifuged. (5000 rpm for 5 min.)

(B) TRANSPORTATION

If the specimen is to be transported, it should be packed in compliance with the current Government regulations regarding transport of aetiologic agents.

11. CAUTION

1. The use of Disposable Gloves and proper Biohazardous clothing is STRONGLY RECOMMENDED while running the test.
2. In case there is a cut or wound in hand, DO NOT PERFORM THE TEST.
3. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.
4. Tests are for *in vitro* diagnostic use only.
5. All the samples to be tested should be handled as though capable of transmitting infection.

6. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. In case of needle prick or other skin puncture or wounds, wash the hands with excess of water and soap.
7. Controls contain Sodium Azide as a preservative. If these material are to be disposed off through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds.
8. All materials used in the assay and samples should be disposed off in the manner that will inactivate virus.
9. ELISA Reader & micropipettes used in testing should be calibrated at regular interval to ensure accurate results.

12. PRECAUTIONS FOR USE

Optimal assay performance requires strict adherence to the assay procedure described in the manual.

1. Do not use kit components beyond the expiration date, which is printed on the kit.
2. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.
3. Stop solution contains sulfuric acid. If sulfuric acid comes in contact with the skin, wash thoroughly with water. In case of contact with eyes, flush with excess of water.
4. Take care while preparing working substrate solution as vials of TMB Substrate & TMB Diluent are of same size.
5. Prepare working substrate solution just 10 minutes prior to adding in the wells.
6. If blue colour or white particles appears in working substrate solution then do not use it. Take fresh containers and tips and prepare it again.
7. Use separate tips for TMB substrate and TMB diluent.
8. Do not allow microwells to dry once the assay has started.
9. Ensure that the microwell strips are levelled in the strip holder. Before reading, wipe the bottom of the microwell strips carefully with soft, absorbent tissue to remove any moisture.
10. If available, a microwell reader which contains a reference filter with settings at 620 or 630 nm should be used. Use of a reference filter minimises interference due to microwells that are opaque, scratched or irregular. However, if a reference filter is unavailable, the absorbance may be read at 450 nm without a reference filter.
11. Distilled or deionised water must be used for wash buffer preparation.
12. Bring all the reagents to room temperature (20-30°C) before use.
13. Do not combine reagents from different batches, as they are optimized for individual batch to give best results.
14. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
15. Run calibrator, negative and positive controls in each assay.
16. Use freshly collected, clean serum samples for assay. Try to avoid Haemolyzed turbid, lipemic serum or plasma samples.
17. Use a separate tip for each sample and then discard it as biohazardous waste.
18. Thorough washing of the wells is critical to the performance of the assay.
19. Avoid strong light exposure during the assay.

13. PREPARATION OF REAGENTS

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30°C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8°C after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator to 37°C.

i) **Anti human IgG antibodies coated strips**

Bring foil pack to room temperature (20-30°C) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that one each of negative & positive control & three calibrator should be included in the run while opening the fresh kit. However for one or two strips, one each of negative, positive control & two calibrator and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8°C, with dessicant in aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8°C from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution : Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

ii) **Sample Preparation:**

TUBE DILUTION: Mark the tubes carefully for the proper identification of the samples. Dilute the serum samples to be tested, with sample diluent 1:100 in separate tubes (1 ml. sample diluent + 10 µl serum samples). Use a separate tip for each sample and then discard as biohazardous waste.

iii) **Preparation of Working Wash Buffer:**

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480 ml. of distilled or deionized water. **Working wash buffer is stable for 2 months when stored at 2-8°C.**

iv) **Preparation of working substrate solution :**

Mix TMB substrate and TMB Diluent in 1:1 to prepare working substrate.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Susbstrate (ml)	05	10	15	20	25	30	35	40	45	50	55	60
TMB Diluent (ml)	05	10	15	20	25	30	35	40	45	50	55	60

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

14. PROCEDURAL NOTES:

1. Material should not be used after the expiry date shown on the labels. Components and test specimen should be at room temperature (20-30°C) before testing begins. Return the reagents to 2-8°C after use.
2. All reagents must be mixed well before use.
3. To avoid contamination, do not touch the top or bottom of strips or edge of wells.
4. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
5. Prevent evaporation during sample incubation by covering the strips with sealer; remove sealer before washing.
6. Routine maintenance of wash system is strongly recommended to prevent carry over from highly reactive specimens to non reactive specimens.

15. TEST PROCEDURE

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the stripholder with the required number of Anti-human IgM coated strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl Negative Control in A-1well.
2. Add 100 µl calibrator in B-1, C-1 & D-1 wells.
3. Add 100 µl Positive Control in E-1 well.
4. Add 100 µl of each sample diluted in sample diluent (1:100), in each well starting from F-1 well. (Refer **TUBE DILUTION**).
5. Apply cover seal.
6. Incubate at 37°C ± 1°C for 60 min. ± 1min.
7. While the samples are incubating, prepare working Wash Solution as specified in preparation of reagents.
8. Take out the plate from the incubator after the incubation time is over and, wash the wells 5 times with working Wash Solution.
9. Add 100 µl of Enzyme Conjugate Solution in each well.
10. Apply cover seal.
11. Incubate at 37°C ± 1°C for 60 min ± 1min.
12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well.
14. Incubate at room temperature (20-30°C) for 30 min. in dark.
15. Add 50 µl of stop solution.
16. Read absorbance at 450 nm and 630 nm (reference filter) within 30 minutes in ELISA READER.

SUMMARY OF PROCEDURE			
Dilute Serum samples		Sample 10 µl	Sample Diluent 1 ml
Add diluted samples & *Calibrator/ Control		100 µl	
Cover the plate & incubate		60 mins. at 37°C	
Wash		5 Cycles	
Add Enzyme Conjugate*		100 µl	
Cover the plate & incubate		60 mins. at 37°C	
Wash		5 Cycles	
Prepare Working Substrate		No of Strips: 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 TMB Diluent (ml): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0	
Add Substrate		100 µl	
Incubate in dark		30 mins. at Room Temp.	
Add Stop Solution		50 µl	
Read Results		450 nm,630 nm.	

* Calibrator/Controls and Enzyme Conjugate are Ready to use

TEST VALIDITY:
Ensure the following is within specified acceptance criteria

i) NC O.D. must be < 0.3. If it is not so, the run is invalid and must be repeated.
ii) PC O.D. must be > 1.0. If it is not so, the run is invalid and must be repeated.
iii) Mean Calibrator O.D. must be ≥ 0.35. If it is not so, the run is invalid and must be repeated.
iv) Cut off value must be ≥1.5 x NC O.D. If it is not so, the run is invalid and must be repeated.
v) Ratio of PC O.D. / cut off must be > 1.1. If it is not so, the run is invalid and must be repeated.

16. CALCULATION OF RESULTS

Imp. Note: The calibration factor detail is batch specific and stamped on back page of instruction manual.

a. Cut off value = mean O.D. of calibrator x calibration factor
b. Calculation of sample O.D. ratio : Calculate sample O.D. ratio as follows:

$$\text{Sample O.D. ratio} = \frac{\text{Sample O.D.}}{\text{Cut off Value}}$$

c. Calculation of Dengue IgG units : Calculate by multiplying the sample O.D. ratio by 10.
Dengue IgG units = sample O.D. ratio x 10.
e.g.: Mean O.D. of calibrator = 0.75
Calibration factor = 0.7
Cut off value = 0.75 x 0.7 = 0.525
e.g.: sample absorbance (O.D.) = 0.925
Cut off value = 0.525
Sample O.D. ratio = 0.925 / 0.525 = 1.761
Dengue IgG units = 1.761 x 10 = 17.61

17. INTERPRETATION OF RESULTS

a. If the Dengue IgG Units is < 9 then interpret the sample as Negative for Dengue IgG antibodies.
b. If the Dengue IgG Units is between 9 - 11 then interpret the sample as Equivocal for Dengue IgG antibodies. Equivocal samples should be repeated in duplicate and calculate the average dengue units. Sample that remain equivocal after repeat testing should be repated by an alternative method or another sample should be collected.
c. If the Dengue IgG Units is > 11 then interpret the sample as Positive for Dengue IgG antibodies.

18. PERFORMANCE CHARACTERISTICS

The kit has been evaluated with the known panel of Dengue IgG positive and Negative samples. The samples included cross-reacting samples; Epstein-BARR virus, Leptospira, Malaria, Hepatitis-A, Influenza A & B, S. typhi. Following is the in-house evaluation.

No. of Samples	Status	Dengue IgG Microlisa		
		Positive	Equivocal	Negative
75	Dengue Positive	75	0	0
5558	Dengue Negative	2	0	5556

Sensitivity : 100% **Specificity** : 99.92%

Precision : Within-run and between-run precisions have been determined by testing 10 replicates of seven samples: three negative and four dengue IgG positive; two weak positive, one medium positive & one strong positive. The C.V.(%) of negative, weak positive, medium positive & strong positive values were within 10%.

19. LIMITATION OF THE TEST

1. The test should be used for detection of IgG antibodies to Dengue in human serum / plasma only.
2. **This is only a screening test** and will only indicate the presence or absence of Dengue antibodies in the specimen. All reactive samples should be confirmed by confirmatory test. Therefore for a definitive diagnosis, the patients clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with the above procedure.
3. False positive results can be obtained due to cross reaction with Epstein-BARR virus, RA, Rubella, Anti-nuclear antibody, Japanese encephalitis, west nile virus disease. This occurs in less then 1% of the sample tested.
4. Immuno-depressive treatments presumably after the immune response to infection, inducing negative results in IgG in Dengue patients.

20. LIMITED EXPRESSED WARRANTY DISCLAIMER

The manufacturer limits the warranty to the test kit, as much as that the test kit will function as an *in vitro* diagnostic assay within the limitations and specifications as described in the product instruction-manual, when used strictly in accordance with the instructions contained therein. The manufacturer disclaims any warranty expressed or implied including such expressed or implied warranty with respect to merchantability, fitness for use or implied utility for any purpose. The manufacture's liability is limited to either replacement of the product or refund of the purchase price of the product and in no case liable to for claim of any kind for an amount greater than the purchase price of the goods in respect of which damages are likely to be claimed.

The manufacturer shall not be liable to the purchaser or third parties for any injury, damage or economic loss, howsoever caused by the product in the use or in the application there of.

21. REFERENCES

1. Pinheiro FP, Corber SJ: Global situation of dengue and dengue haemorrhagic fever and its emergence in the Americas. World Health Stat I 50 (3/4): 161-169, 1997.
2. Gutier DJ, Trent DW: Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. Infect Agents Dis 2:383-393, 1993.
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22. TROUBLE SHOOTING CHART

	PROBLEM	POSSIBLE CAUSE	SOLUTION
1.	Control out of validation limit	a) Incorrect temperature timing or pipetting	Check procedure & repeat assay
		b) Improper preparation of reagents, improper mixing of reagents.	Check procedure & repeat assay
		c) Cross contamination of Controls	Pipette carefully and do not interchange caps. Repeat assay

Detection of HIV by ELISA.

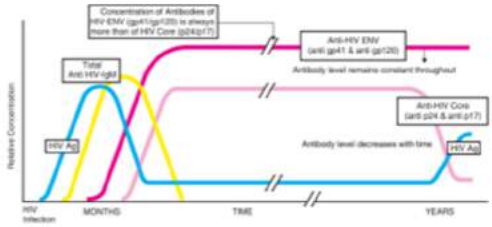
Microlisa - HIV

Microwell ELISA Test for the Detection of Antibodies to HIV-1 (Including Group O & Subtype C) and HIV-2 in Human Serum/ Plasma

1. SUMMARY AND EXPLANATION OF THE TEST

The available research data indicates that Acquired Immunodeficiency Syndrome (AIDS) is caused by HIV virus transmitted by sexual contact, exposure to blood or certain blood products, by an infected mother to her child during pre-natal and post-natal period. The two type of HIV viruses (HIV-1 & HIV-2) have been isolated from patients with AIDS and AIDS related complex (ARC). These two viruses belong to the retrovirus group and are slow viruses.

The serological events following HIV infection are represented graphically in fig.1. In individuals infected with HIV, antigen appears first before anti-HIV but due to seroconversation, the antigen is lost and antibody develops within 1-2 months after infection and thereby the level of the antibody increases.



MICROLISA-HIV is developed to detect anti-HIV ENV (envelope) antibodies to HIV-1 and / or HIV-2 with equal reactivity.

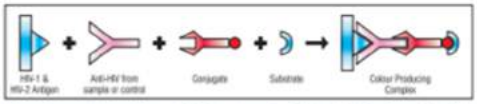
It has been observed that the core protein of HIV-1 and HIV-2 show cross reactivity whereas envelope proteins are more type specific and moreover antibodies against the envelope gene products can be found in almost all infected people. Microlisa-HIV has been developed and designed to be extremely sensitive and specific using recombinant proteins (gp41, C terminus of gp120 and gp36) representing the immunodominant regions of HIV-1 & HIV-2 envelope gene structure respectively.

2. INTENDED USE

Microlisa-HIV is an in-vitro qualitative enzyme immunoassay for the detection of antibodies to HIV-1 and / or HIV-2 in human serum or plasma. It is intended for screening of blood donors or other individuals at risk for HIV-1 and / or HIV-2 infection and for clinical diagnostic testing.

3. PRINCIPLE OF THE TEST

Microlisa HIV test is an enzyme immunoassay based on Indirect ELISA.



HIV envelope proteins proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and / or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

4. DESCRIPTION OF SYMBOLS USED

The following are graphical symbols used in or found on J. Mitra diagnostic products and packing. These symbols are the most common ones appearing on medical devices and their packing. They are explained in more detail in the British and European Standard EN ISO 15223-1:2016.

	Manufactured By	IVD	In vitro diagnostic medical device
	No. of tests		See Instruction for use
LOT	Lot Number Batch Number		Temperature Limitation
	Manufacturing Date		Caution, see instruction for use
	Expiry Date	REF	Catalogue Number
	Keep away from sunlight		Do not use if package is damaged

5. PACK SIZE

- 96 Tests

6. COMPONENTS IN EACH MICROLISA-HIV KIT

Store all components at 2-8°C when not in use. Expiry date on the kit indicates the date beyond which the kit should not be used.

Microlisa-HIV Strip Plates	12 Strips (12 X 8 wells) Breakway microwells coated with HIV-1 & HIV-2 recombinant proteins packed in a pouch with dessicant.
Sample Diluent	1 Bottle (20 ml) Buffer containing protein stabilizers and antimicrobial agents as preservative.
Enzyme Conjugate Concentrate (100x)	1 Vial (0.25 ml) Anti-human IgGs conjugated with horseradish peroxidase with protein stabilizers.
Conjugate Diluent	1 Bottle (15 ml) Buffer containing protein stabilizers.
Wash Buffer Concentrate (25x)	1 Bottle (50 ml) PBS with surfactant. Dilute 1:25 with distilled water before use.
TMB Substrate	1 Bottle (10 ml) To be diluted with TMB diluent before use.
TMB Diluent	1 Bottle (10 ml) Buffer solution containing H_2O_2 with preservative
Control —	1 Vial (2.0 ml) Ready to use, normal human serum negative for HIV, HCV, and HBsAg with preservative.
Control +	1 Vial (2.0 ml) Ready to use, inactivated and diluted human serum; positive for HIV antibodies and non-reactive for HBsAg and HCV with preservative.
Stop Solution	1 Bottle (15 ml) Ready to use, 1N sulfuric acid.
Plate Sealers	Adhesive backed sheets for sealing microwell plate/strips.

7. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips.
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent dilution
- Sodium hypochlorite solution
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37°C
- Vortex Mixer
- Disposable gloves
- Glassware

8. SPECIMEN COLLECTION & PREPARATION

1. Only human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum form the clot as soon as possible to avoid hemolysis. Fresh serum/plasma samples are preferred.
2. Specimens should be free of microbial contamination and may be stored at 2-8°C for one week, or frozen at -20°C or lower. Avoid repeated freezing and thawing.
3. Use of heat inactivated, icteric hyperlipemic and hemolyzed and Icteric hyperlipemic samples should be avoided as may give erroneous results.

9. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

Microlisa-HIV test is best used with fresh samples that have not been frozen and thawed. However most frozen samples will perform well if the procedure suggested below is followed.

Allow the frozen sample to thaw in a vertical position in the rack. Do not shake the sample. This allows particles to settle to the bottom. If a centrifuge is available, the sample should be centrifuged. (10,000 rpm for 15 min.)

(B) TRANSPORTATION

If the specimen is to be transported, it should be packed in compliance with the current Government regulations regarding transport of aetiologic agents.

10. WARNING & PRECAUTION

CAUTION: THIS KIT CONTAINS MATERIALS OF HUMAN ORIGIN. NO TEST METHOD CAN OFFER COMPLETE ASSURANCE THAT HUMAN BLOOD PRODUCTS WILL NOT TRANSMIT INFECTION. NEGATIVE CONTROL, POSITIVE CONTROL & ALL THE SAMPLES TO BE TESTED SHOULD BE HANDLED AS THOUGH CAPABLE OF TRANSMITTING INFECTION.

1. The use of disposable gloves and proper biohazardous clothing is STRONGLY RECOMMENDED while running the test.
2. In case there is a cut or wound in hand, DO NOT PERFORM THE TEST.
3. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.
4. Tests are for in vitro diagnostic use only and should be run by competent person only.
5. Do not pipette by mouth.
6. All materials used in the assay and samples should be decontaminated in 5% sodium hypochlorite solution for 30-60 min. before disposal or by autoclaving at 121°C at 15psi for 60 min. Do not autoclave materials or solution containing sodium hypochlorite. They should be disposed off in accordance with established safety procedures.
7. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. Consult a physician immediately in case of accident or contact with eyes, in the event that contaminated material are ingested or come in contact with skin puncture or wounds.
8. Spills should be decontaminated promptly with Sodium Hypochlorite or any other suitable disinfectant.
9. Controls and Sample diluent contain Sodium Azide as a preservative. If these material are to be disposed off through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds. In addition, consult the manual guideline "Safety Management No. CDC-22", Decontamination of Laboratory Sink Drains to remove Azide salts" (Centre for Disease Control, Atlanta, Georgia, April 30, 1976.)
10. Stop solution contains sulfuric acid. If sulfuric acid comes in contact with the skin, wash thoroughly with water. In case of contact with eyes, flush with excess of water.

11. PRECAUTIONS FOR USE

Optimal assay performance requires strict adherence to the assay procedure described in the manual.

1. Do not use kit components beyond the expiration date which is printed on the kit.
2. Bring all the reagents & samples to room temperature (20-30°C) before use.
3. Do not combine reagents from different batches, as they are optimised for individual batch to give best results.
4. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.
5. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
6. Use freshly collected, clean serum samples for assay. Try to avoid turbid, lipemic serum or plasma samples.
7. Use a separate tip for each sample and then discard it as biohazardous waste.
8. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
9. Do not allow microwells to dry once the assay has started.
10. Run negative and positive controls in each assay to evaluate validity of the kit.
11. Incubation time should not vary by more than ±2 min.
12. Prevent evaporation during sample incubation by covering the strips with strip sealer. Remove sealer before washing.
13. Distilled or deionised water must be used for wash buffer preparation.
14. Thorough washing of the wells is critical to the performance of the assay. Overflowing of reagents or washing to adjacent wells must be prevented during washing, which may lead to incorrect results due to carryover effect.
15. Take care while preparing working substrate solution and use separate tips for TMB Substrate and TMB diluent.
16. Prepare working substrate solution just 10 minutes prior to adding in the wells.
17. If blue colour or white particles appear in working substrate solution then do not use it. Take fresh containers and tips and prepare it again.
18. Avoid strong light exposure during the assay.
19. Ensure that the microwell strips are levelled in the strip holder. Before reading, wipe the bottom of the microwell strips carefully with soft, absorbent tissue to remove any moisture.
20. If available, a microwell reader which contains a reference filter with settings at 620 or 630 nm should be used. Use of a reference filter minimises interference due to microwells that are opaque, scratched or irregular. However, if a reference filter is unavailable, the absorbance may be read at 450 nm without a reference filter.
21. In case of any doubt the run should be repeated.

12. PREPARATION OF REAGENTS

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30°C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8°C after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37°C.

12.1. Microlisa-HIV Strip:

Bring foil pack to room temperature (20-30°C) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that two negative & three positive controls should be included in the run while opening the fresh kit. However for one or two strips, one negative and two positive controls and for more strips at least two negative and three positive controls should be included in each subsequent runs.

b. Unused wells should be stored at 2-8°C, with dessicant in a aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8°C from the date of opening of sealed pouch, when stored with desicant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

12.2. Sample Preparation:
MICROWELL DILUTION:

a) Pipette 100 µl of sample diluent in to the microwell.
b) Add 10 µl of serum sample to be tested.
c) Ensure thorough mixing of the sample to be tested.

12.3. Preparation of Wash Buffer:

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.
b) Prepare at least 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.
c) Mix 20 ml. 25 X wash buffer concentrate with 480 ml. of distilled or deionized water. Working Wash Buffer is stable for 2 months when stored at 2-8°C.

12.4. Preparation of Working Conjugate:

Dilute conjugate concentrate 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme Conjugate Concentrate (µl)	10	20	30	40	50	60	70	80	90	100	110	120
Conjugate Diluent in (ml)	1	2	3	4	5	6	7	8	9	10	11	12

Note: In case any precipitate is found in conjugate diluent/sample diluent, it should be allowed to settle and the supernatant can be used for the test. The precipitate does not interfere with the working of the kit.

12.5. Preparation of working substrate solution:

Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate.

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Substrate (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB Diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

13. WASH PROCEDURE:

1. Incomplete washing will adversely affect the test outcome.
2. Aspirate the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 4 additional times for a total of 5 washes.

3. Automated washer if used should be well adjusted to fill each well completely without overfilling
4. Tap upside down on absorbent sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

14. TEST PROCEDURE

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the stripholder with the required number of Microlisa-HIV strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells so that well A-1 is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100µl sample diluent to A-1 well as blank.
2. Add 100µl Negative Control in each well no. B-1 & C-1 respectively. Negative Control is ready to use and hence no dilution is required.
3. Add 100µl Positive Control in D-1, E-1 & F-1 wells. Positive Control is ready to use and hence no dilution is required.
4. Add 100 µl sample diluent in each well starting from G-1 followed by addition of 10µl sample. (Refer **MICROWELL DILUTION**)
5. Apply cover seal.
6. Incubate at 37°C ± 2°C for 30 min. ± 2 min.
7. While the samples are incubating, prepare Working Wash Solution and Working Conjugate as specified in Preparation of Reagents.
8. Take out the plate form the incubator after the incubation time is over and, wash the wells 5 times with Working Wash solution according to the wash procedure given in the previous section (wash procedure).
9. Add 100µl of Working Conjugate Solution in each well including A-1.
10. Apply cover seal.
11. Incubate at 37°C ± 2°C for 30 min. ± 2 min.
12. Aspirate and wash as described in step no. 8.
13. Add 100 µl of working substrate solution in each well including A-1.
14. Incubate at room temperature (20 - 30°C) for 30 min. in dark.
15. Add 100 µl of stop solution.
16. Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well. (Bichromatic absorbance measurement with a reference wavelength 600 - 650 nm is recommended when available).

15. SUMMARY OF PROCEDURE*

Dilute Serum Sample		Sample 10µl Sample Diluent 100µl
Add control (RTU) *		100µl
Cover the plate & incubate		30 mins. at 37°C
Wash		5 Cycles
Prepare working conjugate		No. of Strips 1 2 3 4 5 6 7 8 9 10 11 12 Enz. conc. (µl) 10 20 30 40 50 60 70 80 90 100 110 120 Diluent (ml.) 1 2 3 4 5 6 7 8 9 10 11 12
Add Conjugate		100µl
Cover the plate & incubate		30 mins. at 37°C
Wash		5 Cycles
Prepare Chromogenic Substrate		No. of Strips 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 TMB Diluent (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Add Substrate		100µl
Incubate in dark		30 mins. at Room Temp.
Add Stop Solution		100µl
Read Results		450 nm./630 nm.

* RTU - Ready to use

16. CALCULATION OF RESULTS

Abbreviations

NC -	Absorbance of the Negative Control
NC̄ -	Mean Negative Control
PC -	Absorbance of the Positive Control
PC̄ -	Mean Positive Control

TEST VALIDITY:

Blank acceptance Criteria

Blank must be < 0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

Negative Control Acceptance Criteria:

NC must be ≤ 0.150. If it is not so, the run is invalid and must be repeated.

Positive Control Acceptance Criteria:

1. PC must be ≥ 0.50
2. Determine the mean (PC̄) value If one of three positive control values is outside of these limits, recalculate PC̄ based upon the two acceptable positive control values.
3. If two of the three positive control values are outside the limits, the assay is invalid and the test must be repeated.

CUT OFF VALUE

Absorbance (O.D.)

NC	-	0.042 B1 Well	PC	-	1.412 D1 Well
	-	0.040 C1 Well		-	1.392 E1 Well
Total:		0.082 2 Wells		-	1.407 F1 Well
			Total :		4.211 3 Wells

NC̄ = 0.082/2 = 0.041 PC̄ = 4.211/3 = 1.403

The cut off value is calculated by adding Mean Negative Control (NC̄) and Mean Positive Control (PC̄) as calculated above and the sum is divided by 6.

$$\text{Cut off Value} = \frac{\overline{NC} + \overline{PC}}{6}$$

e.g. NC̄ = 0.041, PC̄ = 1.403

$$\text{Cut off Value} = \frac{0.041 + 1.403}{6} = \frac{1.444}{6} = 0.240$$

17. INTERPRETATION OF RESULTS

1. Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative for anti-HIV.
2. Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV by Microlisa-HIV.

Note: Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies and should be retested in duplicate.

3. Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be retested in duplicate.
4. If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non reactive.
5. If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. Further confirmation by other EIA assays or confirmation assays including Western Blot or PCR is recommended.

18. LIMITATIONS OF THE ASSAY

1. Microlisa-HIV assay is designed for testing antibodies against HIV-1 and/or HIV-2 in human serum and plasma. Other body fluids and pooled samples are not recommended in this assay. Any result derived from the test of any other body fluid or from test of pooled serum/plasma may not be interpreted correctly based on the current criteria. In establishing infection of HIV-1 and/or HIV-2 or, in evaluating patients with AIDS symptoms, Microlisa-HIV testing alone cannot be used to diagnose AIDS even if antibodies against HIV are present in human serum or plasma. A negative test result at any time does not preclude the possibility of exposure to, or infection with HIV. **This is only a screening test.** All samples detected reactive must be confirmed by using Western Blot. Therefore for a definitive diagnosis, the patient's clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with the above procedure.

2. Some samples show cross reactivity for HIV antibodies. **Following factors are found to cause false positive HIV antibody test results:** Naturally occurring antibodies, Passive immunization, Leprosy, Tuberculosis, Mycobacterium avium, Herpes simplex, Hypergammaglobulinemia, Malignant neoplasms, Rheumatiod arthritis, Tetanus vaccination, Autoimmune diseases, Blood Transfusion, Multiple myeloma, Haemophelia, Heat treated specimens, Lipemic serum, Anti-nuclear antibodies, T-cell eukocyte antigen antibodies, Epstein Barr virus, HLA antibodies and other retroviruses.

19. PERFORMANCE CHARACTERISTICS

Sensitivity and Specificity studies were carried out on samples fresh, as well as frozen, from low risk as well as high risk groups. Performance of the test with reference to sensitivity and specificity has been determined by NATIONAL HIV REFERENCE CENTRES of Govt. of India and WHO Collaborating Centre, using various testing panels.

These evaluations indicates the following Sensitivity and Specificity:

Samples	Indian**	European*	African*	Worldwide* Overall Panel
Sensitivity	100%	100%	100%	100%
Specificity	99.5%	100%	100%	100%

* As per evaluation report dated 20th Feb. 1998, of WHO Collaborating Centre, Instituutvoor Tropische Geneeskunde Nationalestraat 155-B 2000 Antwerpen, Balgium.

** Evaluation Reports of National HIV Reference Laboratories of Govt. of India (CMC Vellore). Drug Controller General (India), Directorate General of Health Services, Govt. of India, New Delhi. (Letter dated 8th May 1997).

Seven HIV-O sera were included in reference serum panel. Microlisa-HIV has detected all the seven HIV-O positive samples are reactive thereby confirming its 100% Sensitivity & Specificity for HIV-O positive samples as well.

A low performance and mixed panel from BBI (Boston Biomedica Inc.) were also tested with Microlisa-HIV were identical with the Western Blot data provided by BBI for the above mentioned low performance and mixed panel.

This information is provided for the Community Enquiring for an independent evaluation other than company's in house evaluation. It is not for commercial or promotion purpose.

Precision: Within-run and between-run precisions have been determined by testing 10 replicates of three specimens : a negative, a weak positive and a strong positive. The C.V.(%) of negative, weak positive and strong positive values were within 10%.

20. LIMITED EXPRESSED WARRANTY DISCLAIMER

The manufacturer limits the warranty to the test kit, as much as that the test kit will function as an in-vitro diagnostic assay within the limitations and specifications as described in the product instruction-manual, when used strictly in accordance with the instructions contained therein. The manufacturer disclaims any warranty expressed or implied including such expressed or implied warranty with respect to merchantability, fitness for use or implied utility for any purpose. The manufacture's liability is limited to either replacement of the product or refund of the purchase price of the product and in no case liable to for claim of any kind for an amount greater than the purchase price of the goods in respect of which damages are likely to be claimed.

The manufacturer shall not be liable to the purchaser or third parties for any injury, damage or economic loss, howsoever caused by the product in the use or in the application there of.

NOTICE: Every effort is made to supply ordered consignment as per the sample submitted but due to continuous development, the company reserves the right to improve/change any specifications/ components without prior information/notice to the buyer.

21. REFERENCES

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22. TROUBLE SHOOTING CHART

	PROBLEM	POSSIBLE CAUSE	SOLUTION
1.	Control out of validation limit	a) Incorrect temperature timing or pipetting	Check procedure & repeat assay
		b) Improper preparation of reagents, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
		c) Cross contamination	Pipette carefully and do not interchange caps. Repeat assay
		d) Incorrect reading filter or readings without blanking the reader.	Check the filter used. It should be 450nm. If no reference filter is used absorbance will increase.
		e) Interference in the optical pathway	Check the reader. Clean or dry the bottom of micro wells, check for bubbles & repeat the readings
		f) Used components from different lots.	Do not use components from different lots as they are adjusted for each batch released.
		g) Expired Reagents	Check the kit expiry date. Use the kit with-in shelf life
2.	No colour or light colour developed at the end of assay	a) Any one reagent has been added in wrong sequence.	Check procedure and repeat assay.
		b) Inactivated conjugate, wrong dilution used, improper conservation	Check for contamination, recheck procedure
		c) Microplate inactivated, due to improper conservation	Keep unused strips in sealable plastic bag, very well closed with the dessicant pouch inside
		d) Inactivated substrate, improper conservation or preparation	Use freshly prepared substrate solution Recheck procedure, repeat assay
3.	Too much colour in all wells of the plate	a) Contaminated substrate use of same container for preparing & dispensing substrate & conjugate.	Check TMB Diluent it should be colourless. If blue in colour then discard and use acid washed or disposable container.
		b) Contaminated or improper dilution of reagents.	Check for contamination, check dilutions.
		c) Contaminated washing solution (1X).	Check the container and quality of water used for dilution.
		d) Over incubation of substrate and delay in addition of stop solution.	Repeat assay.
		e) Insufficient washing. i) Washing not consistent	Check wash device, fill the well close to the top.
		ii) Filling volume not sufficient.	After washing, blot the
		iii) Insufficient no. of wash cycles.	microwells on absorbent tissue.
		iv) Contaminated wash device	
		f) Use of wash buffer from other manufacturer kit.	Use only Microlisa-HIV wash buffer.
4.	Poor reproducibility	a) Washing problems.	
		b) Uncalibrated pipettes or tips not well fitted, improper pipetting.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
		c) Reagent & sera not at room temperature or not well mixed before use.	Equilibrate reagents to room temperature and mix thoroughly before use
		d) Too long time for addition of samples or reagents, Inconsistency in time intervals	Develop consistent and uniform technique.
		e) Interference in optical pathway due to Air bubbles.	1e.
5.	False Positive	Beside 3a, b, c, d, e incorrect interpretation and calculation of final results	Check the calculation part given in the insert and correctly interpret.
6.	False Negative/ low O.D. for PC and positive sample	a) Inadequate addition of substrate/conjugate solution	Recheck the test procedure and reagent volume.
		b) Kit expired, reagent of different kit used.	Check the expiry of the kit before use.
		c) White particles in working substrate solution.	Discard the substrate and prepare the working substrate again in fresh tube.

For in-vitro diagnostic use only, not for medicinal use

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MIS/Ins/12 Rev. Date: Mar. 2019

Collection, presentation and identification of different disease causing arthropods and their whole mount preparation

Collection

Aerial Net: It is designed to collect specific groups of insects, namely – dragonflies, butterflies, moths, bees, wasps, flies, some smaller insect varieties, etc. The collecting bag is usually white in colour.

Presentation

Mouth Parts of House-Fly:
It is convenient to use the whole head for study of mouth parts in a fly. Observe the components under a dissecting binocular.

Preparation:
Separate the head of a fly boil it in 5 to10**%** KOH solution for about 8 to10minutes. Cool it and decant the solution, wash repeatedly with distilled water to remove the last traces of KOH. Mount the whole structure on a slide in glycerin. If required strain in a watch glass and make its permanent slide with single staining technique.

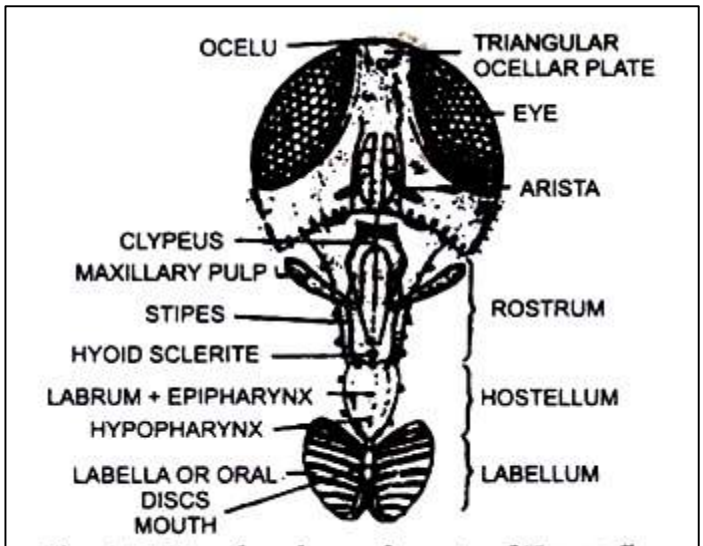


Fig. 4.17. Head and mouth parts of House fly.

Wings of a Fly:

Carefully take out the wings & them in a cavity block containing 70% alcohol. For temporary preparation it is mounted in glycerol. For permanent preparation it is cleared in xylol and then mounted in D.P.X. on a slide.

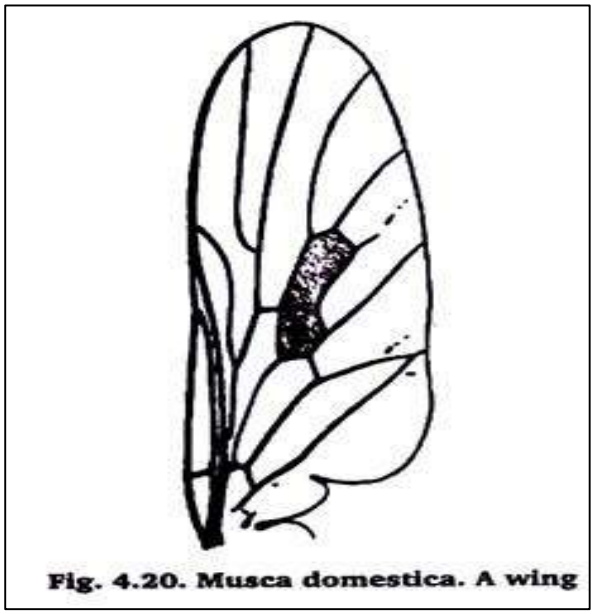


Fig. 4.20. Musca domestica. A wing

Mouth Parts of Mosquitoes:

The basic components of the mouth parts of both the culex and anopheles are same. The size of the proboscis (labium), maxillary palps and antennae differs not only in the two species but also in the male & female members of the same species.

Preparation:

Separate the head of mosquito. Put it in 5 to 10% KOH solution in a watch glass for about 10 minutes. Remove the KOH by repeated washing with distilled water & mount in glycerine.

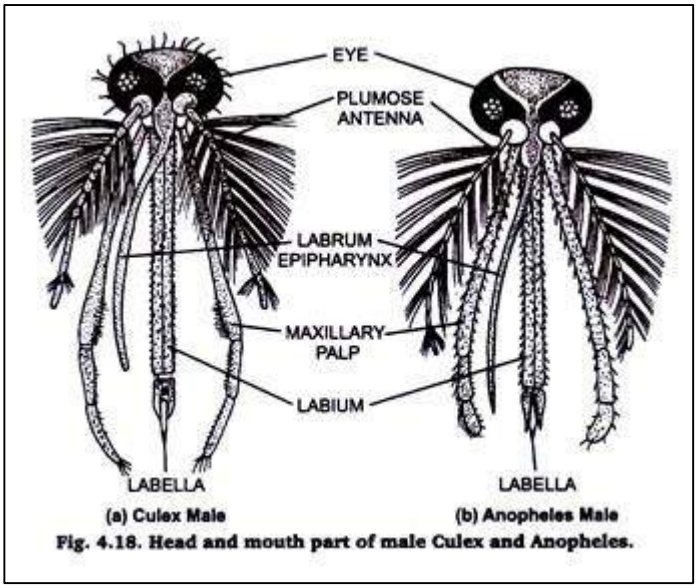


Fig. 4.18. Head and mouth part of male Culex and Anopheles.

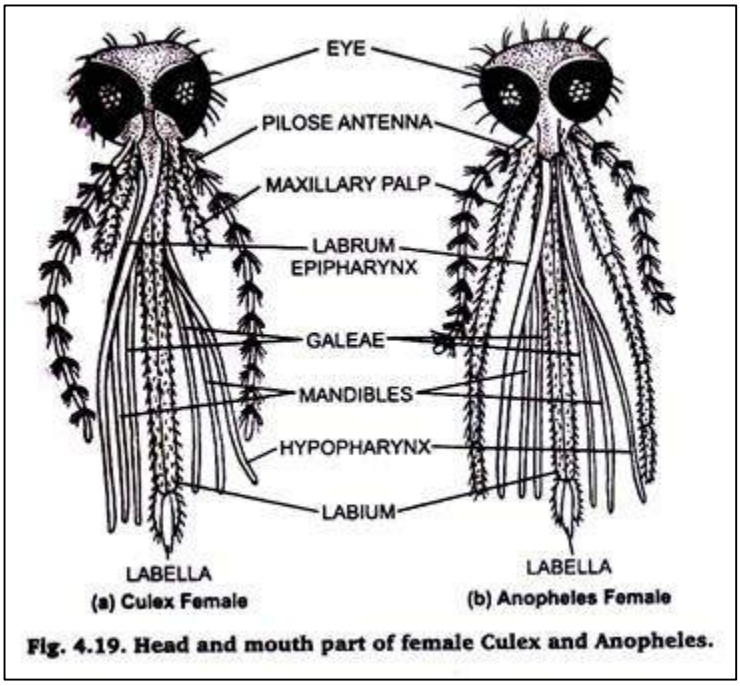


Fig. 4.19. Head and mouth part of female Culex and Anopheles.

Whole mount:

Flies and mosquitoes can be collected during their flights usually occur between sundown and dark with the help of net trap. To prepare the slide put them in 5% KOH solution for some time. Wash off the KOH and proceed for the procedure of single staining.

Identification

Housefly (*Muscadomestica*)

Systematic Position

Phylum- Arthropoda
Sub Phylum- Mandibulata
Class- Insecta
Sub Class- Pterygota
Order- Diptera
Specimen- *Muscadomestica*

Specimen Characters

1. Adult houseflies are usually 6 to 7 mm long with a wingspan of 13 to 15 mm. The females tend to be larger winged than males, while males have relatively longer legs.

2. The head is strongly convex in front and flat and slightly conical behind.

3. The pair of large compound eyes almost touch in the male, but are more widely separated in the female.

4. They have three simple eyes (**ocelli**) and a pair of short antennae.

5. The mouthparts are specially adapted for a liquid diet; the mandibles and maxillae are reduced and not functional, and the other mouthparts form a retractable, flexible proboscis with an enlarged, fleshy tip, the labellum.

6. At the end of each leg is a pair of claws and below them are two adhesive pads, **pulvilli**.

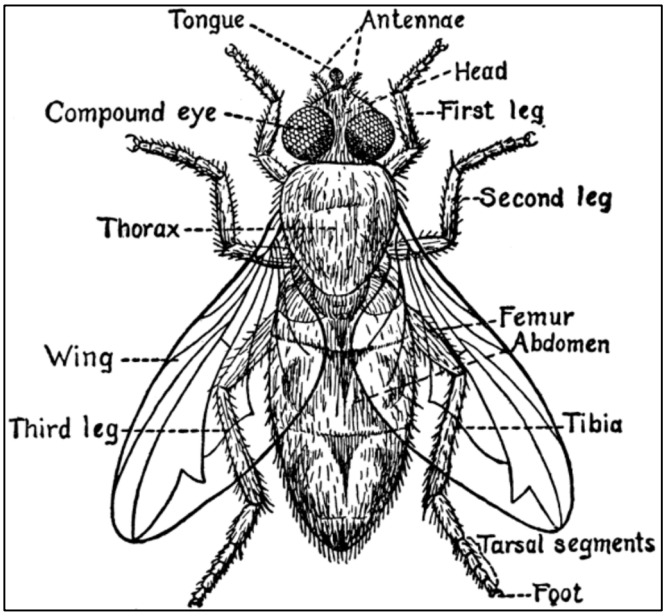
7. The thorax is a shade of grey, sometimes even black, with four dark, longitudinal bands of even width on the dorsal surface. The whole body is covered with short hairs.

8. Houseflies have only one pair of wings; what would be the hind pair is reduced to small **halteres**that aid in flight stability. The wings are translucent with a yellowish tinge at their base.

Significance

Houseflies do not serve as a secondary host or act as a reservoir of any bacteria of medical or veterinary importance, but they do serve as mechanical vectors to over 100 pathogens, such as those causing typhoid, cholera, salmonellosis, bacillary dysentery, tuberculosis, anthrax, ophthalmia, and pyogenic cocci, making them especially problematic in hospitals and during outbreaks of certain diseases.

Status: Harmful organism



Culex sp.

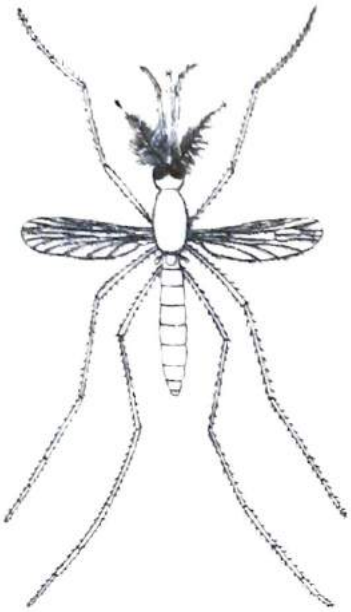


Fig. 1.166 *Culex* sp. (♂)

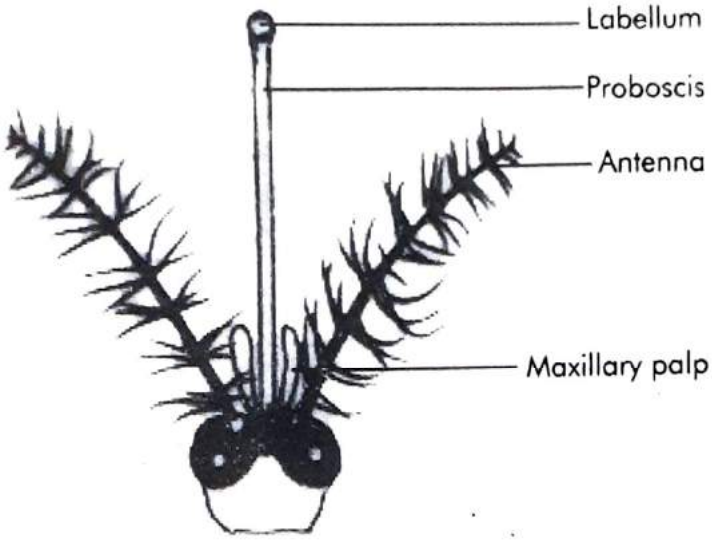


Fig. 1.167 Head of *Culex* sp. (♀)

Systematic Position

(Beck, Liem and Simpson, 1990)

Kingdom	Animalia
Phylum	Arthropoda
Sub Phylum	Mandibulata
Class	Insecta
Sub Class	Pterygota
Order	Diptera
Specimen	*Culex fatigans*
Common name	Mosquito

Specimen Characters

1. At resting position the proboscis and the body are in two axes.
2. Body is longer and broad with stout legs.

3. Bristles on the body are sparse.
4. Forewings are membranous and uniform in colouration.
5. Scutellum is trilobed and has tuft of bristles on either sides.
6. Maxillary palps are as long as proboscis (in males)/are very short (in females).
7. Tip of the abdomen is usually blunt (in females).

Hence, the specimen seems to be *Culex* sp.

Significance

Mosquitoes create annoyance and painful reaction during bites. Chiefly they are important as the intermediate hosts or vectors or carriers of many parasitic and viral diseases of man and domestic animals. *Culex* spp. spread filariasis, dengue fever, encephalitis etc.

Status: Harmful organism

Anopheles sp.

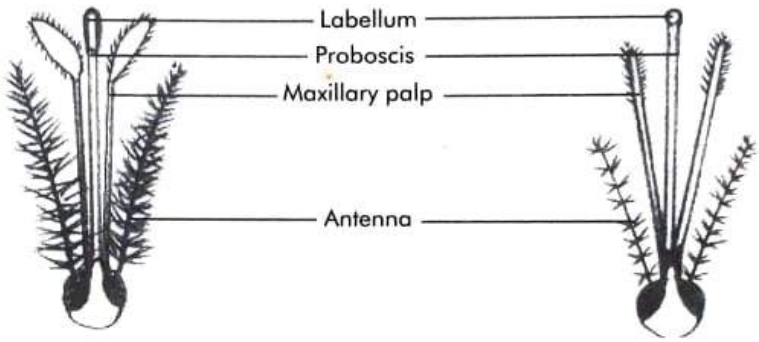


Fig. 1.168 Head of *Anopheles* sp. (♂)

Fig. 1.169 Head of *Anopheles* sp. (♀)

Systematic Position

(Beck, Liem and Simpson, 1990)

Kingdom	Animalia
Phylum	Arthropoda
Sub Phylum	Mandibulata
Class	Insecta
Sub Class	Pterygota
Order	Diptera
Specimen	*Anopheles quadrimaculatus*
Common name	Mosquito

Specimen Characters

1. At resting position, the proboscis and the body are in one axis.
2. Body is small and slender with delicate legs.
3. Dense bristles cover the body.
4. Spots are present on the membranous forewings.
5. Scutellum is evenly rounded and has bristles on its posterior margin.
6. Maxillary palps are as long as proboscis.
7. Maxillary palps are club-shaped (in males).

Hence, the specimen seems to be *Anopheles* sp.

Significance

Mosquitoes create annoyance and painful reactions during bites. Chiefly they are important as the intermediate hosts or vectors or carriers of many parasitic and viral diseases of man and domestic animals. *Anopheles* spp. spread malaria, filaria etc.

Status: Harmful organism

***Aedes* sp.**

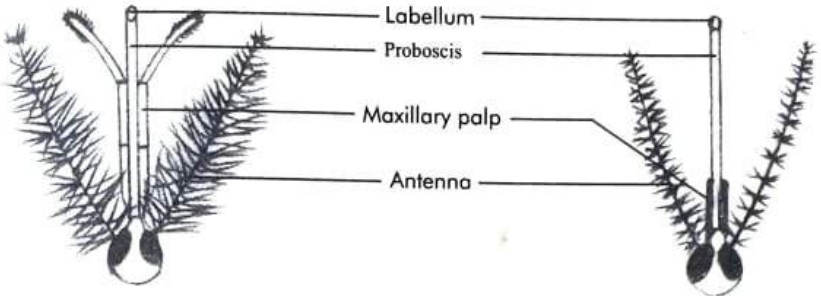


Fig. 1.170 Head of *Aedes* sp. (♂)

Fig. 1.171 Head of *Aedes* sp. (♀)

Systematic Position

(Beck, Liem and Simpson, 1990)

Kingdom	Animalia
Phylum	Arthropoda
Sub Phylum	Mandibulata
Class	Insecta
Sub Class	Pterygota
Order	Diptera
Specimen	*Aedes aegypti*
Common name	Mosquito

Specimen Characters

1. At resting position proboscis and body are in two axes.
2. Body is long and broad with well-built legs.
3. Black and white bands appear on the membranous forewings and on the body.
4. Scutellum is trilobed.
5. Maxillary palps are as long as proboscis (in males)/are shorter than proboscis (in females).
6. Tip of the abdomen is usually pointed (in females).

Hence, the specimen seems to be *Aedes* sp.

Significance

Mosquitoes create annoyance and painful reactions during bites. Chiefly they are important as the intermediate hosts or vectors or carriers of many parasitic and viral diseases of man and domestic animals. *Aedes* spp. spread yellow fever, dengue fever, encephalitis etc. Venereal transmission of some arboviruses by *Aedes* spp. is also reported.

Status: Harmful organism

Identification of different disease causing Helminth and Protozoan parasites

EXPERIMENT 1.3

Finding out Significance vis-à-vis Identification of Selected Species

1.3.1 THEORETICAL FUNDAMENTALS

We are not certain as to how many different species share the planet earth with us. The current biodiversity has been shaped by the same ecological and evolutionary processes that created human beings. Yet, humans are unique in that they are interested to shape their present and future in a way they feel best. The enormous development of human brain have greatly accelerated the renaissance in cultural evolution of human population, we call as 'civilization'. This so called civilization is based primarily on the superb skill of human beings in exploiting nature and natural resources. In his own interest, man gradually learned exploiting a significant portion of the biological diversity in addition to the skills gifted by nature in the form of what is known as instinct. In doing so man had to learn from experience which organisms to be used as food, which are to be used for purposes other than nutrition, and which organisms are to be avoided. Accordingly, man has categorized his fellow creatures as **'beneficial'**, **'harmful'**, **'pest'**, **'parasitic'** and so on. Some of such organisms are considered here for developing an idea how we can meaningfully exploit the biosphere of which we are a part.

1.3.2 FINDING OUT SIGNIFICANCE VIS-À-VIS IDENTIFICATION

Trypanosoma evansi

Systematic Position
(Levine *et al.* 1980)

Kingdom	Protoctista
Phylum	Sarcomastigophora
Sub Phylum	Mastigophora
Class	Zoomastigophorea
Order	Kinetoplastida
Specimen	*Trypanosoma evansi*

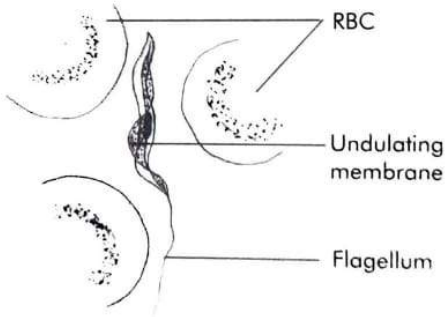


Fig. 1.139 *Trypanosoma evansi*

Specimen Characters

1. Body is 15–34 µm in length (mean being 24 µm) and 1.5–3.0 µm in width.
2. Single nucleus contains a large central nucleolus; endoplasmic reticulum appear to project out from the nuclear membrane.
3. Flagellum arises at the posterior end of body and runs along the free edge of a well-developed undulating membrane.
4. Flagellum is free at the anterior end of the body.
5. Flagellum originates from a complex consisting of a basal body and a strongly basophilic subterminal kinetoplast.
6. Stumpy form may appear sporadically.

Hence, the specimen seems to be *Trypanosoma evansi*.

Significance

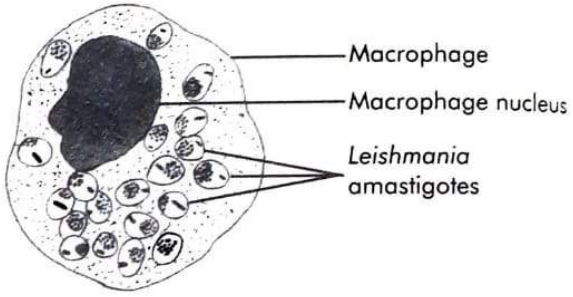
The ailment caused by *Trypanosoma evansi* is most often called 'surra' disease which is widespread among camels, horses, elephants, deer, tapirs and many other mammals. Members of the genus *Trypanosoma* are parasites of all classes of vertebrates. In all probability *T. evansi* was originally a parasite of camels. *T. evansi* is considered a model parasite for the study of evolution of parasites: *T. evansi* probably originated from *T. brucei*, when camels were brought to the tsetse fly belt.

Status: Parasite

Leishmania donovani

Systematic Position
(Levine *et al.* 1980)

Kingdom	Protoctista
Phylum	Sarcomastigophora
Sub Phylum	Mastigophora
Class	Zoomastigophorea
Order	Kinetoplastida
Specimen	*Leishmania donovani*



Amastigote form

Fig. 1.140 *Leishmania donovani*

Specimen Characters

1. Body is small, ovoid or round, about 2–5 μm in diameter, lodged in vertebrate macrophage.
2. Body covered by a periplast.
3. Cytoplasm is differentiated into ecto- and endoplasm.
4. Endoplasm contains a central nucleus, a rod-shaped **kinetoplast**, and a basal body.

Hence, the specimen seems to be the amastigote form of *Leishmania donovani*.

Significance

Leishmania donovani is transmitted by the insect vector *Euphlebotomus argentipes* and related species and housed in the cells of the human reticulo-endothelial system. It causes visceral leishmaniasis or kala-azar (black disease) or 'Dum Dum fever' or 'ponos.' Visceral leishmaniasis is classified as (1) endemic (affects children of age 5–9 years in India), (2) sporadic (affects non-indigenous people entering an epidemic area), and (3) epidemic (all ages are susceptible except those old enough to have been affected during a previous epidemic). The organism is also of special interest to biologists and biochemists on account of metabolic switches which occur in the blood stream and vector stages.

Status: Parasite

Plasmodium vivax

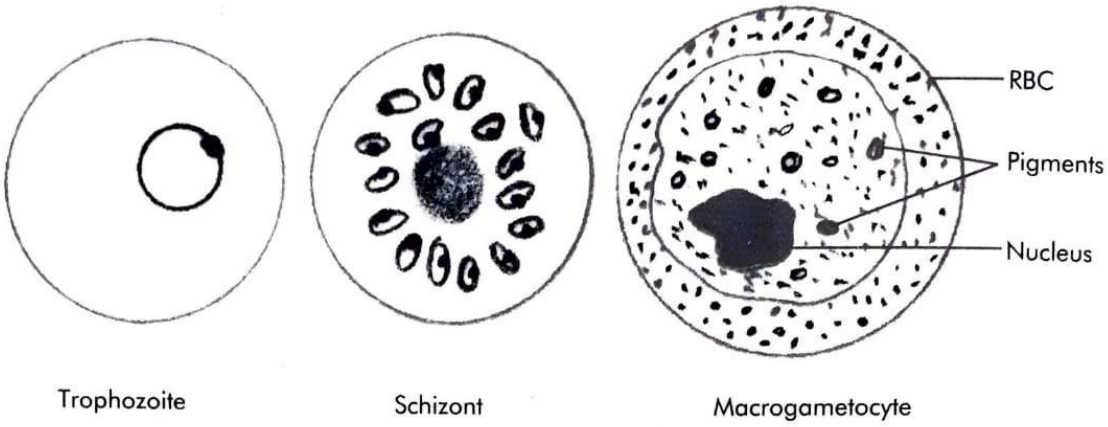


Fig. 1.142 *Plasmodium vivax*

Systematic Position

(Levine *et al.* 1980)

Kingdom	Protoctista
Phylum	Apicomplexa
Class	Sporozoea
Sub Class	Coccidia
Specimen	*Plasmodium vivax*

Specimen Characters

1. Trophozoite is about one-third the diameter of the infected RBC.
2. Cytoplasm opposite to the chromatin dot is thicker.
3. Growing forms are very irregular in shape enclosing a vacuole.
4. Mature schizont is larger than the RBC, and almost completely occupies the enlarged RBC.
5. Merozoites are arranged in grape-like clusters.
6. Presence of the granular yellowish-brown haemozoin pigments.
7. Schuffner's dots are present in enlarged, pale RBCs.
8. Gametocytes are spherical or globular and much larger than RBC.

Hence, the specimen seems to be *Plasmodium vivax*.

Significance

The benign tertian form of malaria (or vivax malaria or tertian ague) is caused by *P. vivax*. Generally, this is not life threatening but can cause severe to acute type of illness. The young and immature red blood corpuscles (reticulocytes) are generally parasitised by *P. vivax*, which account for about 1% of all erythrocytes. About 43% of malaria in the world is caused by *P. vivax*. The concerted effort of the scientists worldwide toward an effective malaria vaccine has largely been a futile exercise mainly due to the following reasons:

1. Antigenic properties (GAM-1, PV200 etc.) of the parasite are highly variable;
2. the parasite mutates so rapidly that today's vaccine may turn out to be ineffective against tomorrow's strain of the parasite;
3. the parasite itself is very complex containing 2000 kb DNA distributed in 14 chromosomes; and
4. immune response against genetically engineered proteins is usually rather poor, and cannot often strengthen the immune response of the vaccines to the level that can endure a long-lasting immunity.

Recent discovery that certain mutations in the genes involved in the eye pigment biosynthetic pathways of insect hosts may be refractory for successful completion of life cycle of *Plasmodium* spp. (Bilkier *et al.*, 1998) has rejuvenated scientists and laymen alike.

Status: Parasite

Plasmodium falciparum

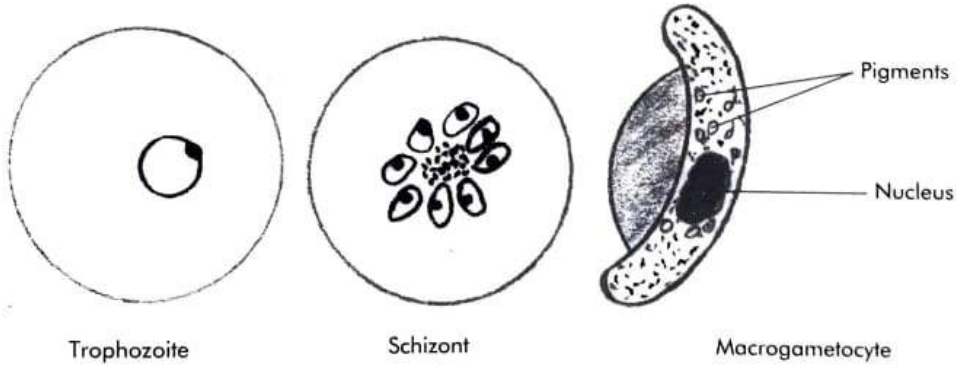


Fig. 1.143 *Plasmodium falciparum*

Systematic Position

(Levine *et al.* 1980)

Kingdom	Protoctista
Phylum	Apicomplexa
Class	Sporozoea
Sub Class	Coccidia
Specimen	*Plasmodium falciparum*

Specimen Characters

1. Trophozoite is about ±1/5th the diameter of the infected RBC.
2. Cytoplasm is fine and regular in outline and often with two chromatin dots.

3. Forms *accole* are seen.
4. Mature schizont is smaller than the RBC and occupies two-third or three-fourth the volume of RBC.
5. Merozoites are arranged in grape-like clusters.
6. One solid block of dark brown or blackish haemozoin pigment is present.
7. **Maurer's** dots may be present in RBC.
8. Gametocytes are crescent-shaped and larger than RBC.

Hence, the specimen seems to be *Plasmodium falciparum.*

Significance

The tertian form of malaria is caused by *P. falciparum* which often terminates fatally. Its infection may cause abortion, stillbirth or even death of non-immuned pregnant women. Erythrocytes of all ages (about 10% of all erythrocytes) are indiscriminately parasitised by *P. falciparum*. In extreme cases about 50% of the RBC may be infected. *P. falciparum* (*Pf*) is a difficult organism for the medical scientists due to certain unique features such as (1) *Pf* suppresses the ability of the dendritic cells of the host to initiate immune responses, (2) antigens produced by *var* and *rif* gene families of *Pf* (*Pf EMP1* and *rifin* respectively) are highly variable, (3) *Pf* exhibits anti-malarial drug resistance by the way of amplification of a gene that encodes an 'ABC transporter' which pumps out anti-malarial drug chloroquine. Overall, of about 250 million clinical cases of malaria that are reported each year all over the world, about 1 million die of *Pf* alone.

Status: Parasite

Wuchereria bancrofti

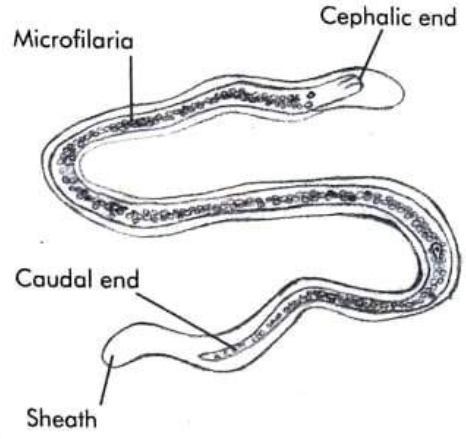


Fig.1.144 A sheathed microfilaria

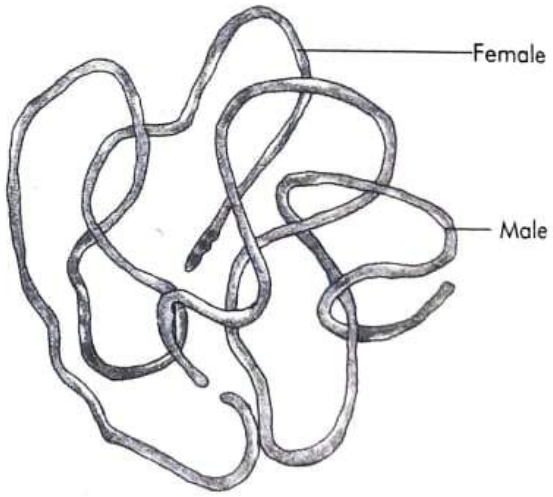


Fig.1.145 Adult male and female worms of *W. bancrofti*

Systematic Position

(Beck, Liem and Simpson, 1990)

Kingdom	Animalia
Phylum	Aschelminthes
Class	Nematoda
Specimen	*Wuchereria bancrofti*
Common name	Filarial worm

Specimen Characters

A. Adult worms

1. Long slender body with smooth cuticle and bluntly rounded ends.
2. Head region is slightly swollen.
3. Presence of two circles of papillae in the head region.
4. Mouth is a small aperture.
5. Presence of a finger-like tail (in males).
6. Vulva is near the middle of the oesophagus (in females).
7. Males measure about 40 mm in length and about 100 µm in width.
8. Females measure 6–10 cm in length and about 300 µm in width.

B. Microfilaria

1. Presence (or absence) of a sheath: a delicate and closely fitting membrane (the egg capsule), which is detectable only when it projects beyond the head or tail of the larva.
2. Presence of several internal nuclei and primordia of organs.
3. Cuticle is thin and striated.
4. While the head is blunt, caudal end is pointed.

Hence, the specimen seems to be *Wuchereria bancrofti.*

Significance

Wuchereria bancrofti causes bancroftian filariasis, resulting in elephantiasis (a misnomer word) in man, causing revolting swellings often in the legs and the genital organs. Bancroftian filariasis is widespread throughout the broad equatorial belt.

Three distinct clinical phases are recognized in bancroftian filariasis: (1) incubation stage, (2) acute or the stage of complications caused by chronic lymphedema. While inguinal lymphadenitis, orchtis, hydrocele, epididymitis and recurrent elephantoid fever are most common symptoms of acute stage, the obstructive stage is marked by lymph varices, lymph scrotum, hydrocele, chyluria and elephantiasis.

It has been estimated that about one billion people in tropical and sub-tropical countries are exposed to infection risk and that at least 200 million are infected with filariasis, primarily with bancroftian type.

Apart from issues related to human health, *W. bancrofti* is important for its characteristic behaviour pattern such as 'rhythm' or 'periodicity': microfilariae make their periodic journey to the peripheral circulation from the blood vessels of the deep tissues between 10 p.m. to 2 a.m. in perfect synchrony with the feeding schedule of the mosquitoes who transmit them from one host to another.

Status: Parasite

Identification of different phases of life cycle of arthropods, protozoa, helminth having medical importance for causing disease

HOUSE FLY (*Musca* sp.)

Introduction

The house fly, Musca domestica Linnaeus, is a well-known cosmopolitan pest of both farm and home. This species is always found in association with humans or the activities of humans. It is the most common species found on hog and poultry farms, horse stables, and ranches. Not only are house flies a nuisance, but they can also transport disease causing organisms. Excessive fly populations are not only an irritant to farm workers but, when there are nearby human habitations, a public health problem could occur.

Distribution

This common fly originated on the steppes of central Asia, but now occurs on all inhabited continents, in all climates from tropical to temperate, and in a variety of environments ranging from rural to urban. It is commonly associated with animal faeces, but has adapted well to feeding on garbage, so it is abundant almost anywhere people live.

Life Cycle

Egg

The white egg, about 1.2 mm in length, is laid singly but eggs are piled in small groups. Each female fly can lay up to 500 eggs in several batches of 75 to 150 eggs over a three to four day period. The number of eggs produced is a function of female size which, itself, is principally a result of larval nutrition. Maximum egg production occurs at intermediate temperatures, 25 to 30°C. Often, several flies will deposit their eggs in close proximity, leading to large masses of larvae and pupae. Eggs must remain moist or they will not hatch.

Larva

Early instar larvae are 3 to 9 mm long, typical creamy whitish in color, cylindrical but tapering toward the head. The head contains one pair of dark hooks. The posterior spiracles are slightly raised and the spiracular openings are sinuous slits which are completely surrounded by an oval black border. The legless maggot emerges from the egg in warm weather within eight to 20 hours. Maggots immediately begin feeding on and developing in the material in which the egg was laid. The larva goes through three instars and a full-grown maggot, 7 to 12 mm long, has a greasy, cream-colored appearance. High-moisture manure favors the survival of the house fly larva. The optimal temperature for larval development is 35 to 38°C, though larval survival is greatest at 17 to 32°C. Larvae complete their development in four to 13 days at optimal temperatures, but require 14 to 30 days at temperatures of 12 to 17°C.

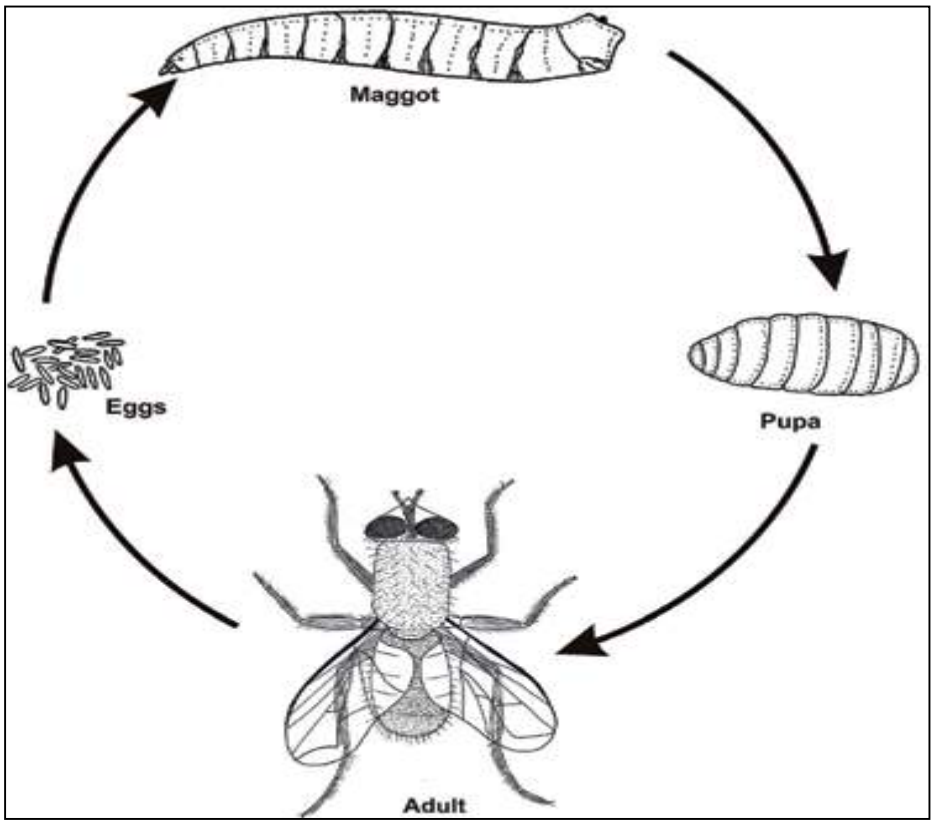
Pupa

The pupal stage, about 8 mm long, is passed in a pupal case formed from the last larval skin which varies in color from yellow, red, brown, to black as the pupa ages. The shape of the pupa is quite different from the larva, being bluntly rounded at both ends. Pupae complete their

development in two to six days at 32 to 37°C, but require 17 to 27 days at about 14°C). The emerging fly escapes from the pupal case through the use of an alternately swelling and shrinking sac, called the ptilinum, on the front of its head which it uses like a pneumatic hammer to break through the case.

Adult

The house fly is 6 to 7 mm long, with the female usually larger than the male. The female can be distinguished from the male by the relatively wide space between the eyes (in males, the eyes almost touch). The head of the adult fly has reddish-eyes and sponging mouthparts. The thorax bears four narrow black stripes and there is a sharp upward bend in the fourth longitudinal wing vein. The abdomen is gray or yellowish with dark midline and irregular dark markings on the sides. The underside of the male is yellowish.



Culex sp.

Eggs

- Adult, female mosquitoes lay eggs on the surface of fresh or stagnant water. Water sources can include barrels, horse troughs, ornamental ponds, unmaintained swimming pools, puddles, creeks, ditches, and marshy areas.
- A female *Culex* mosquito lays eggs one at a time. Eggs stick together to form a raft of 100 to 300 eggs. The raft floats on the water.

Larva

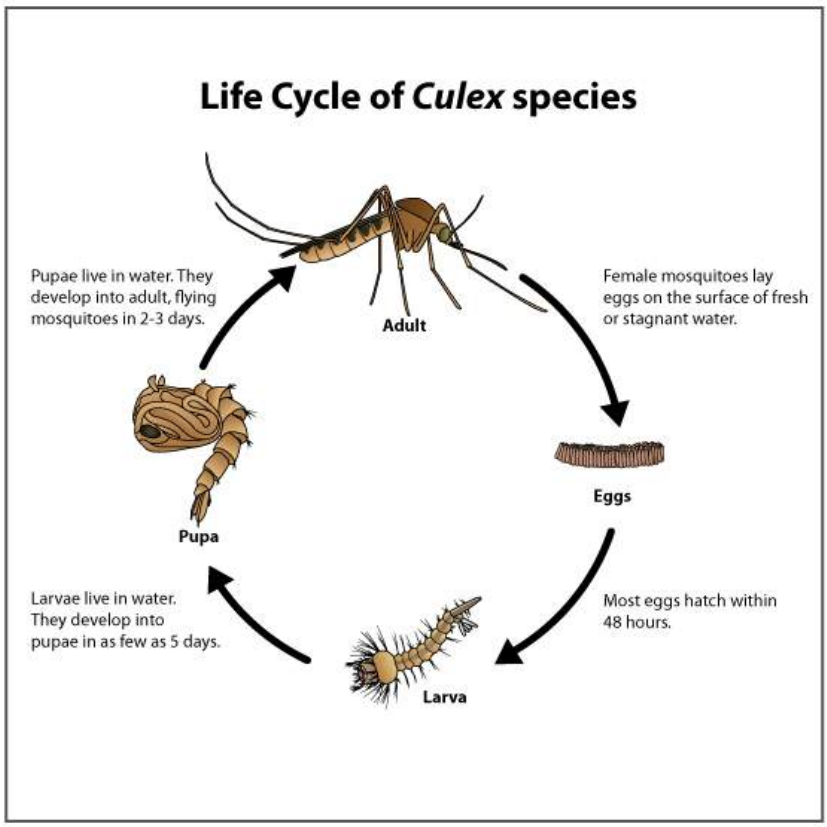
- Larvae hatch from mosquito eggs and live in water.
- Larvae can be seen in the water. They are very active and are often called “wigglers.”
- They feed on a variety of things found in the water.
- Larvae shed their skin (molt) several times during this stage.

Pupa

- Pupae live in water. Pupae do not have external mouthparts and do not feed during this stage.
- An adult mosquito emerges from a pupa and flies away.

Adult

- Adult female mosquitoes bite people and animals. Mosquitoes need blood to produce eggs.
- After blood feeding, female mosquitoes look for water sources to lay eggs. Several days pass between feeding and looking for a place to lay eggs.
- *Culex* mosquitoes don’t fly long distances but have been known to fly up to 2 miles (3.2 km).
- Some *Culex* mosquitoes prefer to live near and bite birds. They bite people when other animals are not nearby.
- Because *Culex* bites animals and people, they live outdoors or near homes.



Anopheles sp.

Life Stages

- Like all mosquitoes, anophelines go through two phases. The first is aquatic and lasts 5-14 days depending on the species and the ambient temperature, and comprises the egg, larval and pupa stages.
- The second is aerial and involves the adult. The adult females can live up to a month (or more in a laboratory setting) but the majority live 2 weeks or less in nature. The adult stage is when the female Anopheles mosquito acts as a malaria vector.

Eggs

- One or two days after blood-feeding, adult females lay 50 to 300 eggs per oviposition and can lay between 800 and 1000 eggs during their life.
- Eggs are laid singly directly on water and are unique in having floats on either side. Eggs are not resistant to drying and hatch within 2-3 days.

Larva

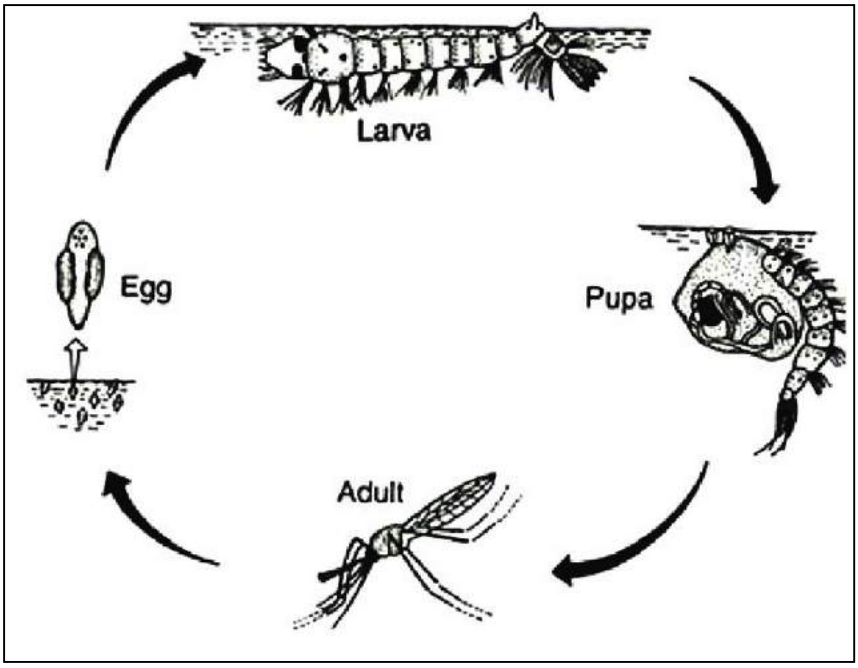
- A larva emerges from each egg and floats parallel to the surface of the water to breathe. It feeds on particles present in the water.
- Measuring just 1mm, the larva undergoes three moults to reach 5mm in the fourth stage.
- Larvae occur in a wide range of habitats but most species prefer clean, unpolluted water. Larvae of Anopheles mosquitoes have been found in fresh or salt-water marshes, mangrove swamps, rice fields, grassy ponds, tree trenches, canals, ditches, the edges of streams and rivers, and small temporary rain pools.
- After the 4th stage, the larva turns into an intermediate stage between larva and adult, the pupa.

Pupa

- The pupa is comma-shaped when viewed from the side. As with the larvae, pupae must come to the surface frequently to breathe, which they do through respiratory trumpets present on their cephalothorax.
- After a few days the adult mosquito emerges from the pupa. The complete cycle from egg to adult typically takes between 9 and 20 days.

Adult

- Adult mosquitoes usually mate within a few days after emerging from the pupal stage.
- The males form swarms around dusk often nearby huts within villages, and the females fly into the swarms to mate.
- *Anopheles* sp. females usually only mate once in their lifespan. Males and females feed on nectar and other plant exudates. Only females feed on blood - males do not have the right mouthparts to do this.



Aedes sp.

Eggs

- Adult, female mosquitoes lay eggs on the inner walls of containers with water, above the waterline.
- Eggs stick to container walls like glue. They can survive drying out for up to 8 months. Mosquito eggs can even survive a winter in the southern United States.
- Mosquitoes only need a small amount of water to lay eggs. Bowls, cups, fountains, tires, barrels, vases, and any other container storing water make a great “nursery.”

Larvae

- Larvae live in the water. They hatch from mosquito eggs. This happens when water (from rain or a sprinkler) covers the eggs.

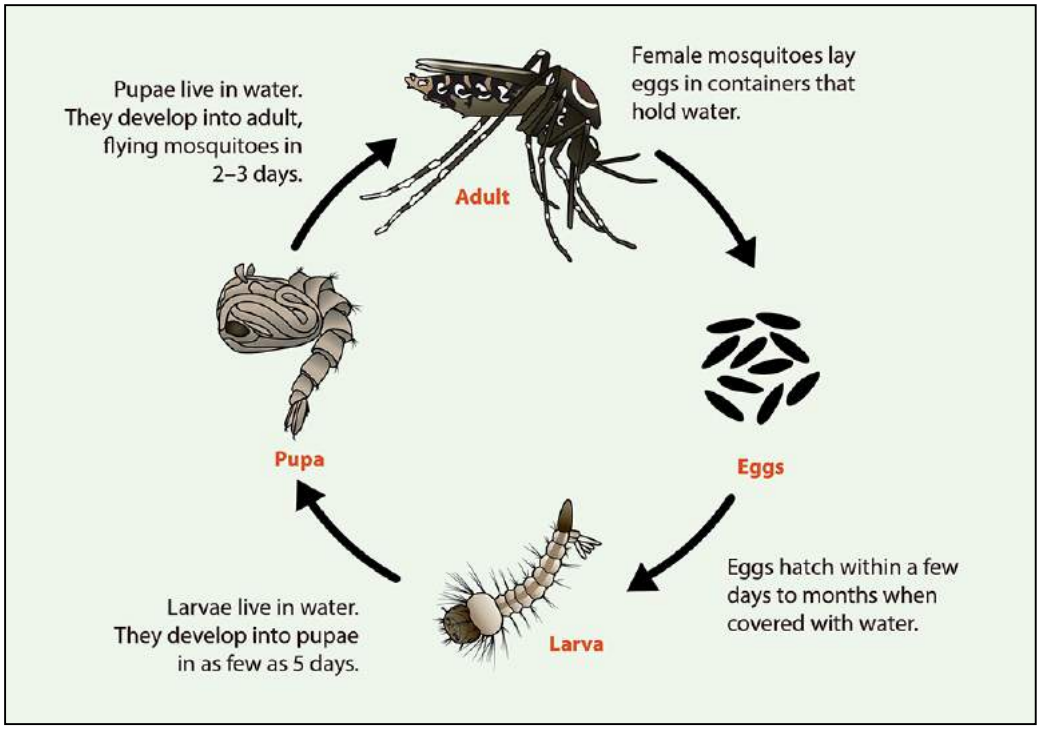
- Larvae can be seen in the water. They are very active and are often called “wigglers.”

Pupae

- Pupae live in the water. An adult mosquito emerges from the pupa and flies away.

Adult

- Adult female mosquitoes bite people and animals. Mosquitoes need blood to produce eggs.
- After feeding, female mosquitoes look for water sources to lay eggs.
- *Ae. aegypti* and *Ae. albopictus* don’t fly long distances. In its lifetime, these mosquitoes will only fly within a few blocks.
- *Ae. aegypti* mosquitoes prefer to live near and bite people.
- Because *Ae. albopictus* mosquitoes bite people and animals, they can live in or near homes or in neighboring woods.
- *Ae. aegypti* mosquitoes live indoors and outdoors, while *Ae. albopictus* live outdoors.



Wuchereria bancrofti

Life Cycle

Different species of the following genera of mosquitoes are vectors of *W. bancrofti* filariasis depending on geographical distribution.

During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound

1. They develop in adults that commonly reside in the lymphatics

2. The female worms measure 80 to 100 mm in length and 0.24 to 0.30 mm in diameter, while the males measure about 40 mm by .1 mm. Adults produce microfilariae measuring 244 to 296 µm by 7.5 to 10 µm, which are sheathed and have nocturnal periodicity, except the South Pacific microfilariae which have the absence of marked periodicity. The microfilariae migrate into lymph and blood channels moving actively through lymph and blood

3. A mosquito ingests the microfilariae during a blood meal

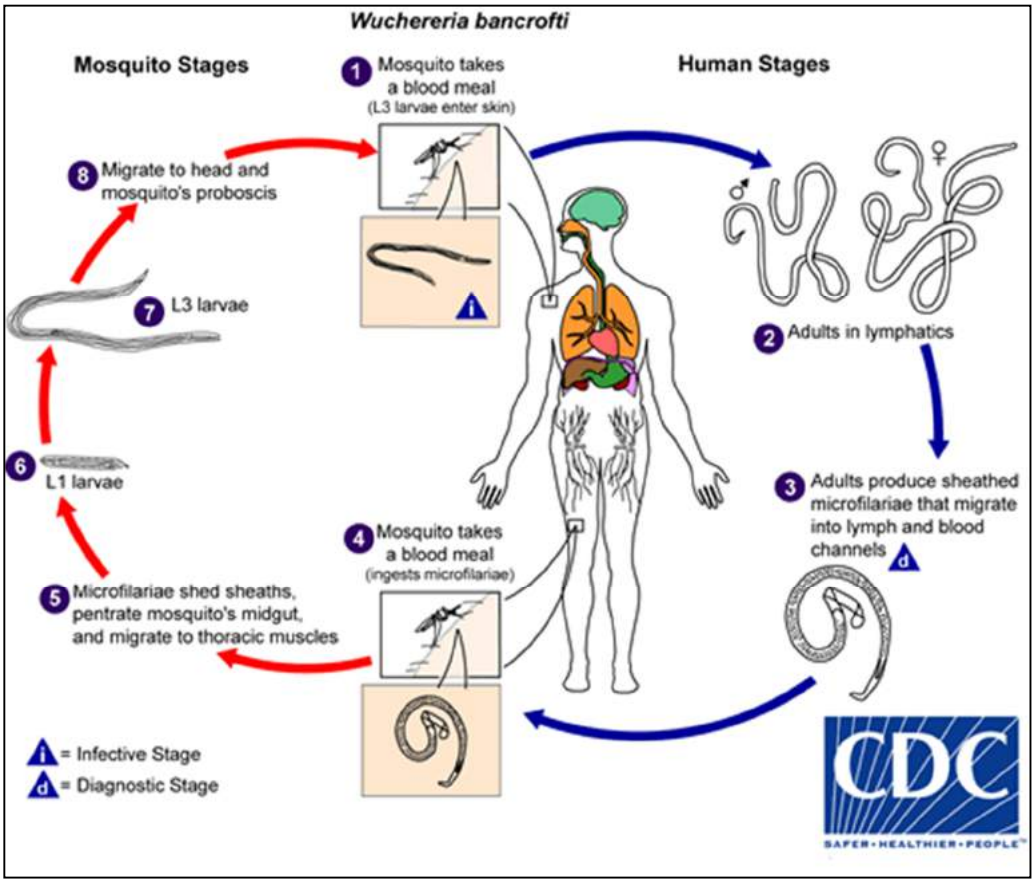
4. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles

5. There the microfilariae develop into first-stage larvae

6. and subsequently into third-stage infective larvae

7. The third-stage infective larvae migrate through the hemocoel to the mosquito's prosbocis

8. and can infect another human when the mosquito takes a blood meal.



Trypanosoma sp.

During their passage through insect and vertebrate hosts, flagellates undergo developmental change. Trypanosoma exists in two forms: Epimastigotes and Trypomastigotes. Within the gut

of the insect (and in culture media), the organism assumes the promastigote (Leishmania) or epimastigote (Trypanosoma) form. Epimastigote/ Promastigote- These protozoa are motile and fusiform and have a blunt posterior end and a pointed anterior end from which a single flagellum projects. They measure 15 to 30 µm in length and 1.5 to 4.0 µm in width. The kinetoplast complex of the epimastigote form, in contrast, is located centrally, just in front of the vesicular nucleus. The flagellum runs anteriorly in the free edge of an undulating membrane before passing out of the cell. In the mammalian host, hemoflagellates appear as trypomastigotes (Trypanosoma) or amastigotes (Leishmania, T cruzi). The former circulates in the bloodstream and closely resemble the epimastigote form, except that the kinetoplast complex is in the posterior end of the parasite.

Life Cycle

1. During a blood meal on the mammalian host, an infected tsetse fly (genus Glossina) injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream.

2. Inside the host, they transform into bloodstream trypomastigotes,

3. are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue the replication by binary fission

4. The entire life cycle of African trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host

5. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission

6. leave the midgut, and transform into epimastigotes

7. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission

8. The cycle in the fly takes approximately 3 weeks. Rarely, T. b. gambiense may be acquired congenitally if the mother is infected during pregnancy.

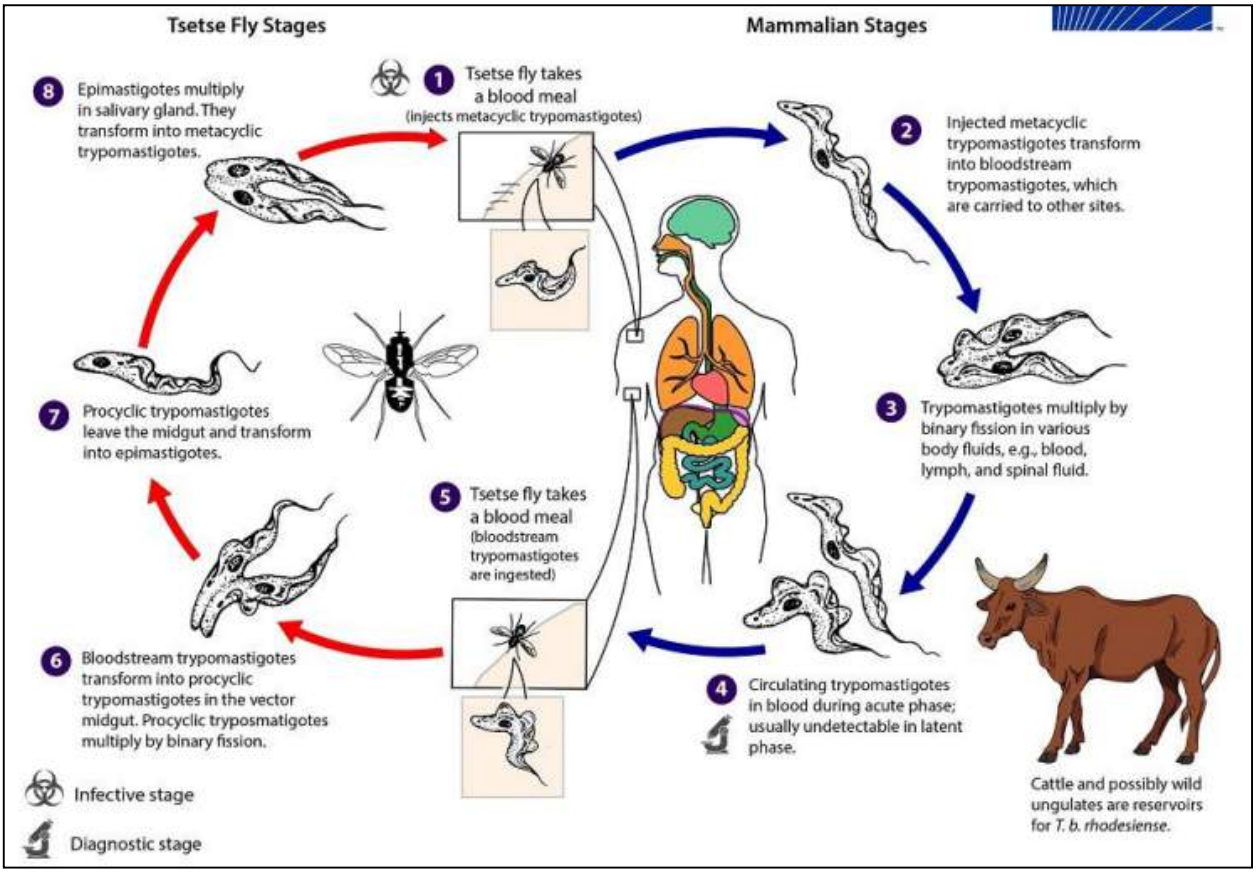
➢ Newly emerged and young flies are more efficient transmitters of the disease than older flies. A highly variable surface glycoprotein (VSG) coat, which is acquired in the tsetse fly, accounts for this organism's ability to undergo a process of antigenic variation in its mammalian host.

➢ The parasite enters the bloodstream and trypomastigote stage parasites referred to as slender forms divide by longitudinal fission every 5 to 10 hours. For reasons independent of the host's immune response, multiplication eventually slows and some parasites of a dominant population of organisms assume a short, stumpy appearance. These forms have a more developed kinetoplast–mitochondrial complex and constitute the parasites that are infective to the tsetse fly. Near the end of the episode of parasitemia, both slender and stumpy types may be seen in a single blood specimen.

➢ Metacyclic trypomastigotes inoculated by a tsetse fly usually contain a population of organisms dominated by a distinctive antigenic type. After a period of time in the vertebrate

host, usually a week or so, the antigenic variant type changes. This change is under the control of up to 1000 genes that have been identified in some strains of these organisms that can account for a change in the variant surface glycoprotein antigenic type. Each dominant population usually contains a few organisms that have already undergone antigenic change so that when the host responds immunologically to the dominant population there will be survivors that give rise to the next dominant population.

➢ Epimastigote and trypomastigote forms develop in tsetse fly. Infectious trypomastigote form injected into the bloodstream of mammalian host from the fly's saliva. Antigenic variation of glycoprotein coat of trypomastigotes is due to shifting expression of preexisting genes.



Leishmania sp.

Leishmaniasis is a vectorborne disease that is transmitted by sand flies and caused by obligate intracellular protozoa of the genus *Leishmania*. Human infection is caused by more than 20 species. These include the *L. donovani* complex with 2 species (*L. donovani*, *L. infantum)*

Life Cycle:

1. Leishmaniasis is transmitted by the bite of infected female phlebotomine sand flies. The sand flies inject the infective stage (i.e., promastigotes) from their proboscis during blood meals.

2. Promastigotes that reach the puncture wound are phagocytized by macrophages

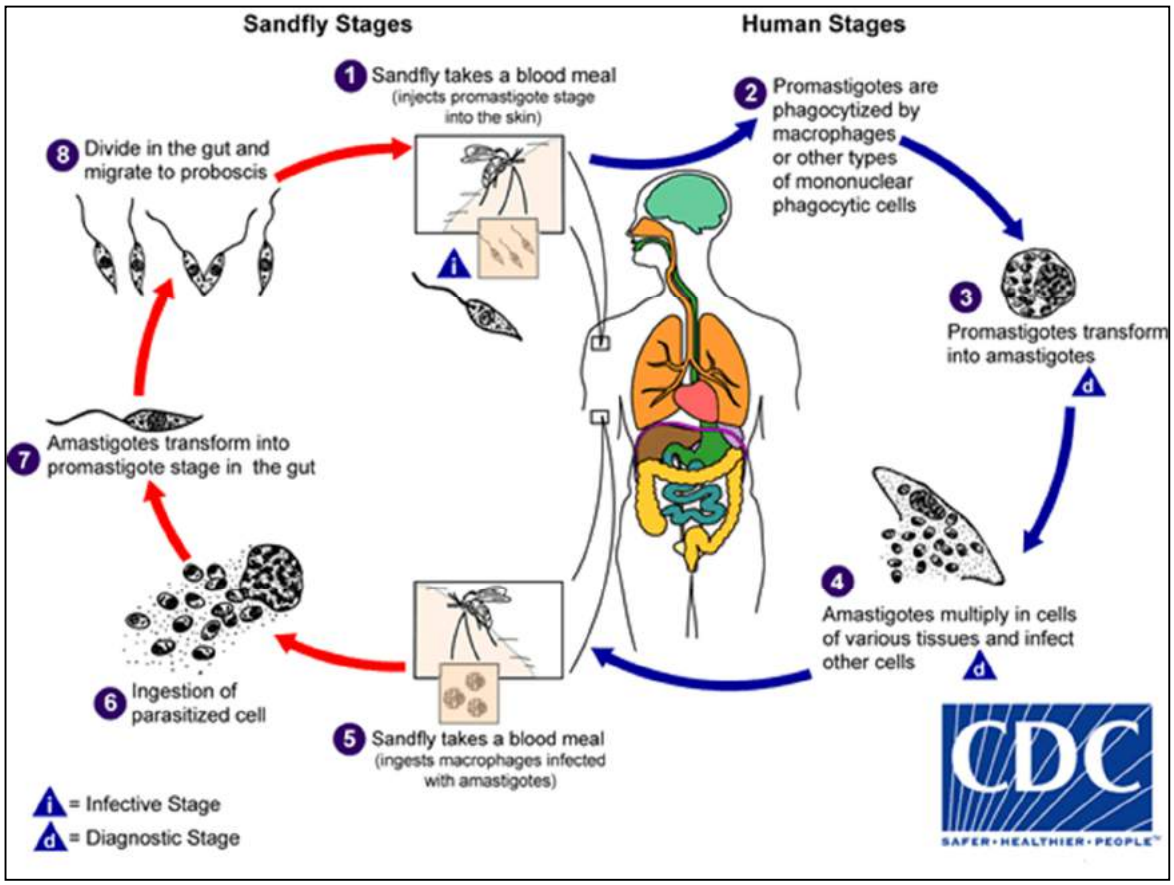
3. and other types of mononuclear phagocytic cells. Promastigotes transform in these cells into the tissue stage of the parasite (i.e., amastigotes)

4. which multiply by simple division and proceed to infect other mononuclear phagocytic cells

5. and 6. Parasite, host, and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sand flies become infected by ingesting infected cells during blood meals

7. In sand flies, amastigotes transform into promastigotes, develop in the gut

8. (In the hindgut for leishmanial organisms in the *Viannia* subgenus; in the midgut for organisms in the *Leishmania* subgenus), and migrate to the proboscis.



Plasmodium vivax

The *P. vivax* lifecycle is complex, including more than ten stages of cellular differentiation, with the parasite invading at least four types of cells within two different hosts.

Mosquito to human transmission

- During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host.
- Sporozoites from the mosquito migrate to the liver, where they mature into schizonts, which rupture and release merozoites.
- Another stage of the malaria parasite, called the hypnozoite, can remain dormant and persist in the liver for months.

Hypnozoites

- Undetectable with current diagnostic methods, hypnozoites in the human liver may reactivate weeks or months after the initial infection, leading to multiple clinical relapses and onward transmission.
- It is unknown how many people in *P. vivax* malaria endemic areas are hypnozoite carriers. Thus, hypnozoites represent a silent transmission reservoir.

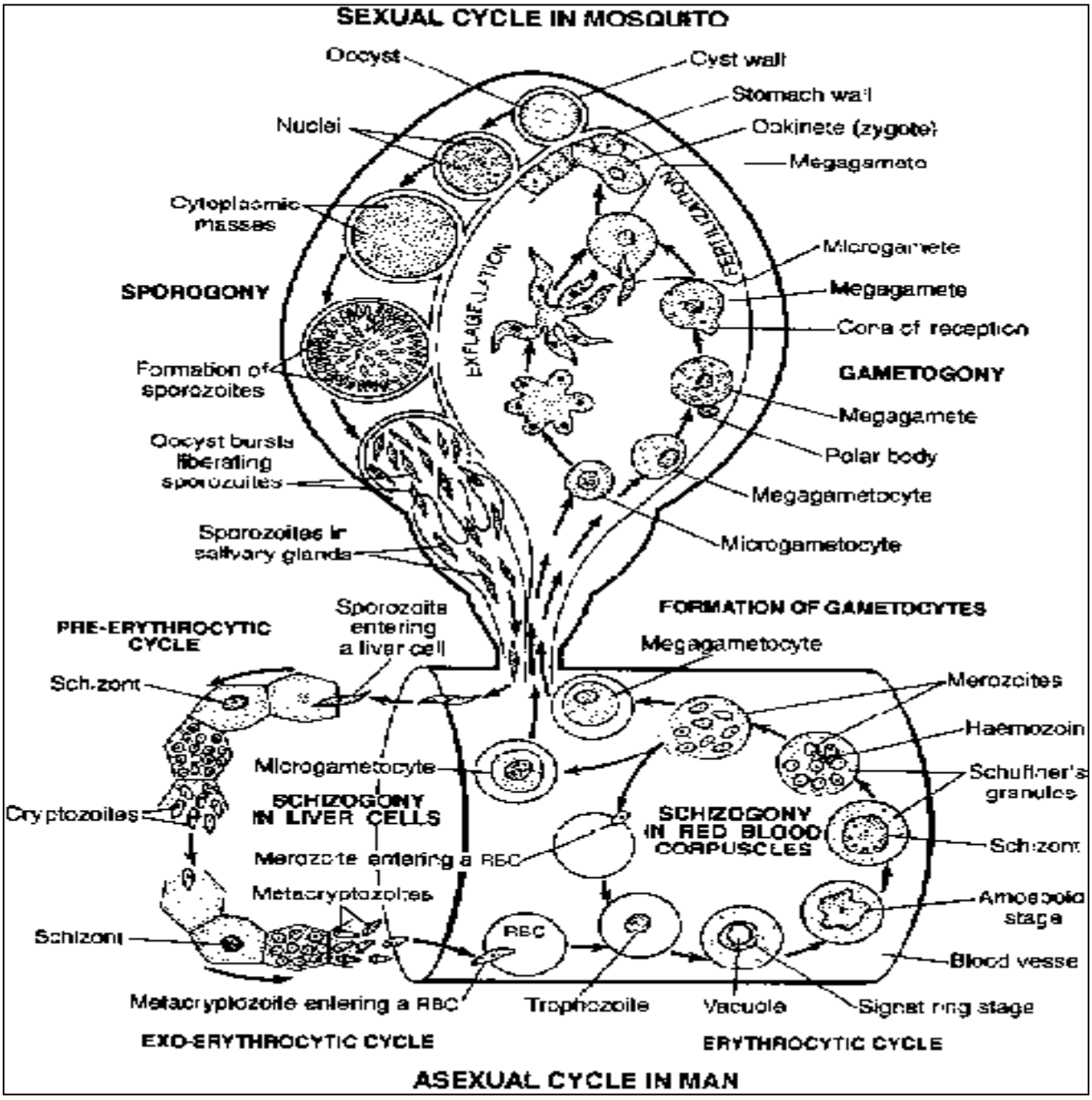
Asexual blood stage infection

- Following their release into the blood, merozoites invade reticulocytes (immature red blood cells), and mature into schizonts which rupture to accelerate the infection. This asexual erythrocytic stage (or blood stage) causes the symptoms of malaria which appear about 48 h after infection.
- The blood stage causes the elimination of infected and uninfected red cells resulting in anaemia, which can be severe.

Sexual stage infection

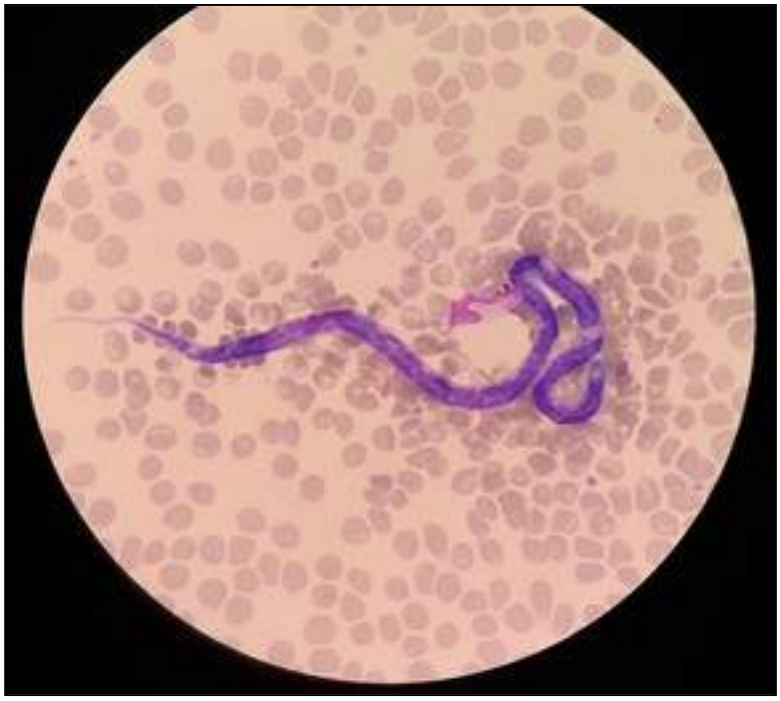
- Merozoites also differentiate into the infectious stage (sexual stage), gametocytes.

- In *P. vivax malaria*, gametocytes can be produced before clinical symptoms, which may allow transmission before the host feels unwell or receives treatment.



Slide identification of microfilaria, *Taenia solium*, *Ascaris*, and developmental stages of malaria

Wuchereria bancrofti (microfilarial stage)



Systematic Position
(Beck, Liem and Simpson, 1990)

Kingdom	Animalia
Phylum	Aschelminthes
Class	Nematoda
Specimen	*Wuchereria bancrofti*
Common name	Filarial worm

B. Microfilaria

1. Presence (or absence) of a sheath: a delicate and closely fitting membrane (the egg capsule), which is detectable only when it projects beyond the head or tail of the larva.
2. Presence of several internal nuclei and primordia of organs.
3. Cuticle is th
4. While the h

Hence, the speci

Taenia solium

Systematic Position

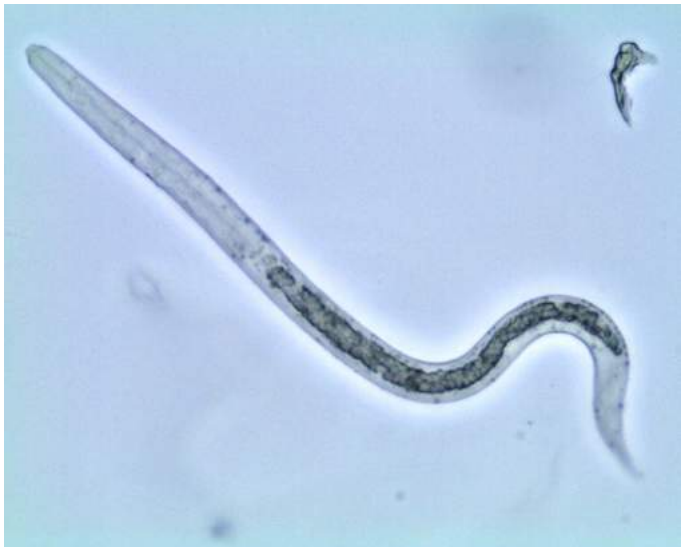
Kingdom- Animalia
Phylum- Platyhelminthes
Class- Cestoda
Subclass- Eucestoda
Specimen- Taenia solium
Common name- Pork tapeworm

Identifying characters

1. Rostellum is surrounded by two rows of hooks.
2. Head bear four suckers.
3. The proglottids gradually increase in size towards the posterior end.

Hence, the specimen seems to be Taenia solium.

Ascaris lumbricoides



Systematic Position

Kingdom- Animalia
Phylum- Aschelminthes
Class- Nematoda
Specimen- Ascaris lumbricoides

Common name- Roundworm

1. Terminal mouth is bound by three lips.
2. Body surface is marked by single dorsal and ventral midlines and two lateral lines.
3. Tail is curved and pointed (in male) or straight and blunt (in female).
4. Anal opening is transverse near the posterior end.
5. In male, a pair of **penial setae** are projecting out from the **cloaca**.
6. In female, a ventral genital pore is present near the 1/3 distance from the anterior end.
7. Excretory pore is present on anteroventral line.

Hence, the specimen seems to be *Ascaris* sp.

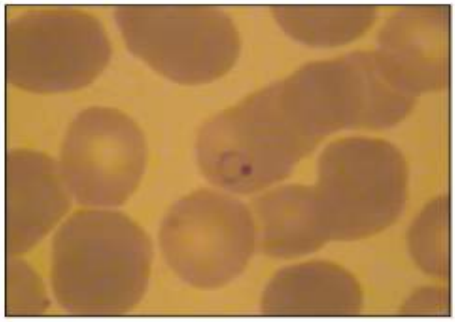
Plasmodium vivax

Plasmodium vivax

- Early Trophozoite (ring form, R.) (Pv. R.):
  - Ring form one-third the diameter of a red blood cell;
  - Heavy chromatin dot; Cytoplasma circle around vacuole.

间日疟原虫环状滋养体

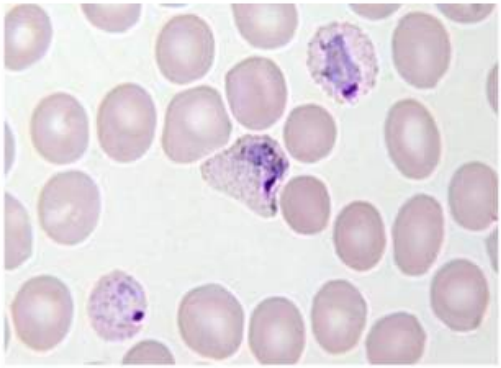
Young ring form trophozoite of *Plasmodium vivax*



- Late Trophozoite (Pv. T.):
  - Irregular ameboid mass that almost fills the entire red blood cell.
  - Fine, golden-brown pigment may be present.
  - One or more small vacuoles retained until schizont stage.
  - Schüffner's stirpling may be seen on Giemsa-stained smears.

间日疟原虫大滋养体

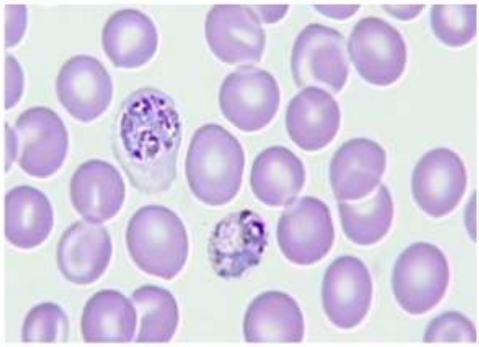
Mature trophozoite of *Plasmodium vivax*



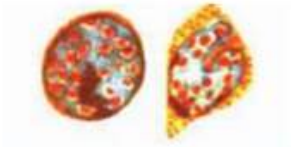
- Early Schizon (Pv. S.):
  - Division of chromatin apparent.
  - Cytoplasmic bands may contain clumps of brown pigment.

间日疟原虫未成熟裂殖体

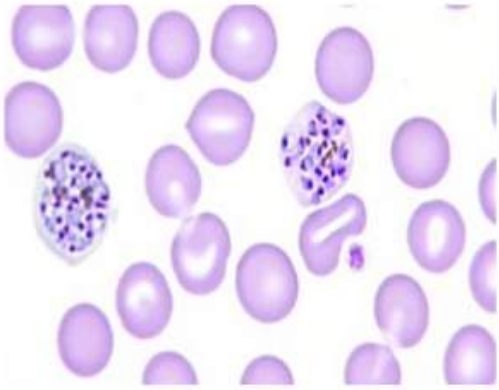
Immature schizont of
Plasmodium vivax



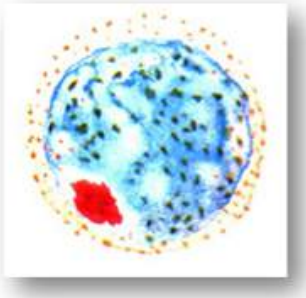
- Late Schizont (Pv. S.):
  - 12 to 24 merozoites (16 average), each with distinct cytoplasm and chromatin.
  - Schizont fills entire red blood cell, which becomes thin and transparent.

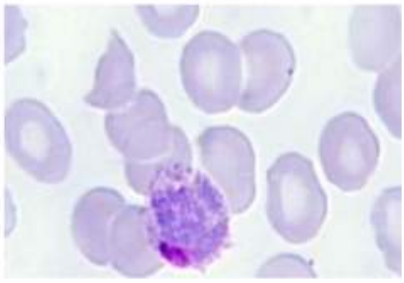


间日疟原虫成熟裂殖体
mature schizont of
Plasmodium vivax

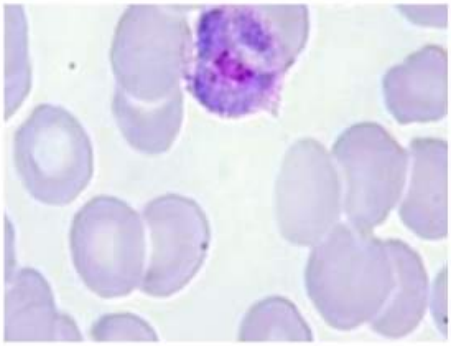


- female gametocytes (♀ G.):
  - ✓ Rounded or oval with homogeneous cytoplasm.
  - ✓ Diffuse delicate golden to light brown malarial pigment throughout parasite.
  - ✓ The macrogametocyte displays a compact, deep red staining chromatin mass arranged near the cell periphery and cytoplasm which stains bright blue.





- male gametocytes (♂G.):
  - ✓ Rounded or oval with homogeneous cytoplasm.
  - ✓ Diffuse delicate golden to light brown malarial pigment throughout parasite.
  - ✓ The microgametocyte displays a large chromatin mass which stains pink to purple and cytoplasm which stains pale blue.



Examination of stool for OPV (Ova parasite cyst)

1. **Saline wet mount:** It is used to detect worms, bile stained eggs, larvae, protozoan trophozoites and cysts. In addition, it can reveal the presence of RBCs and WBCs.

2. **Iodine wet mount:** It is used to stain the glycogen and nuclei of the cysts. A cyst is appreciated better in an iodine preparation, but the motility of the trophozoite is inhibited in the iodine preparation.

Procedure

• Place a drop of saline on the left half of the slide and one drop of iodine on the right half.

• With an applicator stick, pick up a small portion of the specimen (equivalent to the size of a match head) and mix it with a saline drop.

• Similarly, pick up a similar amount and mix with a drop of iodine.

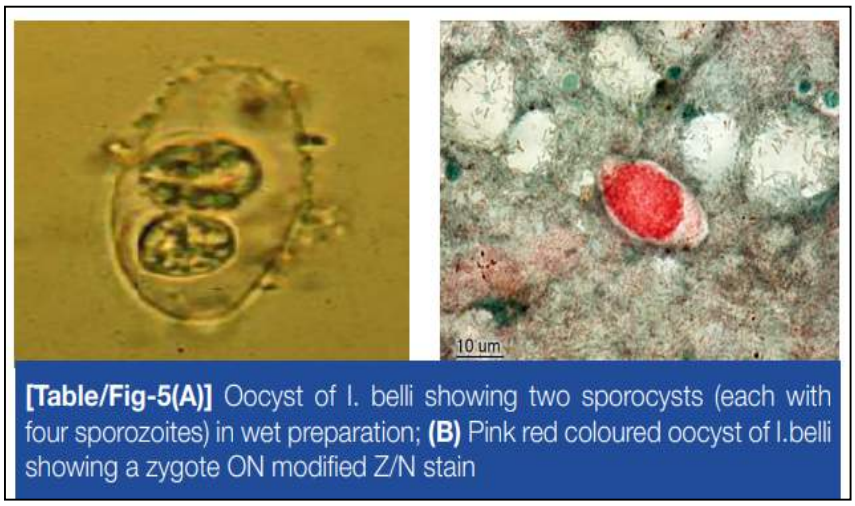
• Put the cover slip separately on both and examine under the microscope.

• The ova, cysts, trophozoites and adult worms can be identified as per their characteristic features.

Identification

A. The oocyst of *Isospora belli*

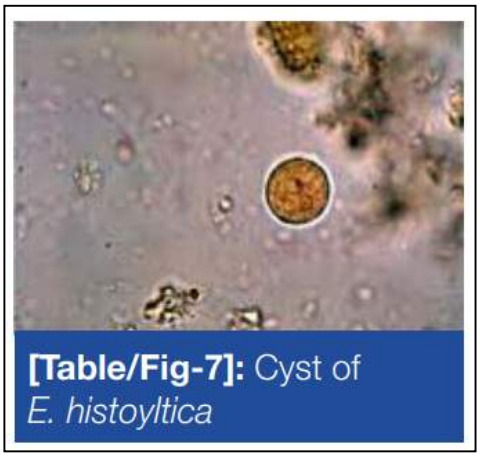
It is oval, measuring 20-22 μm in diameter and it usually shows a granular zygote. Occasionally, the oocyst may show two sporocysts (each with four sporozoites).



[Table/Fig-5(A)] Oocyst of I. belli showing two sporocysts (each with four sporozoites) in wet preparation; **(B)** Pink red coloured oocyst of I.belli showing a zygote ON modified Z/N stain

B. *Entamoeba histolytica* cyst

It is spherical and it measures 10- 20 µ. It is a mature cyst with four nuclei, with a compact, centrally located karyosome; the chromatin is delicate. Some cysts may have chromatoid bars. This is the infective stage of the parasite.



[Table/Fig-7]: Cyst of *E. histoyltica*

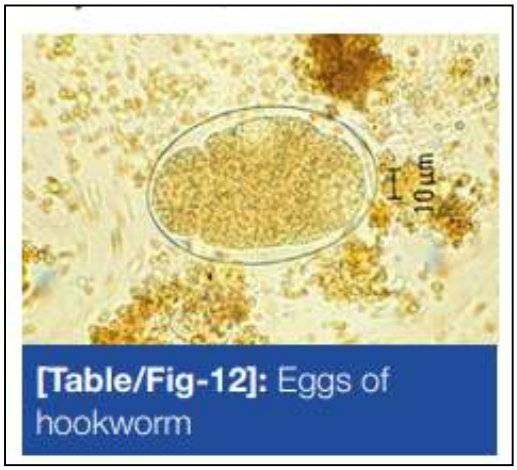
C. Tapeworm egg (*Taenia* spp)

It is spherical, it measures 31-43µ and it has a thick shell with prominent radial striations. An embryonated oncosphere which possesses 3 pairs of hooklets within the shell is diagnostic of the genus. Species identification on the basis of morphology is not possible.

[Table/Fig-15]: Eggs of *Taenia* spp

D. *Hookworm egg*

They are oval and ellipsoid and they measure 60x40 µ. Their shells are thin walled, smooth and colourless. Their internal cleavages are well developed at the 4-8 cell stage, which pull away from the shell, leaving an empty space.



[Table/Fig-12]: Eggs of hookworm

E. Fertile egg of roundworms

It measures 60×45 µ and is round or ovoid with a thick shell. It is covered by a thick albuminous coat, its inner cell is in various stages of cleavage and it is brown in colour.

[Table/Fig-10]: Fertile egg of round worm

HAEMOGLOBINOPATHIES AND SEROLOGY LAB

Code: MMLT 396C

1. Determination of Blood groups

PRINCIPLE

The surfaces of red cell membrane contain a variety of genetically determined antigens, called **isoantigens** or **agglutinogens,** while the plasma contains antibodies **(agglutinins).** To determine the blood group of a person, his/her red cells are made to react with commercially available antisera containing known agglutinins. The slide is then examined under the microscope to detect the presence or absence of clumping and hemolysis (agglutination) of red cells which occurs as a result of antigen-antibody reaction.

APPARATUS AND MATERIALS

1. Microscope. •Glass dropper with a long nozzle. •Sterile blood lancet or needle. •Sterile cotton/ gauze swabs. •Alcohol. •5 ml test tube. •Toothpicks.
2. Clean, dry microscope slides. (A special porcelain tile with 12 depressions is available for this purpose and may be used in place of glass slides.)
3. 1% sodium citrate in normal saline (or normal saline alone).
4. **Anti-A serum:** [contains monoclonal anti-A antibodies (against human); these antibodies are also called anti-A or alpha (α) agglutinins]. The anti-A serum can also be obtained from a person with blood group B.
5. **Anti-B serum:** [contains monoclonal anti-B antibodies (against human); these antibodies are also called anti-B or beta (β) agglutinins]. The anti-B serum can also be obtained from a person with blood group A.
6. Anti-D (anti-Rh) serum: [Contains monoclonal anti-Rh (D) antibodies (against human). These antibodies are also called anti-D agglutinins.

PROCEDURES

1. Using a glass-marking pencil, divide 3 slides, each into two halves by a line drawn down the middle (the left sides will act as “test sides” and right sides as the “control sides”). Mark the left corner of 1st slide anti-A, left corner of 2nd slide “anti-B, and the left corner of 3rd slide “anti-D”. Mark the right corners of these 3 slides ‘C’ (for control).
2. Mark another slide (4th) ‘S’ (for only red cell suspension in saline, i.e. no antiserum will be added on this slide).
3. Place 8–10 drops of saline in the center of slide ‘S’.
4. **Preparation of red cell suspension.** A suspension of red cells in saline should preferably be prepared and used instead of adding blood drops directly from the fingerpick to the antisera for the following reasons:
   a. Dilution of blood permits easy detection of agglutination and hemolysis, if present. (Red cells in undiluted blood tend to form large rouleaux and masses. These may be difficult to disperse and may be mistaken for agglutination).
   b. Plasma factors likely to interfere with agglutination are eliminated.
5. Get a finger-prick under aseptic conditions, and add 2 drops of blood to the saline on the slide marked ‘S’. Mix the saline and blood with a clean glass dropper to get a suspension of red cells. You may use a toothpick for this purpose.
   • A better method is to place 2 ml of saline in a small (5 ml) test tube. Then get a finger pricked and allow a blood drop to form. Now place the pricked fingertip on top of the test tube and invert it. Mix the blood and saline by inverting the tube 2 or 3 times. A suspension of red cells is now ready.

• Washed red cell suspension gives the best results. The red cells are "washed" in saline by centrifuging the diluted blood, removing the
supernatant, and adding fresh saline to get a suspension of "washed" red cells.

6. **Determination of Blood group.**

Put one drop of anti-A serum on the left half ("test side") of 1st slide (marked anti-A), one drop of anti-B serum on the left half of 2nd slide (marked anti-B), and one drop of anti-D serum on the left half of 3rd slide (marked anti-D).

7. Put one drop each of normal saline on the "control" sides (right halves) of the 3 slides (i.e. areas marked 'C').

8. Add a drop each of red cell suspension (from the slide 'S', or from the test tube of red cell suspension) on anti-A, one drop on anti-B and one drop on anti-D sera, and one drop each on the normal saline taken on the "control" sides of the 3 slides.
In this way, the red cells-saline mixture on the "control" sides of each slide will act as a control to confirm agglutination or no agglutination on the corresponding test side **(Figure)**.

9. Mix the anti-sera and red cells, and saline and red cells on each slide by gently tilting it first one way and then the other a few times. Take utmost care that the "test" mixtures and "control" mixtures do not flow into each other and get mixed up.

• The red cells and sera can also be mixed by gently blowing on them. You may use 3 separate toothpicks to transfer red cell suspension to the three anti-sera, and for mixing them, and 3 toothpicks to transfer red cells to saline drops taken on the "control" sides of the 3 slides.

10. Wait for 8–10 minutes, then inspect the 3 antisera-red cell mixtures ("test" mixtures) and "control" mixtures, first with the naked eye to see whether agglutination (clumping and hemolysis of red cells) has taken place or not. Then confirm under low magnification microscope, comparing each "test mixture" with its corresponding "control mixture".

OBSERVATIONS AND RESULTS

It is essential that you should be able to distinguish between ***"agglutination"*** and ***"no agglutination"***. The features of each are:

Agglutination.

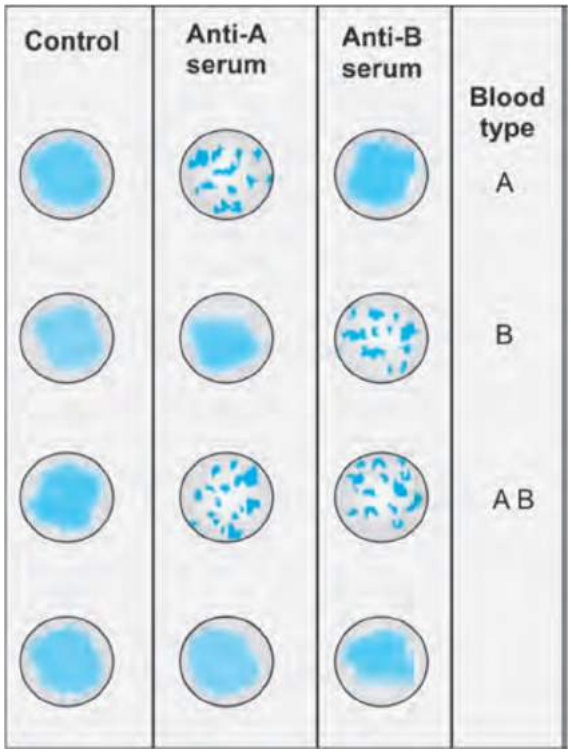
i. If agglutination occurs, it is usually visible to the naked eye. The hemolysed red cells appear as isolated (separate), dark-red masses (clumps) of different sizes and shapes.

ii. There is brick-red coloring of the serum by the hemoglobin released from ruptured red cells.

iii. Tilting or rocking the slide a few times, or blowing on it does not break or disperse the clumps.

iv. Under LP objective, the clumps are visible as dark masses and the outline of the red cells cannot be seen.

Grading of Agglutination Reaction. The agglutination reaction may be graded according to whether there is a single large agglutinate (4+), or a number of large (3+), medium or small masses with no free red cells (2+), or many small aggregates in the background of free red cells (1+).



No Agglutination

i. In the "control" mixtures, the red cells may form a bunch, or rouleaux. These sedimented red cells give an orange tinge of a suspension of red cells rather than "isolated dark red masses" of ruptured red cells.

ii. The red cells will disperse if you gently blow on the slides, or tilt them a few times.

Confirm all these features of "no agglutination" under the microscope.

2. Cross matching technique

Cross matching is a procedure performed prior to transfusion of blood or blood products to detect any serological incompatibilities in the blood of donor and recipient. Before a donor's blood is transfused into a recipient, there should be no antigens or antibodies in both, that would react with each other resulting in transfusion reaction. Cross matching is designed to prevent such transfusion reactions which may occur after transfusion.
Cross matching plays important role to detect:

- Most recipient antibodies directed against donor's red blood cell antigens.
- Most donor antibodies directed against recipient's red blood cell antigens.
- Major errors in **ABO grouping**, labeling and identification of donor and recipients.

Principle of Cross Matching

Cross matching is based on the principle of serological detection of any clinically significant irregular/unexpected antibodies in either donor or recipient's blood. There are two types of cross matches:

1. **Major Cross Match**: It involves testing the donor's red cells with recipient's serum to determine the presence of any antibody which may cause hemolysis or agglutination of donor red cells.This is more important than minor cross match.
2. **Minor Cross Match**: It involves testing of donor's plasma with recipient's red cells to determine the presence of any antibody which may cause hemolysis or agglutination of recipient's red cells.

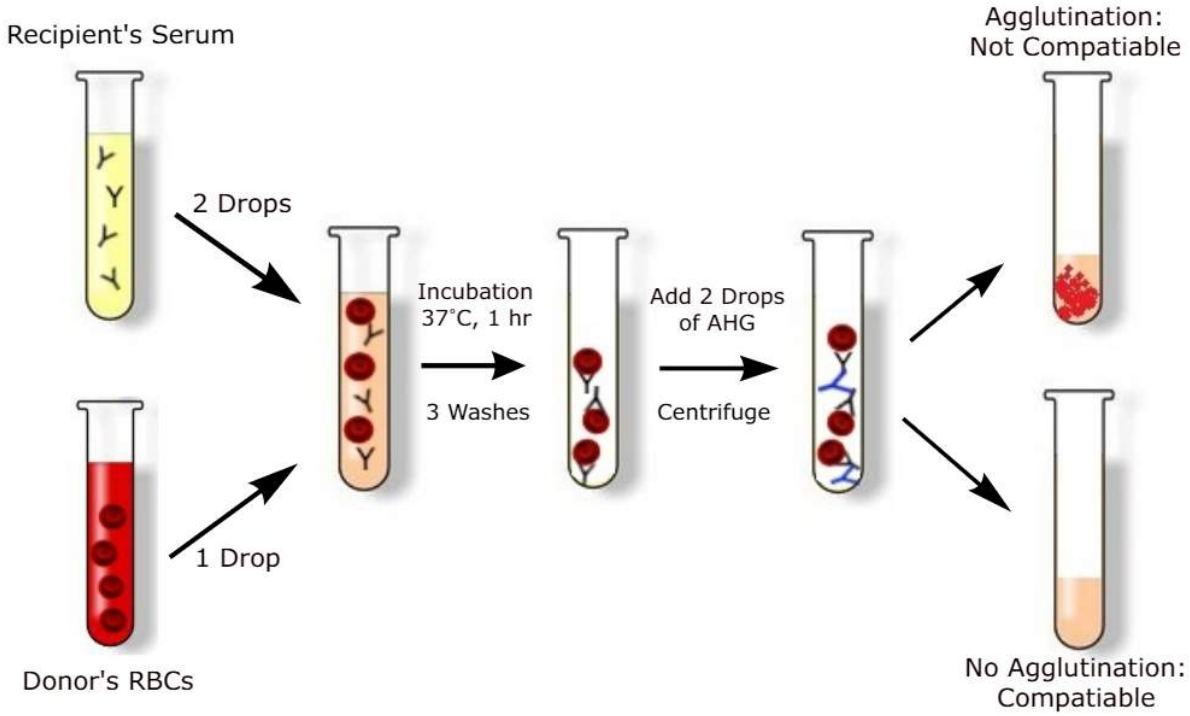
Procedure of Cross Matching

There are different methods for cross matching, as shown in table. Among them most commonly used technique is Anti-human globulin (AHG) cross match.

Major Cross Match

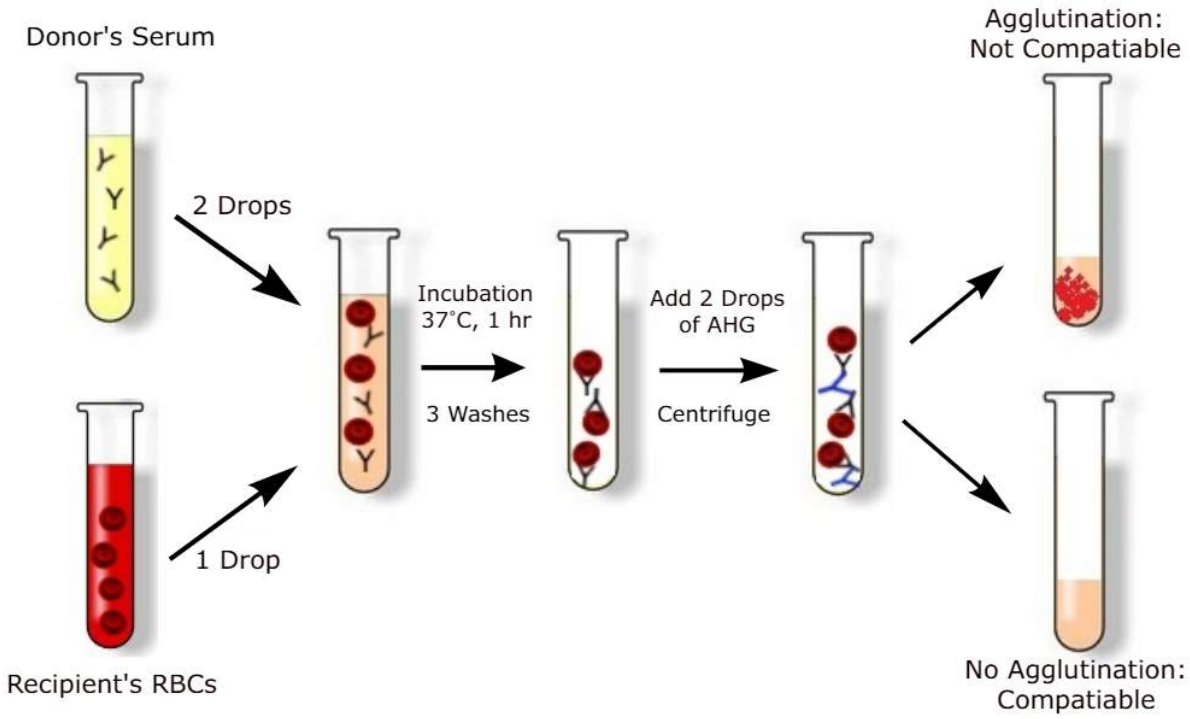
1. Prepare donor and recipient's blood sample: **Donor's red cells and recipient's serum/plasma**.
2. Prepare 3-5% saline cell suspension of red cells.
3. Label a test tube.
4. Add two drops of recipient's serum and one drop of donor cell suspension.
5. Mix and incubate the tubes at 37 degree Celsius for about 60 minutes.
6. Decant the serum completely and wash the cells three times in saline.
7. Add two drops of Anti-human Globulin (AHG) and mix. Allow to stand at room temperature for 5 minutes.
8. Centrifuge at 1500 rpm for 1 minute.
9. Observe macroscopically and microscopically for agglutination.

10. If macroscopic agglutination is not observed, transfer a small amount onto a glass slide and examine for microscopic agglutination. Rouleaux is not an indication of incompatibility.



Minor Cross Match

1. Prepare donor and recipient's blood sample: **Recipient's red cells and donor's serum/plasma**.
2. Label a test tube.
3. Add two drops of donor's serum and one drop of recipient's cell suspension.
4. Mix and incubate the tubes at 37 degree Celsius for about 60 minutes.
5. Decant the serum completely and wash the cells three times in saline.
6. Add two drops of Anti-human Globulin (AHG) and mix. Allow to stand at room temperature for 5 minutes.
7. Centrifuge at 1500 rpm for 1 minute.
8. Observe macroscopically and microscopically for agglutination.
9. If macroscopic agglutination is not observed, transfer a small amount onto a glass slide and examine for microscopic agglutination. Rouleaux is not an indication of incompatibility.



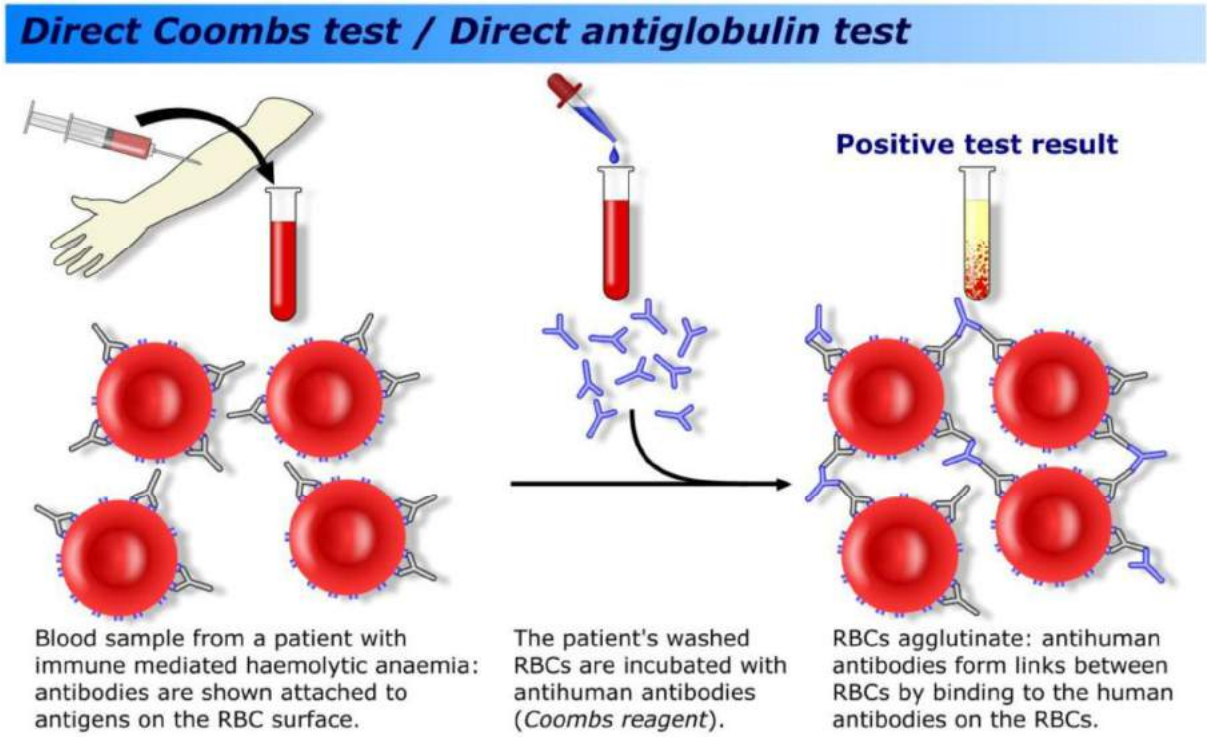
Results and Interpretation

Compatible donor and recipient blood should show **no agglutination** in both major and minor cross match. Blood which shows incompatibility in major cross match should never be transfused, because the large plasma volume of the recipient blood containing antibodies can destroy the donor's red cells easily. The minor incompatibility is **less important** because the donor's serum which contains the antibodies is diluted in the recipient's own plasma, making the antibodies very dilute and ineffective.

3. Erythrocyte Hemolysis Assessments and Direct Coomb's Test

Coomb's Test (The Antiglobulin Test) It refers to clinical blood tests that used in immunohematology to find certain antibodies which cause autoimmune hemolysis of RBCs.

Types of Coomb's Test 1. Direct Coomb's Test -The patient's cells, after careful washing are tested for sensitization that has occurred in vivo (inside the body). -This test is done on the newborn's blood sample, usually in the setting of a newborn with jaundice. -The test is looking for foreign antibodies that are already adhered to the infant's RBCs, a potential cause of hemolysis.
-A positive DAT may also be caused by the presence of allo-antibodies (e.g. owing to a delayed hemolytic transfusion reaction), so details of any transfusion in the past months must be checked for.



2. Indirect Coomb's Test
Normal RBCs are incubated with a serum suspected of containing an antibody and subsequently tested, after washing for in vitro-bound antibody.

Aims of the Test -To detect incomplete Rh antibodies. -IgG antibodies sensitizing RBCs but cannot agglutinate RBCs suspended in saline, while IgM antibodies agglutinate saline-suspended RBCs completely.

Principle of the Test -In certain diseases or conditions, an individual's blood may contain IgG antibodies that can specifically bind to antigens on the RBC surface membrane. -RBCs coated

with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement. -In order for agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the C3b or C3d component of complement, must be added to the system. -Because antibodies are gamma globulins, they can form bridges between RBCs sensitized with antibodies and cause them to agglutinate.

Materials Phosphate Buffered Saline (PBS): NaCl 0.9%, pH 7.0 ± 0.2 at 22ºC ± 1ºC. IgG sensitized red cells Inert antibody serum Weak anti-D Water bath or dry heat incubator equilibrated to 37ºC ± 2ºC. Coomb's cell washer Low Ionic Strength Solution (LISS)

Procedure

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

4. Fraction collection from Blood and its storage.

The collection of blood from donors may take place within the blood transfusion centre or hospital blood bank.

It is also often collected from donors during mobile blood collection sessions.

The blood is then taken to a laboratory for testing and processing into components and for storage and distribution as the need arises.

Blood is collected at body temperature, i.e. +37 °C. But in order to maintain its vital properties, it must be cooled to below +10 °C to be transported, and stored at refrigeration temperatures of around +4 °C until use.

What is blood cold chain

The term, blood cold chain, which begins the moment the blood is collected and continues until it is transfused.

The blood cold chain is a series of interconnected activities involving equipment, personnel and processes that are critical for the safe storage and transportation of blood from collection to transfusion.

Harmful effects of Improper Storage.

If blood is stored or transported outside of these temperatures for long, it loses its ability to transport oxygen or carbon dioxide to and from tissues respectively upon transfusion.

Other factors of serious concern are the risk of bacterial contamination if blood is exposed to warm temperatures.

Conversely, blood exposed to temperatures below freezing may get hemolysed and can lead to a fatal transfusion reaction.

Whole blood and red cells must always be stored at a temperature between +2 °C and +6 °C.

If blood is not stored at between +2 °C and +6 °C, its oxygen- carrying ability is greatly reduced.

The anticoagulant/preservative solution in the blood bag contains nutrients for the blood during storage and stops the blood from clotting.

The red cells can carry and deliver oxygen only if they remain viable.

Fresh frozen plasma

Fresh frozen plasma (FFP) is plasma which is separated from a unit of whole blood within 6 hours of collection, and has been rapidly frozen and maintained at all times at a temperature of minus –30 °C or lower.

FFP, once thawed has a shelf life of 24 hours at 1^0C to 6^0C.

Plasma contains water, electrolytes, clotting factors and other proteins (mostly albumin), most of which are stable at refrigerator temperature, i.e. +2 °C to +6 °C.

Storage and transport of Red Cells

Condition	Temperature range	Storage Time
Transport of pre- processed blood	+20 °C to +24 °C	Less than 6 hours
Storage of pre- processed or processed blood	+2 °C to +6 °C	Approx. 35 days
Transport of processed blood	+2 °C to +10 °C	Less than 24 hours

Permitted Storage Time According To Temperature Used To Store FFP and Cryoprecipitate

Product	Storage temperature	Maximum storage time
FFP	–30 °C or below	1 year
Cryoprecipitate	–30 °C or below	1 year

Platelet concentrates

Platelet-rich plasma (PRP)/ Platelet concentrate (PC) must be separated from whole blood by centrifugation within 6 hours of collection.

whole blood should be kept at between +20 °C and +24 °C until it is processed into platelet concentrates and other blood components.

Platelet concentrates should be stored at a temperature of between +20 °C and +24 °C i.e 22±2 °C with continuous gentle agitation. This is essential to prevent platelet aggregation which results in loss of viability.

Length of Time Permitted For The Storage And Transportation of Platelet Concentrates Within The Temperature Range +20 °C To +24 °C

Process	Maximum Storage Time
Storage	Upto 5 days
After issue, before transfusion	30 minutes
Open system and/or pooled platelet prepared in open system.	4 hours
Pooled platelets prepared in closed system.	5 days.

Plasma derivatives

Unlike blood components, plasma derivatives such as albumin or immunoglobulin are concentrated, sterile specific proteins, obtained from large pools of donor plasma through a complex pharmaceutical process called plasma fractionation

It is essential to store all plasma derivatives according to the manufacturer's instructions

Cold chain samples and reagents

The storage and transportation of reagents or blood samples is as critical as that for blood.

Manufacturers of laboratory reagents recommend methods for their safe storage and transportation.

The recommendations in the package inserts must be followed to avoid deterioration of the reagents and subsequent poor performance in use.

Testing of the blood samples should be carried out rapidly after collection. The longer that testing is delayed, the poorer the results.

The method of collection, storage and transportation of blood samples will depend on the type of laboratory test to be carried out.

5.Pre-transfusion testing

Resolution of anomalous grouping

ABO grouping is the most important pre-transfusion serological test performed. Fully automated ABO and D grouping procedures have significantly improved the accuracy and security of results, and should be used wherever possible.

When anomalous ABO groups are encountered laboratory protocols should support investigation of the following findings.

Missing agglutinins in reverse grouping:

- obtain the patient's history, and review for information which may explain missing agglutinin (e.g. age, immunodeficiency)
- repeat the reverse group, increasing the sensitivity of the test, consider the use of tube techniques, lower incubation temperature, increased plasma:cell ratio and enzyme-treated red cells.

Unexpected additional reactions in the reverse group:

- investigate the presence of allo- or autoantibodies active at temperatures below 37°C
- consider repeating the reverse group at 37°C
- consider repeating the reverse group using cells negative for any identified alloantibody.

Unexpected reactions in the forward or D grouping, including positive diluent control:

- check for immunoglobulin coating of the patient's cells by performing a direct antiglobulin test (DAT)
- consider repeating tests using unpotentiated reagents in tube techniques
- consider techniques to remove or reduce immunoglobulin coating (e.g. warm wash to remove IgM) and repeat tests with appropriate controls.

Unexpectedly weak or mixed field reactions in forward or D group:

- obtain the patient's history, and review for information which may explain results (e.g. recent non-ABO identical transfusion, haemopoietic cell transplant)
- consider additional investigations which may include adsorption/elution, and flow cytometry

- panels of monoclonal anti-D reagents are commercially available for the investigation of partial and weak D phenotypes.

Genotyping is useful in resolving grouping problems, particularly weak and partial D types (see section 15.2). Genotyping alone must not be used to determine the ABO group for use in selection of blood for transfusion. Where the patient ABO group cannot confidently be assigned by serology, group O (high-titre negative) blood must be selected.

Antibody identification

In all cases of the investigation of alloantibodies laboratories should focus on:

- secure identification of alloantibodies detected
- exclusion of additional specificities to those identified
- selection of blood for transfusion.

When antibodies which cannot be identified have been detected, laboratories should consider referral to the International Blood Group Reference Laboratory (IBGRL).

When patients with rare phenotypes are encountered, laboratories should, when practicable, exchange material with other RCI departments via the UK Rare Red Cell Exchange to ensure continued supply of valuable materials.

Antibody identification techniques and protocols are described in BCSH guidelines and should be adhered to. More complex problems encountered by RCI laboratories and not covered by BCSH are considered below.

Complex antibody mixtures

When investigating complex antibody mixtures RCI laboratories should consider:

- extended phenotyping of the patient, e.g. C, c, D, E, e, K, M, N, S, s, P_1, Le^a, Le^b, Fy^a, Fy^b, Jk^a, Jk^b
- if this is impossible due to previous transfusion or heavy IgG sensitisation, genotyping offers an alternative source of information
- extending the range of techniques and incubation temperature to identify component antibodies
- using cells matching the patient's phenotype/genotype to confirm the presence of multiple antibodies rather than an antibody to a high-frequency antigen
- careful use of alloadsorption techniques to confirm the specificity of elements of the mixture.

Antibodies known as high-titre low-avidity (HTLA)

Antibodies traditionally known as HTLA include anti-Ch, –Rg, –Kn^{a}, McC^{a}, –Yk^{a}, –Cs^{a} and –Sl^{a}. Typically HTLA antibodies present as reacting with most panel cells by indirect autoglobulin test (IAT) with variable strength, with or without similar patterns using enzyme-treated cells. Experienced operators can recognise characteristic agglutination by microscopic examination of tube IAT, which have been described as 'loose', 'stringy' or 'gritty'. In investigating samples suspected to contain HTLA antibodies RCI laboratories should consider:

Neutralising anti-Ch or -Rg specificities by incubating the patient's plasma with pooled group AB donor plasma before IAT is undertaken. Reactivity of these antibodies is usually abolished. A dilution control in which the patient's plasma is incubated with phosphate-buffered saline should be prepared and tested in parallel with the neutralised plasma.

The use of a panel of cells lacking HTLA antigens.

Antibodies to high-frequency antigens (HFA)

Typically antibodies to HFA present with positive reactions of similar strength against all routine screen and identification panel cells. The most commonly encountered specificities include anti-k, –Lu^{b}, –Kp^{b}, –Vel, –Co^{a}, –Yt^{a}, –Fy3, –U and –In^{b}. In investigating samples suspected to contain antibodies to HFA, RCI laboratories should consider:

- the ethnicity of the patient
- typing the patient's red cells with antibodies to HFA. Where possible, CE-marked reagents must be used, otherwise results must be considered in context of the reliability of the reagent in use, supported by adequate controls.

Antibodies to low-frequency antigens (LFA)

Typically antibodies to LFA present with negative antibody screen and are detected in crossmatch. The most commonly encountered specificities include anti-Kp^{a}, –Wr^{a} and –Co^{b}. In investigating samples suspected to contain antibodies to LFA, RCI laboratories should consider:

- testing the patient's plasma with a panel of red cells expressing LFA
- phenotyping the incompatible unit(s) for LFA.

Autoantibodies

Autoantibodies are frequently encountered in pre-transfusion testing, and may be the cause of autoimmune red cell destruction, or may be clinically benign. In either case autoantibodies may interfere with pre-transfusion testing, either due to coating of patient's cells with

immunoglobulin, or as pan-reactive antibody in patient's plasma. In providing safe transfusion in the presence of autoantibodies, RCI laboratories may adopt the following strategies.

ABO and Rh grouping in the presence of autoantibodies

Most modern test systems support routine, accurate grouping of the majority of patients whose cells are coated with immunoglobulin and who give a positive DAT. Cases which are problematic may present with reaction patterns that cannot be assigned to an ABO group, weak additional reactions and positive reagent controls.

Laboratories should make a clear documented assessment, based on the recommendations of reagent and test system suppliers, how to manage cases with anomalous ABO and D groups. This is particularly important when potentiated reagents are included in test systems.

Alloantibody detection and identification in the presence of autoantibodies

In dealing with cross-reacting autoantibodies, which complicate the detection and identification of underlying alloantibodies, RCI laboratories should consider:

- The characteristics of available, validated IAT in testing patient plasma-containing pan-reacting autoantibodies. Some workers consider tube IAT to be less prone to interference by autoantibodies than column technologies.
- The use of the patient's own cells to adsorb autoantibody from the plasma, permitting detection and identification of alloantibodies.
- The use of cells from two or more selected donors to adsorb autoantibody. Typically these cells are enzyme treated to optimise removal of autoantibody.

Management of patients with autoantibodies

Consideration should be given to close matching of recipient and donor red cell types. This is to safeguard against the presence of alloantibodies undetected by tests on modified plasma, and to prevent further alloimmunisation. In patients who cannot be grouped by conventional serology, due to sensitisation of red cells or previous transfusion, genotyping offers a solution.

6. The Widal Test

Widal test was devised by Frank Widal in 1896. Widal originally described the test to diagnose *Salmonella enterica* serotype Paratyphi B infection.

Patients infected with *S. enterica* serotype Typhi and Paratyphi produce serum antibodies to the O and H antigens of these pathogens, and the detection of these specific antibodies forms the basis of this test, the standardized protocol of which was established in 1950 (Felix, 1950).

Widal test is the most widely used diagnostic test for typhoid fever in developing countries. The Widal test has been in use for more than a century as an aid in the diagnosis of typhoid fever. It measures agglutinating antibody levels against O and H antigens. The Widal test is positive after the tenth day of the disease and may be false positive if an individual previously received a TAB vaccine.

Unfortunately, *S. enterica* serotype Typhi shares these antigens with other Salmonella serotypes and shares cross-reacting epitopes with other *Enterobacteriaceae*. The levels are measured by using serial dilutions of sera. Tube agglutination method is the recommended method of performing the Widal test; where serial two-fold dilutions of the subject's serum from 1:20 to 1:1280 are tested.

After its development, the **rapid slide test** became the most commonly used technique in local laboratories because of its convenience.

Ideally, the Widal test should be run on **both acute and convalescent-phase** sera to detect an increase in the agglutination titre but patient management cannot wait for results obtained with a convalescent-phase sample. For practical purposes, a treatment decision must be made on the basis of the results obtained with a single acute-phase sample. It is, therefore, important to establish the antibody level in the normal population in a particular locality in order to determine a threshold above which the antibody titre is considered significant.

In a situation where second sample collection is not feasible, knowledge of the agglutinin levels in the sera of normal subjects from the patients' community can form the baseline on which a diagnosis can be made. For practical purposes, titres occurring in more than 5% of the subjects under study were not diagnostically significant and should be regarded as normal in that population.

Widal test is simple and inexpensive, so it has gained widespread use despite shortcomings of both sensitivity and specificity that compromise its utility as a diagnostic test.

Serological response with Widal agglutinins

The earliest serological response in acute typhoid fever is a rise in the titre of the O antibody, with an elevation of the H- antibody titre developing more slowly but persisting longer than that of the O- antibody cutoff titre. Usually, O antibodies appear on days 6-8 and H antibodies on days 10-12 after the onset of the disease. The O antibody concentrations fall about 6 months after previous exposure to typhoid. Patients from communities where typhoid is endemic have higher H-antibody titres than do those not previously exposed to the antigens.

Some authors have reported that the level of H agglutinins is unhelpful in the diagnosis of typhoid, mentioning that the H-agglutinin titre remains elevated for a longer period than the O-agglutinin titre after an episode of typhoid fever and also may rise as a nonspecific response to other infections. Others, however, have proposed that the H-agglutinin titre is as useful as or more useful than the O-agglutinin titre. Some of the studies found O titre to be of greater diagnostic significance.

Although **simple to perform, the Widal test is difficult to interpret**, requiring detailed knowledge of the patient's medical, travel and vaccination history. The interpretation of a Widal test is greatly affected by the nature and extent of the patient's previous contact with typhoid antigens, whether the contact depends on a clinical or subclinical infection with typhoid or related organisms or is from TAB vaccination. The less the degree of the **previous contact, the greater the possibility that the findings of a Widal test may be usefully interpreted.** In countries where typhoid is endemic and prevalent the test can be interpreted only if the reporting laboratory has information about the basic level of O and H agglutinin in the population. Figure well in excess of the known titres especially if the amount of antibody rises during the illness are highly suggestive of infection.

The **value of the Widal test in diagnosing enteric fever in endemic areas remains controversial.** Some express the view that the test lacks standardization and adequate sensitivity and specificity to be clinically useful, while others consider the test to have diagnostic value when judged with clinical findings and knowledge of the 'normal' O and H agglutinin titres in the local population ('baseline titres'). The results from a single sample are difficult to interpret because high background rates of circulating antibodies to *Salmonella enterica* serotype Typhi or other Salmonella serotypes may produce a false-positive result. If paired sera are not available a single serum may be valuable if it yields an antibody concentration significantly in excess of the community norm for the test.

The sensitivity and specificity of the Widal test are known to vary widely, and a specific cutoff value should be established for each specific situation.

Materials required

- Widal test kit (killed colored suspension of *S. enterica* serotype Typhi O antigen, *S. enterica* serotype Typhi H antigen and *S. enterica* serotype Paratyphi AH antigen and *S. enterica* serotype Paratyphi BH antigen).
- Incubator
- Normal saline
- Refrigerator
- Applicator stick
- Graduated pipette

The Widal agglutination test was performed using standardized suspension of *S. enterica* serotype Typhi 'O' and 'H' and *S. enterica* serotype Paratyphi A 'H' and *S. enterica* serotype Paratyphi B 'H' antigen.

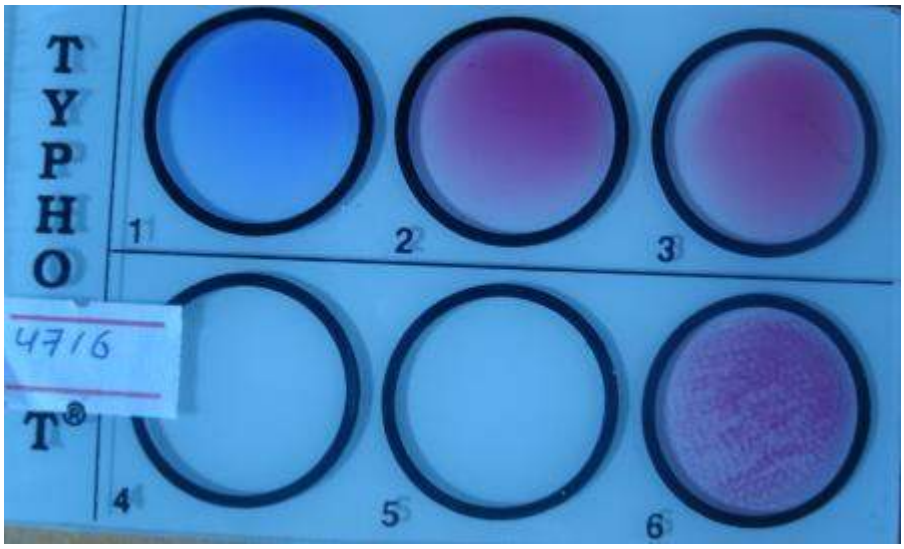
Before use, the antigen suspensions were allowed to warm to room temperature and were well-mixed.

Rapid screening test

- Using a micropipette dispense one drop (80 µl) of undiluted serum was onto 4 different circles.
- Dispense one drop of O, H, AH, and BH antigens on these circles respectively.
- Mix it thoroughly with the aid of the applicator stick and rotate the slide gently.

Result:

- Agglutination was observed within a minute (+ve test).
- No agglutination (-ve test)



Widal Test Result: Sample (6) showing BH positive in screening test

Slide titration

Rapid slide titration needs to perform for the samples which showed positive titre during rapid screening.

- Using a micropipette, dispense 40, 20, 10 and 5µl of undiluted serum onto a row of 3 cm diameter circles.
- Shake the reagent bottle rigorously shaken and add a drop (0.03ml) of the undiluted antigen suspension to each serum aliquot.
- Mix it thoroughly mixed with the aid of a stirring stick and rotate the slide gently.
- Observe the reactions after a minute.

Result:

- Agglutination was observed within a minute (+ve test).
- No agglutination (-ve test)

Reporting Widal test

The Widal test was reported by giving the titre for both O and H antibodies. The titre of each serum was read as the highest serum dilution giving visible agglutination.

The agglutination observed in any circle was indicative of the following results in a test tube.

Quality control

Quality control was done using the positive polyspecific control of the same dilutions as the test sample. Normal saline was used for a negative control.

Significance of Widal test in typhoid endemic and non endemic areas

Epidemiologic studies in an endemic country have shown that at least seven subclinical cases of typhoid fever occur for each clinical case. Therefore a positive Widal test may be seen in apparently healthy persons from an endemic area as a result of previous subclinical infection.

Factors affecting Widal agglutinin titre (Limitation of Widal Test)

- Previous typhoid vaccination may contribute to elevated agglutinins in the non-infected population.
- Cross reaction between malaria parasites and salmonella antigens may cause false-positive Widal agglutination test
- False-positive Widal tests have also been reported for patients with non-enteric salmonella infection, for example, typhus, immunological disorders, chronic liver disease, and cryptococcal meningitis.
- Prior use of antibiotics can dampen antibody response giving a low titre in the Widal test even in the face of bacteriologically confirmed typhoid fever resulting in misdiagnosis
- The Widal agglutination titre varies with the geographic location based on the endemicity of the enteric fever, the prevalence of non-typhoid salmonellae infection and other infections which cross-react with salmonella antigen.
- Past infection with serotype Typhi or another nontyphoidal Salmonella serotype that shares common antigens gives false positive Widal test.

The value of the Widal test in diagnosing enteric fever in endemic areas remains controversial. But is still useful and widely available test in endemic areas. Most of the researchers reported that Widal test has a diagnostic value when judged with clinical findings and knowledge of the baseline O and H agglutinin titres in the local population. Although there was no consensus on the diagnostic titre for a single Widal test, all the studies reported a “positive” Widal test based on a fourfold rise in O agglutinins in repeated tests or a titre of >1:80 or greater in a single test. Some researchers found that the level of H agglutinins is more helpful than the level of O agglutinins.

Elevated levels of both O and H agglutinin titre are more helpful than either of them alone, in making a presumptive diagnosis. When blood cultures are not available or impractical, a single Widal test can still have diagnostic significance, if the results are interpreted with relevant clinical findings and prevailing O and H agglutinin titres in the local population.

❖ **General inflammatory marks and specific therapeutic bio indicators.**

1. C-Reactive Protein (CRP)

C-reactive protein (CRP) is a protein produced by the liver in response to inflammation. Because high levels of CRP in the blood are reliable indicators of inflammation, a CRP test can be an important first step in diagnosing medical conditions that cause inflammation. This includes infections and autoimmune diseases, such as lupus.

Although the CRP test cannot reveal where the inflammation is occurring or what is causing it, results can point your healthcare provider in the direction of the likely suspects.

This article walks you through the uses of the CRP test, how it is performed, and what to expect before, during, and after the test. It also offers insights into how the results are interpreted so that you can participate in treatment decisions.

Purpose of the Test

The CRP test is a general marker for inflammation. It is used to determine if someone's symptoms are related to an inflammatory or non-inflammatory condition. The results, along with other findings, can help narrow the possible causes.

The CRP level can also tell if the inflammation is acute (severe and sudden, such as with an allergic reaction) or chronic (persistent, such as with diabetes).

Although there are limitations to what the test can reveal, it is a relatively reliable way to measure inflammation. The higher the CRP levels, the greater amount of inflammation in the body.

The CRP test can help identify a wide array of medical conditions, including:

- Allergic reaction
- Asthma
- Autoimmune diseases like rheumatoid arthritis and lupus
- Bacterial infection
- Bronchitis
- Cancer
- Celiac disease
- Chronic obstructive pulmonary disease (COPD)
- Connective tissue disease
- Diabetes
- Heart attack
- Heart disease, including atherosclerosis or myocarditis
- High blood pressure (hypertension)
- Inflammatory bowel disease (IBD)
- Major trauma

- Pancreatitis
- Pelvic inflammatory disease (PID)
- Pneumonia
- Systemic (body-wide) fungal infections
- Vascular diseases like aortic aneurysm and vasculitis
- Viral infections

Other Tests

The CRP test is often performed with another blood test called the erythrocyte sedimentation rate **(ESR)**. Both are non-specific markers for inflammation but, together, can offer important clues as to what is going on in the body.

The main difference between the two tests is that changes occur more quickly with CRP. For instance, CRP may drop to normal levels quickly once an infection has cleared, while ESR will remain elevated. In such cases, the ESR can help reveal the "footprint" of an illness even as the symptoms resolve.

There is also a **high-sensitivity CRP (hs-CRP) test** that measures very low amounts of CRP in order to help predict a person's risk of heart attack and stroke. Together with a cholesterol test, the hs-CRP can help determine if preventive measures, like statin drugs, are needed.

Risks

There are very few risks involved with blood tests. You may experience bruising, swelling, or a hematoma (a pooling of blood under the skin) after the blood draw.

Some people feel dizzy, lightheaded, or even faint.

There is a very small risk of infection from the needle puncture.

Before the Test

Before getting a CRP test, let your healthcare provider know about any medications you take, since some can affect CRP levels.

Timing

A blood draw usually takes less than five minutes. You will be able to leave as soon as the test is complete as long as you're not feeling faint or sick.

Location

The CRP test can be performed in your doctor's office, at a local hospital or clinic, or at a dedicated lab facility.

What to Wear

It is helpful to wear a short-sleeved shirt for the blood draw. Avoid tight sleeves that are difficult to roll or push up.

Food and Drink

A CRP test doesn't require fasting beforehand. However, other blood tests may be performed at the same time that do, such as a fasting cholesterol test. Speak with your healthcare provider or the lab to double-check.

Cost and Health Insurance

A CRP test is relatively inexpensive—around $12 to $16, on average.[1] If you have health insurance, your plan should cover the cost at least in part.

You can find out what your out-of-pocket costs are by calling the number on the back of your insurance card.

What to Bring

Bring a form of ID (such as your driver's license) as well as your insurance card and an approved form of payment, if needed. Check with the lab in advance to find out what kinds of payment they accept.

Recap

The CRP test requires a blood draw performed at a doctor's office or lab. You do not need to fast for this test, although fasting may be needed if other blood tests are being performed. Check with your doctor.

During the Test

The CRP test may be performed by a lab technician, a nurse, or a phlebotomist, a professional who is specially trained to draw blood.

Pre-Test

You may have to fill out some routine paperwork before your test. The receptionist will let you know once you check in.

Throughout the Test

The CRP test takes just a few minutes. Once you're called into the lab, you will sit in a chair. The technician will ask you which arm you want to use.

After a vein, typically one near the crook of your elbow, is chosen. Them the blood draw is performed as follows:

1. An elastic band is tied around your upper arm to help the vein swell.
2. The skin is cleaned with an alcohol swab.
3. A small needle is inserted into the vein. You may feel a slight pinch or poke. If the pain is considerable, let the technician know.
4. Blood is drawn into a vacuum tube via a thin tube connected to the needle.
5. After enough blood is taken, the elastic band is taken off and the needle is removed.
6. Pressure is placed on the puncture site with a cotton ball, after which an adhesive bandage is applied.

Post-Test

Once you feel well enough to do so, you can leave.

If you are feeling lightheaded or faint. let the technician or a member of the staff know.

Recap

The blood draw needed to run a CRP test is performed by a lab technician, nurse, or phlebotomist. If you feel pain, lightheadedness, or dizziness afterward, let the technician know.

After the Test

When you've finished having your blood drawn, you can resume normal activities.

Although there may be swelling, bruising, or pain at the injection site, the side effects tend to be mild and go away within a few days. If they don't or get worse, call your healthcare provider.

Interpreting Results

The results of a CRP or hs-CRP test are typically returned within a day or two, depending on the lab.

CRP Test

The CRP test results may be reported in milligrams per liter (mg/L) or milligrams per deciliter (mg/dL).[2]

Based on the level, a doctor can begin to narrow down the possible causes of an illness. Some of the likely causes can be broken down by the following CRP results:[3]

- **Less than 6 mg/L (0.3 mg/dL):** Normal level seen in healthy people
- **3 to 10 mg/L (0.3 to 1.0 mg/dL):** Normal to moderate inflammation (often seen in people who are obese, pregnant, smoke, or have issues like diabetes or the common cold)
- **10 to 100 mg/L (1.0 to 10 mg/dL):** Whole-body inflammation due to autoimmune disease, bronchitis, pancreatitis, heart attack, cancer, or another cause
- **Over 100 mg/L (10 mg/dL):** Marked whole-body inflammation due to acute bacterial infections, acute viral infections, systemic vasculitis, or major trauma, among other causes

- **Over 500 mg/L (50 mg/dL):** Severe body-wide inflammation most often due to severe bacterial infections

hs-CRP Test

The results of a CRP test are classified as follows to offer a sense of one's risk of a heart attack or stroke:[4]

- **Low risk:** Lower than 1.0 mg/L
- **Average risk:** 1.0 and 3.0 mg/L
- **High risk:** Above 3.0 mg/L

Recap

A CRP test result of 1.0 mg/dL or greater indicates body-wide inflammation. Moderate elevation is anything that falls between 1.0 and 10 mg/dL. Anything more is high or severe elevation.

An hs-CRP result above 3.0 mg/L puts you at high risk for a heart attack or stroke.

Follow-Up

The follow-up of a CRP test can vary based on your diagnosis. Your healthcare provider will consider your CRP results along with your symptoms and medical history, a physical exam, and other lab tests and procedures. The treatment or next steps will depend on that diagnosis.

With respect to the hs-CRP test, a repeat test may be ordered within two weeks since the results can fluctuate. If the results are borderline, the healthcare provider may take a watch-and-wait approach, re-testing after conservative measures like diet and exercise are tried.

If the hs-CRP results are high, they may recommend drugs to help reduce your blood pressure or cholesterol.

7.Immunological test for pregnancy

There are immunological tests for many different medical conditions and purposes – for instance, to test for an allergy, to screen for bowel cancer or to find out if a woman is pregnant. They can be used to carry out routine tests in hospitals and laboratories, to do quick tests yourself at home, as well as in family doctors' and specialists' practices.

How do they work?

Certain substances or pathogens (germs) in your body can be detected with the help of immunological techniques. The things that can be detected include viruses, hormones and the blood pigment hemoglobin. The tests take advantage of the body's immune system: In order to fight germs or foreign substances, the immune system produces antibodies. Antibodies are proteins that can bind to a specific germ or substance, just like a key fits into a specific keyhole. They "catch" the germs or substances, neutralize them and attract other immune cells.

The immunological tests used in laboratories are made by producing artificial antibodies that exactly "match" the substance or germ in question. When these antibodies come into contact with a sample of blood, urine or stool, they bind to the matching substance or germ if found in the sample. This reaction shows that the germ or substance is present.

What happens during the test?

As mentioned above, immunological tests contain specific antibodies that bind to the substance or germ that is being looked for. In some tests this reaction is visible to the naked eye. For example, in tests to determine your blood group, the blood coagulates (clumps together) on the test card. In other tests, the reaction has to be made visible using a fluorescent dye or an enzyme.

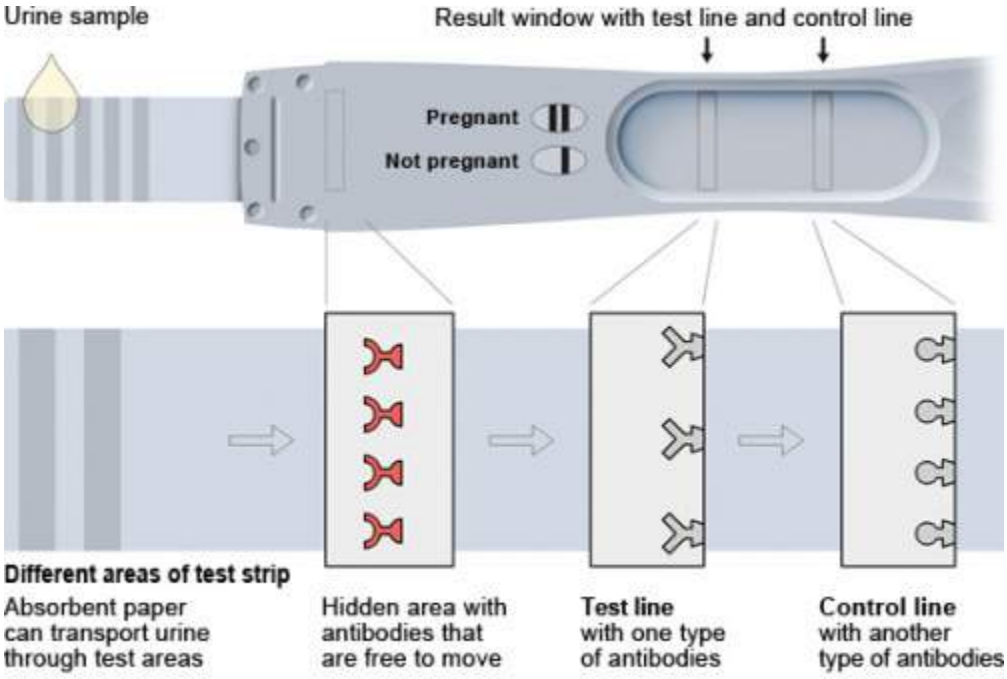
Immunological tests can generally be divided into rapid tests and laboratory tests.

Laboratory tests

In laboratory tests, sensitive devices measure the amount of bound antibodies based on the extent of a light or color reaction. The greater the reaction, the more of the substance or germ is present. Laboratory tests take longer than rapid tests but they are also more accurate.

Rapid test

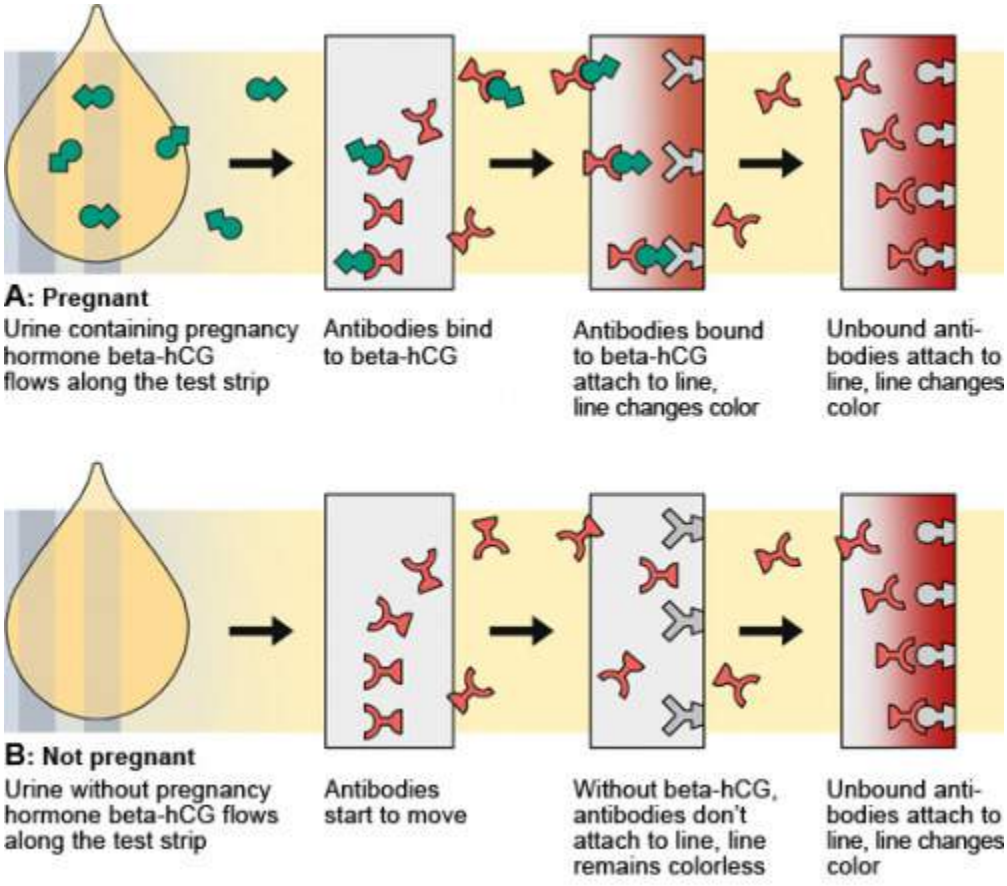
In rapid tests, the antibodies are usually found on paper strips (test strips), but sometimes glass is used too. Rapid tests are easy to use and provide instant results. But they are not as sensitive as laboratory tests and can't determine exactly how much of the substance or germ is present.



Paper strip for rapid test

Rapid tests work based on the principle of "lateral flow" (flowing sideways):

When a liquid sample (such as urine) is placed on one end of the test strip, the antibodies on the test strip bind to the substance you are looking for if it is present. Then the liquid slowly moves along the absorbent paper towards the other end of the strip. The antibodies continue to bind to the substance you are looking for, and this reaction causes a change in color. If enough of the liquid sample is used, it flows all the way along the paper strip until it reaches a control line at the other end. If the control line changes color too, the test was carried out properly.



Paper strip tests: positive and negative reactions

What are immunological tests used for?

Immunological tests are widely used. Their areas of application include:

- **Bowel cancer screening:** This test looks for the blood pigment hemoglobin, a sign of blood in stool. Blood in stool can be caused by various things, such as hemorrhoids, polyps or even bowel cancer.
- **Allergy tests:** to detect antibodies against allergy-triggering substances like grass pollen or certain foods.
- **Detecting germs causing an infection:** If it is thought someone has bacterial tonsillitis or scarlet fever, the test looks for Streptococcus bacteria. In the case of Lyme disease following a tick bite, there are tests that can detect the Borrelia bacteria that cause it, and there are tests that can detect the antibodies to Borrelia bacteria. Immunological tests can also be used to detect viruses. Examples include hepatitis C, HIV or HPV viruses. Pregnant women can have a blood test to find out whether they are protected from (immune to) toxoplasmosis.
- **Diagnosing heart attacks and thrombosis:** Shortly after a heart attack or if someone has thrombosis , higher levels of a certain protein are found in the blood. These can be detected using an immunological test.

- **Urine test:** If sugar, blood, proteins or inflammatory cells are found in urine using this rapid test, it could be a sign of diabetes, a urinary tract infection or kidney damage.
- **Pregnancy test:** Women can use this rapid test to find out whether their urine contains the "pregnancy hormone" beta-hCG.
- **Rapid tests for drugs and medication:** Immunological tests can also be used to look for recreational drugs such as cannabis, ecstasy and cocaine. Medical drugs that affect the central nervous system can also be detected in this way. These include sleeping pills (benzodiazepines), amphetamines and morphine.
- **Determining your blood group:** When blood transfusions are done, the person donating the blood and the person receiving the blood have to have the same blood group. Immunological tests can be used to determine the blood groups before a blood transfusion.

Immunological tests can also be used to diagnose congenital or acquired immune diseases, differentiate between different forms of rheumatoid arthritis, or monitor the progression of an existing medical condition, such as certain tumors (in prostate cancer the PSA levels in blood are monitored).

The antibody principle is also applied in doping tests, food hygiene tests, and tests for toxic substances.

8. Haemagglutination test

Introduction

All strains of Newcastle disease virus will agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination.

Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles. The test does not discriminate between viral particles that are infectious and particles that are degraded and no longer able to infect cells. Both can cause the agglutination of red blood cells.

Note that some other viruses and some bacteria will also agglutinate chicken red blood cells. To demonstrate that the haemagglutinating agent is Newcastle disease virus, it is necessary to use a specific Newcastle disease virus antiserum to inhibit the haemagglutinating activity.

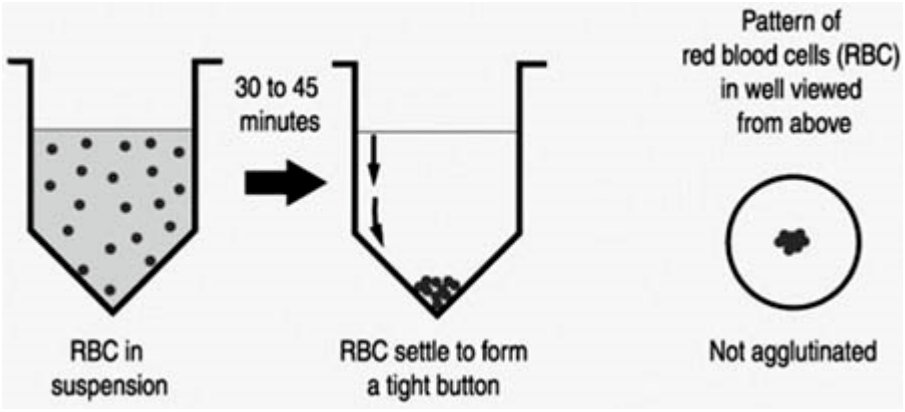
Substances that agglutinate red blood cells are referred to as haemagglutinins.

Note:

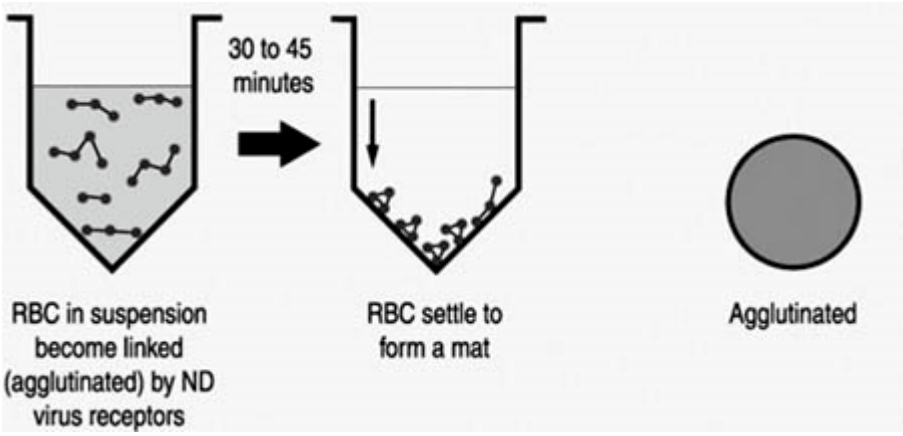
➢ The abbreviation **HA** is used in this manual for haemagglutinin and haemagglutination.

➢ Two tests are described, the rapid test which takes one minute and the micro test which takes 45 minutes.

Principle of the haemagglutination test



A. Negative control well (no haemagglutinin)



B. Positive control well (contains haemagglutinin)

Red blood cell control in the haemagglutination test

Every time a haemagglutination test is carried out, it is necessary to test the settling pattern of the suspension of red blood cells. This involves mixing diluent with red blood cells and allowing the cells to settle.

1. Dispense diluent.

2. Add red blood cells and mix by gently shaking.

3. Allow the red blood cells to settle and observe the pattern.

4. Observe if the cells have a normal settling pattern and there is no auto-agglutination. This will be a distinct button of cells in the micro test and an even suspension with no signs of clumping in the rapid test.

Note:

➢ The diluent used for haemagglutination tests in this manual is PBS.

➢ There should be no signs of haemolysis in the red blood cell suspension. If there are signs of haemolysis, a fresh suspension must be prepared.

➢ There should not be any sign of auto-agglutination in the red blood cell control. If an agglutination pattern is observed, discard the suspension of red blood cells. Prepare a fresh suspension and test again.

Control allantoic fluid samples

Negative and positive control samples are tested in both the rapid and micro haemagglutination tests to ensure the validity of the test.

Negative control allantoic fluid is harvested from 14-day old embryonated eggs that have not been inoculated with Newcastle disease virus. It should always test negative for the presence of haemagglutinins. There should not be any sign of haemagglutination.

Positive control allantoic fluid is known to contain a high infectivity titre of Newcastle disease virus. It should always test positive for the presence of haemagglutinins. Haemagglutination should be visible.

Rapid haemagglutination test

This test can determine the presence of a haemagglutinating agent in one minute. If testing many samples at the same time, it is necessary to test the negative and positive control samples only once.

Materials

- Clean glass microscope slide or a clean white ceramic tile.
- 10 percent suspension of washed chicken red blood cells.
- Micropipette and tips, glass Pasteur pipette or a wire loop.
- PBS.
- Negative and positive control allantoic fluid samples.
- Sample to be tested for the presence of Newcastle disease virus, for example allantoic fluid.

Method

1. Place 4 separate drops of 10 percent chicken red blood cells onto a glass slide or a white tile.

2. To each drop of blood, add one drop of the control and test samples as follows. Use separate tips, pipettes or a flamed loop to dispense each sample.

Drop 1 PBS

Drop 2 Negative control allantoic fluid (no haemagglutinin)

Drop 3 Positive control allantoic fluid (contains haemagglutinin)

Drop 4 Unknown sample to be tested

3. Mix by rotating the slide or tile for one minute.

4. Observe and record results. Compare results of the test samples with the control samples.

Results

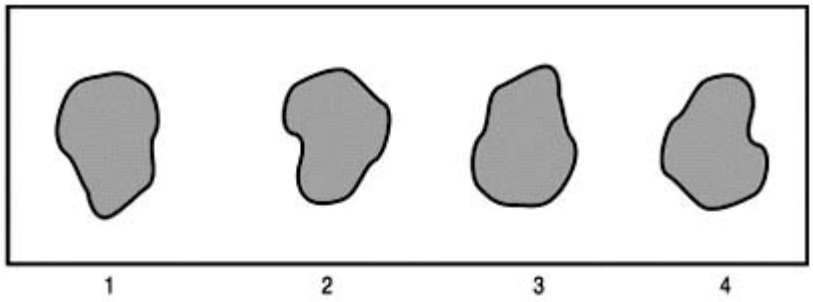
❖ Agglutinated red blood cells in suspension have a clumped appearance distinct from non-agglutinated red blood cells.

❖ The red blood cells mixed with the positive control allantoic fluid will clump within one minute.

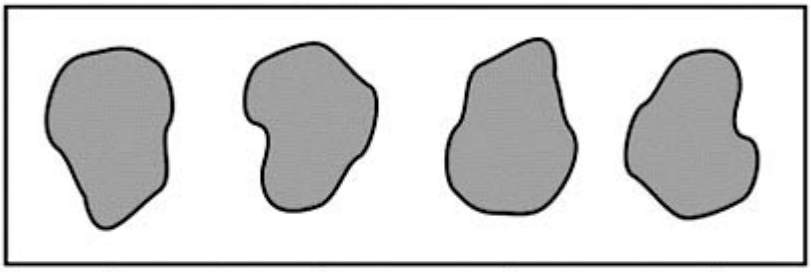
❖ The red blood cells mixed with the PBS and negative control allantoic fluid remain as an even suspension and do not clump.

❖ Judge the results of the test sample by comparison with the positive and negative controls.

❖ The PBS and negative allantoic fluid controls are used to detect clumping of the red blood cells in the absence of virus. This is unlikely to occur. If it does occur, the test is invalid.

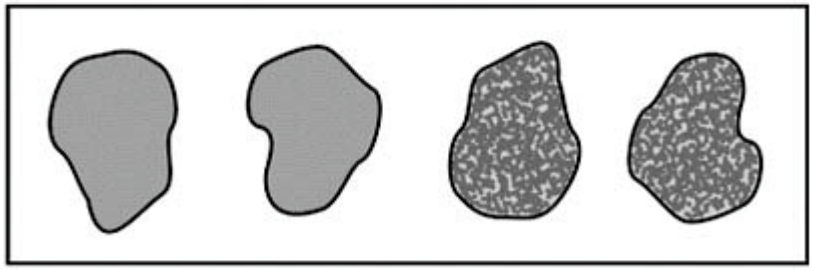


Step 1 Place 4 drops of 10% red blood cells on a slide



	1	2	3	4
Step 2	Add 1 drop of PBS	Add 1 drop of negative control	Add 1 drop of positive control	Add 1 drop of test sample

Step 3 Mix by gently rotating the slide for 1 minute



	1	2	3	4
Step 4	No agglutination	No agglutination	Agglutination	Agglutination

Step 5 Observe and read the results. Note that this particular test sample is positive for haemagglutinin

Rapid haemagglutination test

Micro haemagglutination test in a V-bottom microwell plate

This method is convenient when testing allantoic fluid from a large number of embryonated eggs for the presence or absence of haemagglutinin. A 1 percent solution of red blood cells is used. The cells settle faster in V-bottom plates and there is a better contrast between positive and negative results than observed in U-bottom plates.

Materials

- Inoculated eggs, chilled for at least 2 hours, preferably overnight
- Negative and positive control samples
- V-bottom microwell plate and lid
- Micropipette and tips to measure 50 µL
- 1 percent suspension of red blood cells
- 70 percent alcohol solution
- Cotton wool
- Forceps and/or small scissors
- Absolute alcohol
- Discard tray
- Microwell plate recording sheet.

Method

1. Fill in the details of samples being tested on a recording sheet. Samples and controls will be distributed into the wells as indicated on this sheet.

2. Use a micropipette to remove 50 mL of allantoic fluid from each egg and dispense into a well of the microwell plate. Use a separate tip for each sample.

3. Include negative and positive control allantoic fluid samples on one of the plates.

4. Dispense 50 mL of PBS into two wells. These wells will be the red blood cells controls for auto-agglutination.

5. Add 25 mL of 1 percent red blood cells to each well.

6. Gently tap sides of the plate to mix. Place a cover on the plate.

7. Allow the plate to stand for 45 minutes at room temperature.

8. Observe and record the results.

Results

❖ The settling patterns of single and agglutinated red blood cells are different. Single cells roll down the sides of the V-bottom well and settle as a sharp button. Agglutinated cells do not roll down the sides of the well to form a button. Instead they settle as a diffuse film.

❖ Negative HA result = a sharp button

❖ Positive HA result = a diffuse film

❖ Red blood cell control = a sharp button

❖ Mark the HA results on microwell recording sheet.

See **Appendix 10** for an example completed microwell plate recording sheet.

Table : Summary of Haemagglutination tests

Tests	Result	Interpretation
Rapid HA, Micro HA	Positive	Presence of viral particles that may or may not be infectious.
Rapid HA, Micro HA	Negative	Absence of viral particles or presence of viral particles in levels too low to detect.

Titration to establish haemagglutinin (HA) titre of a suspension of virus

The haemagglutination test is used to quantify the amount of Newcastle disease virus in a suspension. This is done by carrying out two-fold serial dilutions of the viral suspension in a microwell plate and then testing to determine an end point. This result can then be used to determine the amount of haemagglutinin in the suspension and is expressed as a HA titre.

Materials

- 96 well V-bottom microwell plate and cover
- 25 mL single and multi-channel micropipettes and tips
- PBS
- 1 percent chicken red blood cells
- Sample to be titrated
- Reagent troughs
- Microwell plate recording sheet. See Appendix 9.

Method

1. Record on recording sheets how samples will be dispensed in microwell plate.

2. Dispense 25 µL of PBS into each well of the microwell plate.

3. Place 25 µL of test samples in first well of each row of column 1. Samples can be tested in duplicate or triplicate if necessary.

4. Use a multichannel pipette to carry out two-fold serial dilutions across the plate until Column 11.

5. Add 25 µL of PBS to each well.

6. Add 25 µL of 1 percent red blood cells to each well including Column 12. The wells in this column are control wells that contain only PBS and red blood cells.

7. Gently tap sides of the plate to mix. Place a cover on the plate.

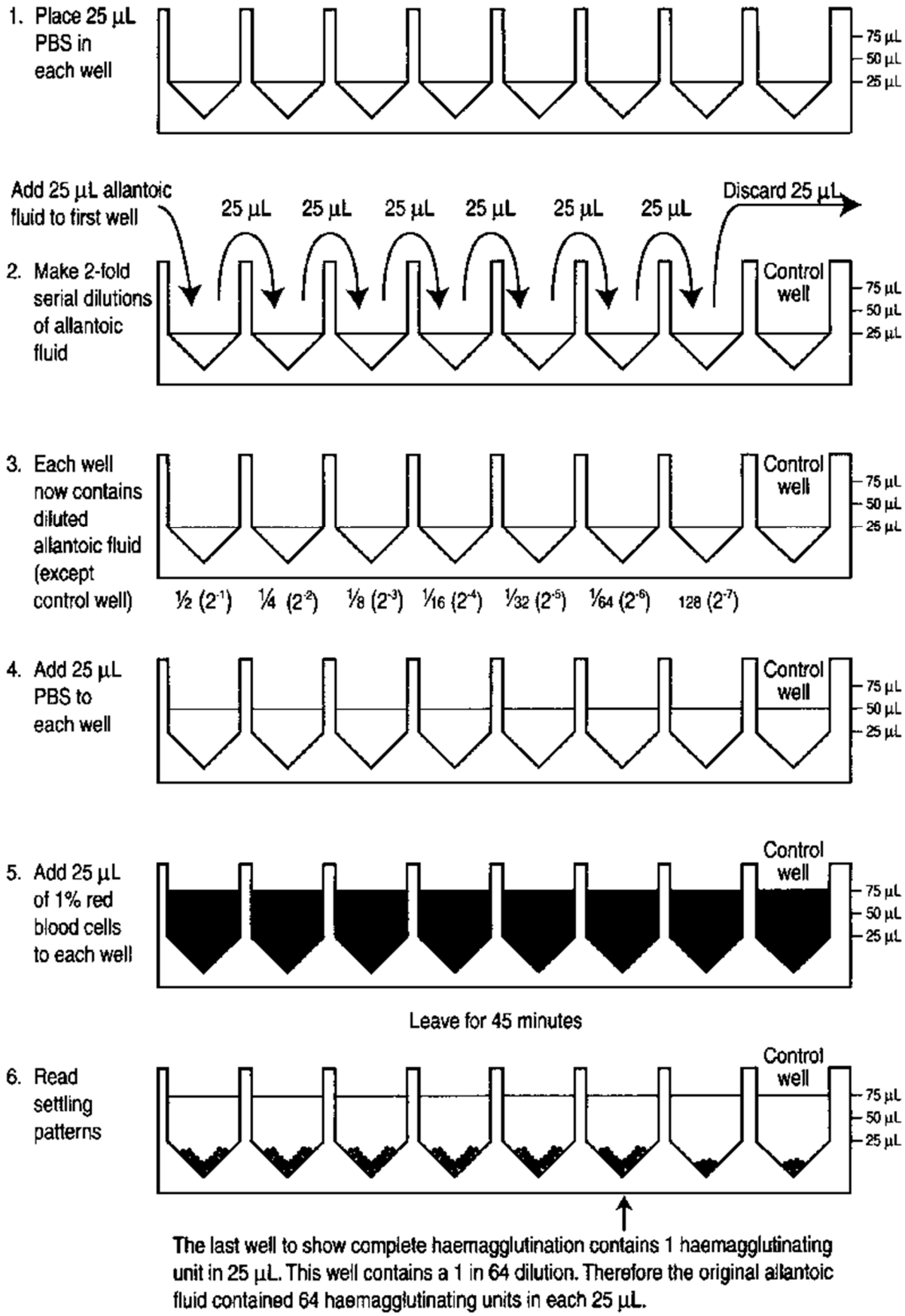
8. Allow the plate to stand for 45 minutes at room temperature.

9. Read and record the results in each well. All the control wells should be HA negative.

10. **HA negative:** A sharp button of red blood cells at the bottom of the V-bottom well.

11. **HA positive:** A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V-bottom well.

12. Identify the end point. This will be the last well to show complete haemagglutination and contains one haemagglutinating unit.



[Titration to determine HA titre of allantoic fluid sample]

Definition of one HA unit

One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the red blood cells. The last well that shows complete agglutination is the well that contains one HA unit.

Calculation of the HA titre of the test sample

The HA titre is the reciprocal of the dilution that produces one HA unit.

A 1 in 64 (1/64) dilution contains 1 HA unit.

❖ The HA titre of the test sample is therefore the reciprocal of 1/64 = 64 = 2^6

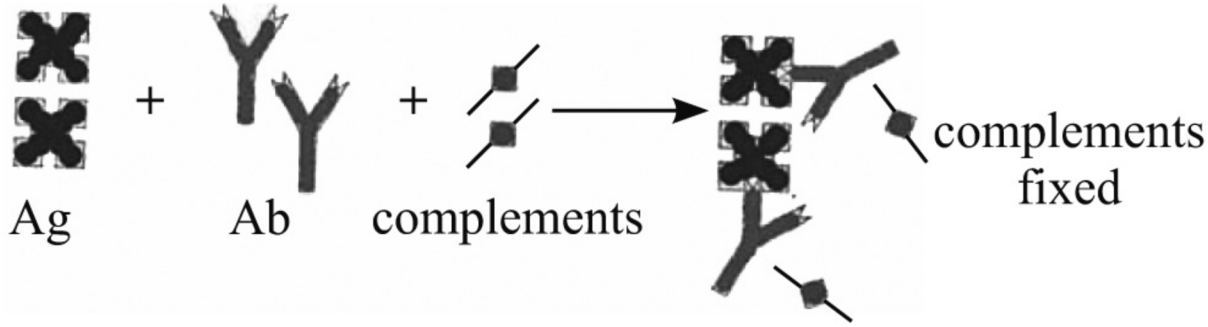
❖ The titre of the suspension of Newcastle disease virus can be expressed as 64 or 2^6 HA units in 25 mL.

8. Compliment fixation test

In 1894, Richard Pfeiffer, a German scientist, had discovered that when cholera bacteria were injected into the peritoneum of a guinea pig immunized against the infection, the pig would rapidly die. This bacteriolysis, Bordet discovered, did not occur when the bacteria was injected into a non-immunized guinea pig, but did so when the same animal received the antiserum from an immunized animal. Moreover, the bacteriolysis did not take place when the bacteria and the antiserum were mixed in a test tube unless fresh antiserum was used. However, when Bordet heated the antiserum to 55 degrees centigrade, it lost its power to kill bacteria. Finding that he could restore the bacteriolytic power of the antiserum if he added a little fresh serum from a non-immunized animal, Bordet concluded that the bacteria-killing phenomenon was due to the combined action of two distinct substances: an antibody in the antiserum, which specifically acted against a particular kind of bacterium; and a non-specific substance, sensitive to heat, found in all animal serums, which Bordet called "alexine" (later named "complement").
In a series of experiments conducted later, Bordet also learned that injecting red blood cells from one animal species (rabbit cells in the initial experiments) into another species (guinea pigs) caused the serum of the second species to quickly destroy the red cells of the first. And although the serum lost its power to kill the red cells when heated to 55degrees centigrade, its potency was restored when alexine (or complement) was added. It became apparent to Bordet that haemolytic (red cell destroying) serums acted exactly as bacteriolytic serums; thus, he had uncovered the basic mechanism by which animal bodies defend or immunize themselves against the invasion of foreign elements. Eventually, Bordet and his colleagues found a way to implement their discoveries. They determined that alexine was bound or fixed to red blood cells or to bacteria during the immunizing process. When red cells were added to a normal serum mixed with a specific form of bacteria in a test tube, the bacteria remained active while the red cells were destroyed through the fixation of alexine. However, when serum containing the antibody specific to the bacteria was destroyed, the alexine and the solution separated into a layer of clear serum overlaying the intact red cells. Hence, it was possible to visually determine the presence of bacteria in a patient's blood serum. This process became known as a complement fixation test. Bordet and his associates applied these findings to various other infections, like typhoid fever, carbuncle, and hog cholera. August Von Wasserman eventually used a form of the test (later known as the Wasserman test) to determine the presence of syphilis bacteria in the human blood**.** The complement fixation test (CFT) was extensively used in syphilis serology after being introduced by Wasserman in 1909. It took a number of decades before the CFT was adapted for routine use in virology.
CFT meet the following criteria

- it is convenient and rapid to perform
- the demand on equipment and reagents is small
- a large variety of test antigens are readily available.

However, there is now a trend to replace the CFT with more direct, sensitive and rapid techniques, such as RIAs and EIAs. Although CFT is considered to be a relatively simple test, it is a very exacting procedure because variables are involved Guinea pig is the commonest source of fresh complement. The serum should be collected from guinea pig just before the test because complement is easily destroyed by heat. However, complement can be preserved either by lipophilizing, freezing or by adding preservatives. Preserved complement is also obtained from commercial sources. Complement should be titrated for its haemolytic activity. One unit or minimum haemolytic dose (MHD) is the highest dilution of the guinea pig serum that lyses one unit volume of washed sheep red blood cells in the presence of excess of haemolysin (amboceptor) in either 30 or 60 minutes, at 37C. Physiological saline with added magnesium and calcium ions is used as the diluent for titration and CFT.



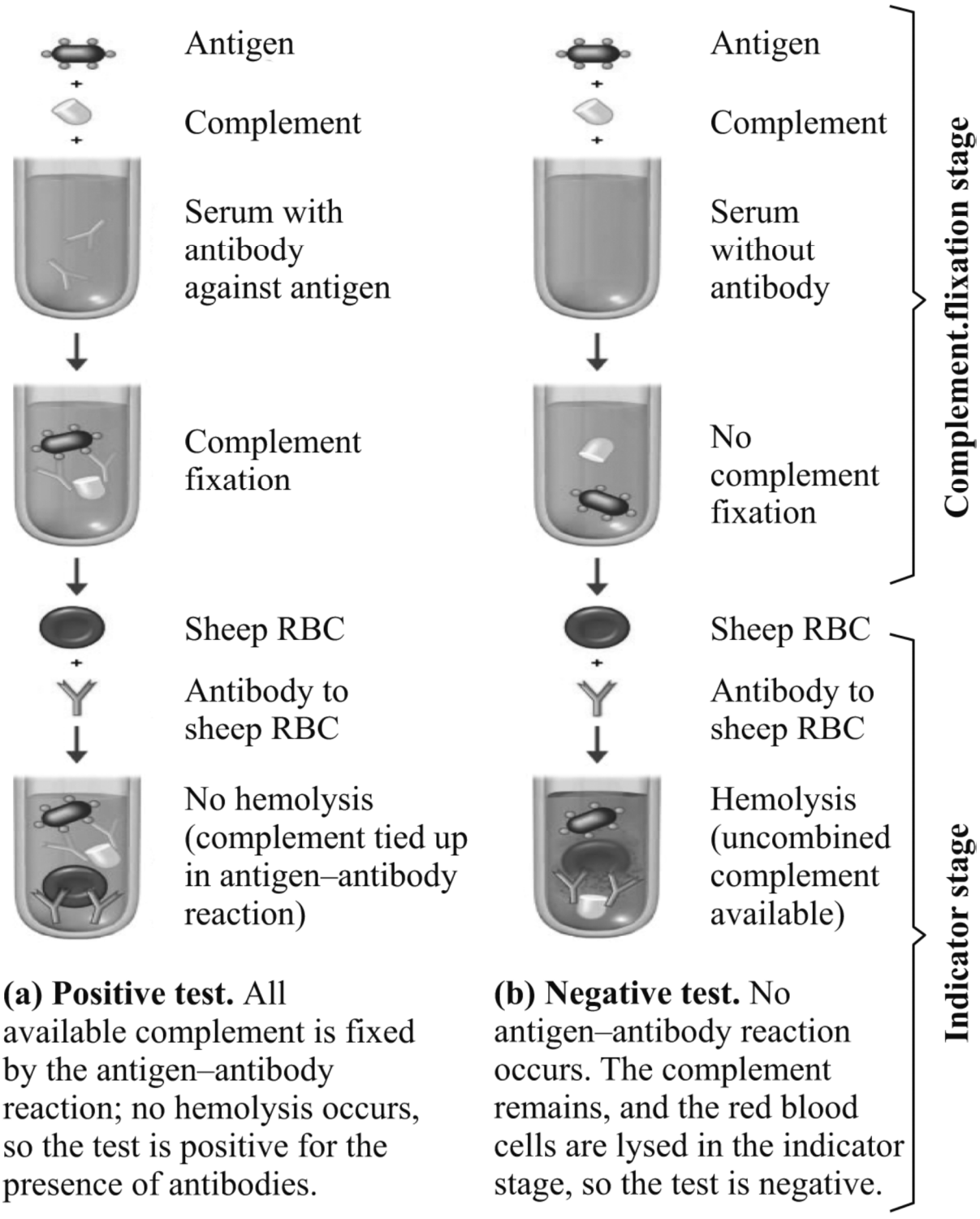
Complement fixation test consists of a test system and an indicator system, both of which can activate complement. When used to detect the presence of an antibody the test system is formed by the patient's serum and a known antigen. The indicator system is formed by sheep red blood cells coated with rabbit antibody to sheep red cells (amboceptors). The sheep red blood cells will lyse in the presence of complement.

 Sheep red cells: 5% suspension of washed sheep red blood cells should be used.

 Haemolysin (amboceptors): it is an antibody to sheep red cells which raised in rabbit. It should also be titrated for haemolytic activity. The MHD of the amboceptor is the highest dilution of the inactivated an amboceptor, which lyses one unit volume of sheep red blood cells in the presence of excess complement in 30 or 60 minutes at 37°C.

CFT consists of two steps:

Step 1: a known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free.



(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis. Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.

Controls should be used along with the test to ensure that
(a) Antigen and serum are not anti complimentary (b) The appropriate amount of complement is used and
(c) The sheep red blood cells do not undergo autolysis.
Complement Fixation Test in Microtiter Plate, rows 1 and 2 exhibit complement fixation obtained with acute and convalescent phase serum specimens, respectively. (2-fold serum dilutions were used) The observed 4-fold increase is significant and indicates infection.

Advantages of CFT

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical.

Disadvantages of CFT

1. Not sensitive - cannot be used for immunity screening
2. Time consuming and labor intensive
3. Often non-specific e.g. cross-reactivity between HSV and VZV

Modifications of complement fixation test

(a) **Indirect complement fixation test:** This modification is used when serums which don't fix guinea pig complement is to be tested. Here, the test is set up in duplicate. After step 1, standard antiserum to antigen which is known to fix complement is added to one set. If antibodies were not present in the test serum then the antigen would react with the standard antiserum fixing the complement. On the other hand if antibodies are present in the test serum the antigen would be utilized in the first step. So, no reaction would occur between the standard antiserum and the antigen and therefore no fixation of complement would cause lysis of sheep red blood cells. Thus in this case haemolysis indicates a positive result.

(**b) Congulatinating complement absorption test**: Here horse complement which is non-haemolytic is used. The indicator system used is sensitized sheep red blood cells mixed with bovine serum. Bovine serum contains a beta globulin called conglutinin would also combine with this complement causing agglutination (conglutination) of the sheep red blood cells, indicating a negative result.

(**c) Immune adherence**: When some bacteria (such as vibrio cholera or treponemapallidum) combine with their specific antibody in the presence of complement and some particles such as erythrocytes or platelets, they adhere to the erythrocytes or platelets. This is called immune adherence.

(d) Immobilisation test: Here antigen is incubated with patient's serum in presence of complement. If specific antibody is present it would immobilize the antigen. Eg.Treponema palladium immobilization test, considered gold standard for the serodiagnosis of syphilis.

(e) Cytolytic tests: The incubation of a live bacterium with its specific antibody in the presence of complement leads to the lysis of the bacteria cells. This is the basis of vibriocidal antibody test used to measure anticholera antibodies.

9. Bone Marrow Examination

Red blood cells, most white blood cells, and platelets are produced in the bone marrow, the soft fatty tissue inside bone cavities. Sometimes a sample of bone marrow must be examined to determine why blood cells are abnormal or why there are too few or too many of a specific kind of blood cell. A doctor can take two different types of bone marrow samples:

- Bone marrow aspirate: Removes fluid and cells by inserting a needle into the bone marrow and sucking out (aspirating) fluid and cells

- Bone marrow core biopsy: Removes an intact piece of bone marrow using a coring device (similar to a large diameter needle)

The bone marrow aspirate shows what cells, normal and abnormal, are present in the bone marrow and provides information about their size, volume, and other characteristics. Special tests, such as cultures for bacteria, fungi, or viruses, chromosomal analysis, and analysis of cell surface proteins can be done on the sample.

The core biopsy removes an entire piece of bone marrow and shows not only what cells are present but also how full the bone marrow is with cells and where the cells are located within the marrow.

Although the aspirate often provides enough information for a diagnosis to be made, the process of drawing the marrow into the syringe breaks up the fragile bone marrow. As a result, determining the original arrangement of the cells is difficult.

When the exact anatomic relationships of cells must be determined and the structure of the tissues evaluated, the doctor also does a core biopsy. A small core of intact bone marrow is removed with a special bone marrow biopsy needle and sliced into thin sections that are examined under a microscope.

Both types of samples are usually taken from the hipbone (iliac crest), often during a single procedure. Aspirates are rarely taken from the breastbone (sternum). In very young children, bone marrow samples are occasionally taken from one of the bones in the lower leg (tibia).

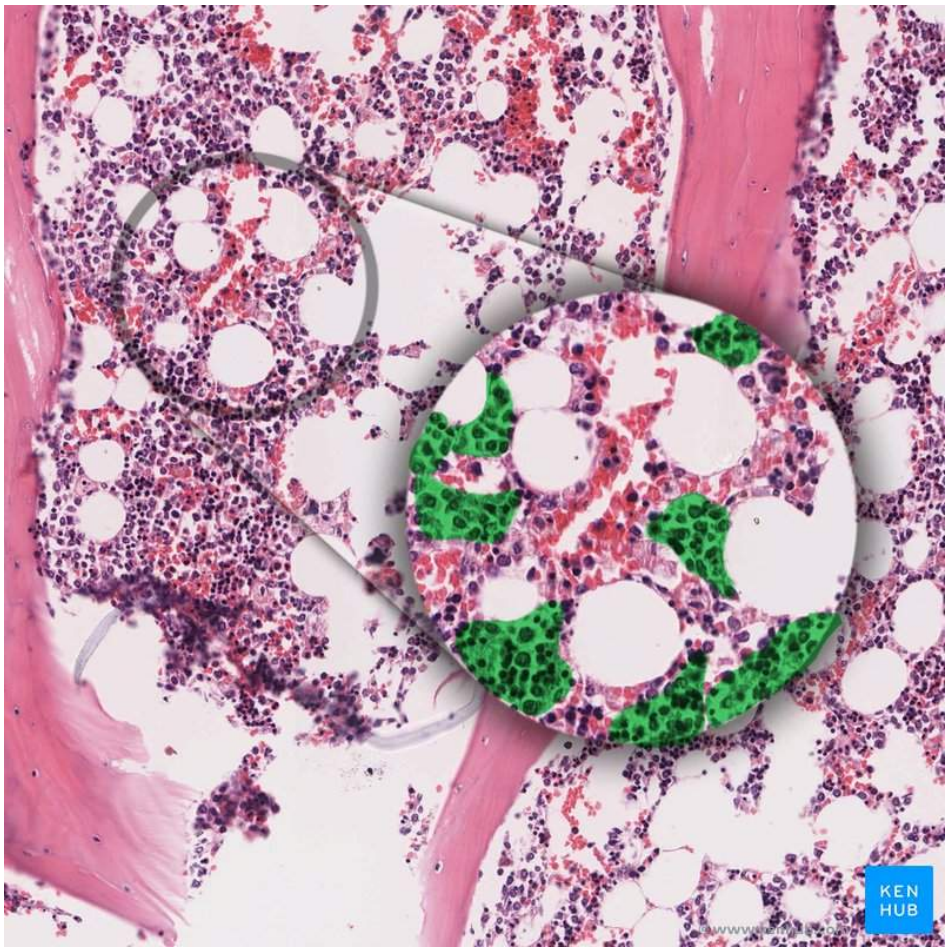
A bone marrow sampling begins with cleaning, sterilizing, and anesthetizing the skin over the bone. The procedure generally involves a slight jolt of pain, followed by minimal discomfort. The procedure takes a few minutes and causes no lasting damage to the bone.

Clinical Significance

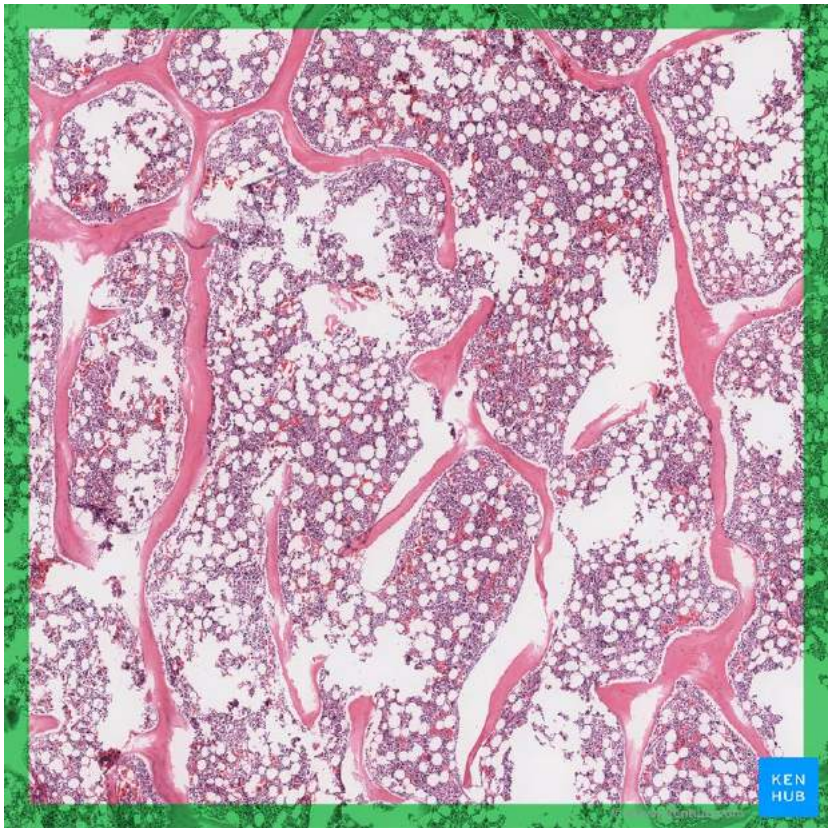
Bone marrow aspirations and biopsies remain a mainstay for the workup of hematologic malignancies and their definitive diagnosis. These methods avoid more invasive procedures, can yield an accurate diagnosis in a relatively short amount of time, and are relatively uncomplicated to perform. The quick nature of the procedure, the potential for avoidance of sedation or general anesthesia, and the ability to perform the procedure on an outpatient basis facilitate an efficient path towards diagnosis and treatment as well as a nontraumatic experience for the patient.

Red bone marrow

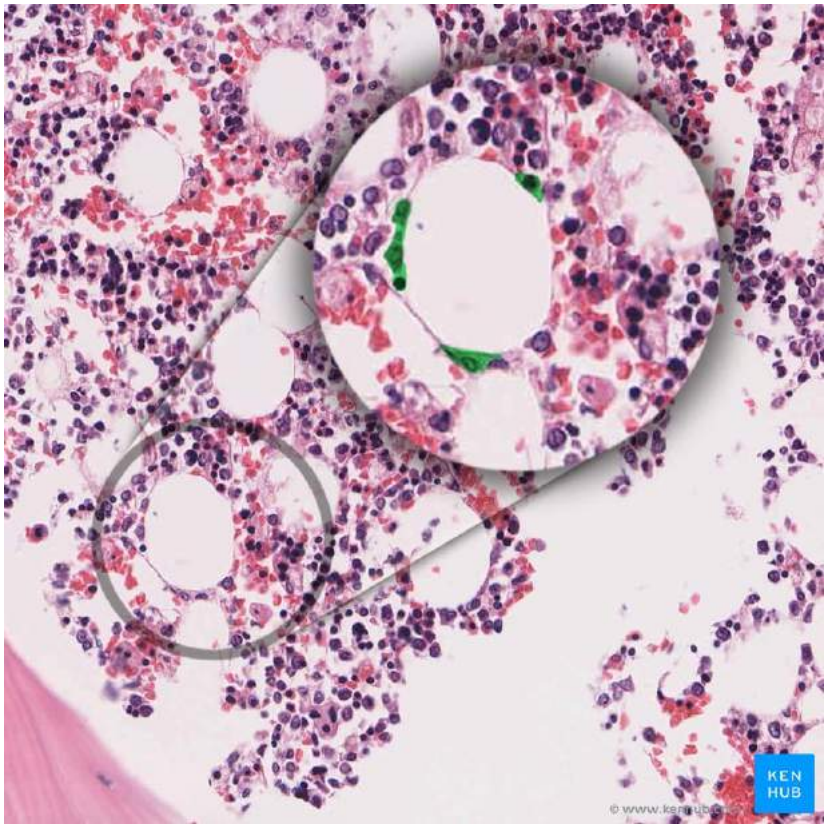
Clusters of haematopoietic cells known as **haematopoietic islands** are widely distributed throughout the loose connective tissue network observed in red marrow. These islands are found next to relatively large, yet thin walled, sinusoids that also communicate with **nutrient vessels** of the bone. The **sinusoids** are situated at a central part of a roundabout circulation such that the nutrient arteries that leave the **nutrient canals** to supply the bones anastomose in the bone marrow and subsequently terminate in arterioles that coalesce to form the sinusoids. The sinusoids then drain to significantly larger veins that form **nutrient veins**, which then leave the bone via the same nutrient canals that the arteries enter by.



Red marrow is most abundant in all skeletal structures from intrauterine life up until around the 5th year of life. As time progresses, red marrow is restricted to the central flat bones (i.e. cranial bones, clavicle, sternum, ribs, scapula, vertebrae, and pelvis) and the proximal ends of the proximal long bones of the upper and lower limbs.

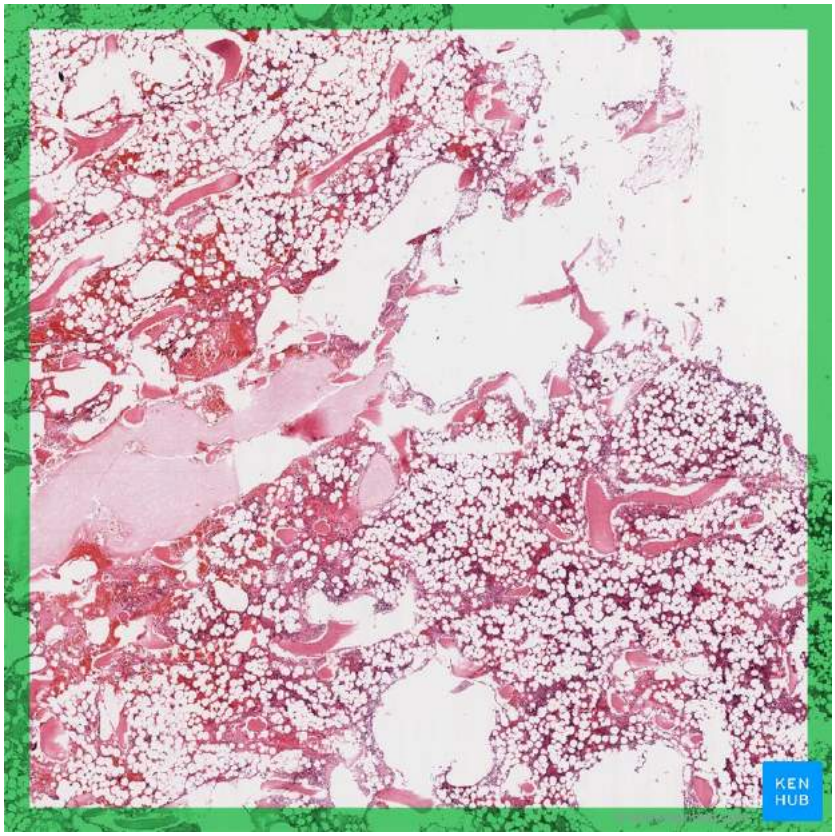


The supporting substance that supports the haematopoietic and adipocyte cells in the marrow is made up of **reticulin**. This is a fine **type III collagen** that is produced by mesenchyme derived **reticular cells** (fibroblast-like cells). Other housekeeping cells like macrophages exist in the stroma and facilitate haematopoiesis by phagocytosing cellular debris generated from the process.



Yellow bone marrow

Depending on the age and haematological demand of an individual, the reticular cells become swollen as a result of increased lipid uptake. Subsequently, yellow marrow is formed. It contains mainly supportive connective tissue that provides scaffolding for the neurovascular structures that traverse the cavitation. There are also numerous **adipocytes** in addition to very few dormant haematopoietic clusters. These latent **haematopoietic centres** can be reactivated in the event of an increase demand for red blood cells.



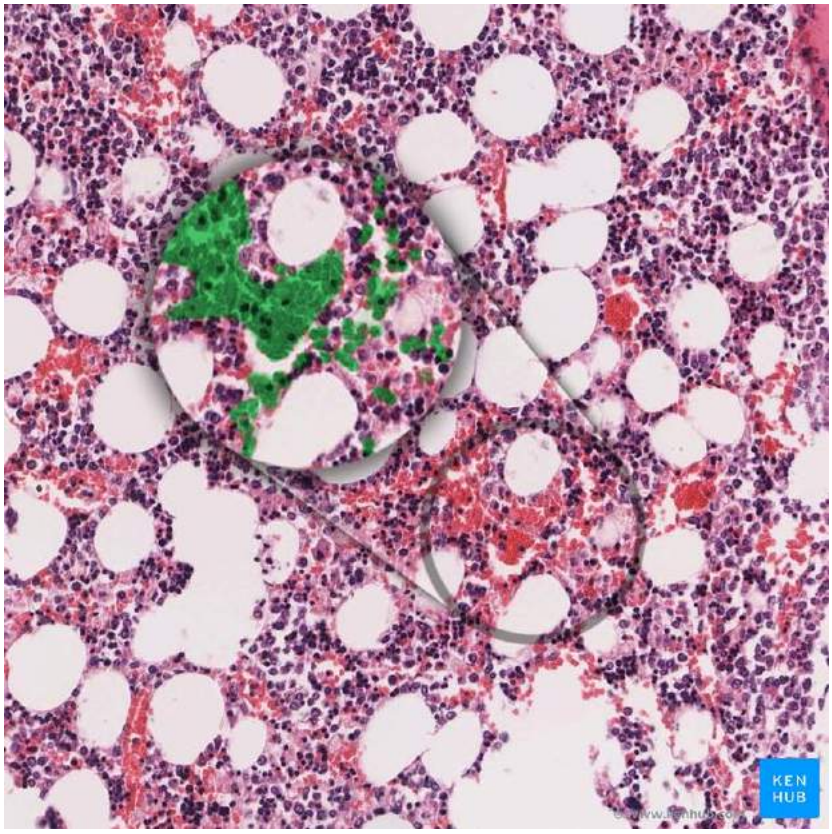
Cell types

Histological analysis of the bone marrow will reveal an abundance of **progenitor cells** and their derivatives at different stages of development. Typically, the progenitor cells are larger than their end products. The suffix "-blast" is often used to denote that the cell line being referenced are the stem cells for that series (i.e. erythroblasts are the precursor cells for red blood cells [erythrocytes]). The following is a list of the cell lines found in the bone marrow:

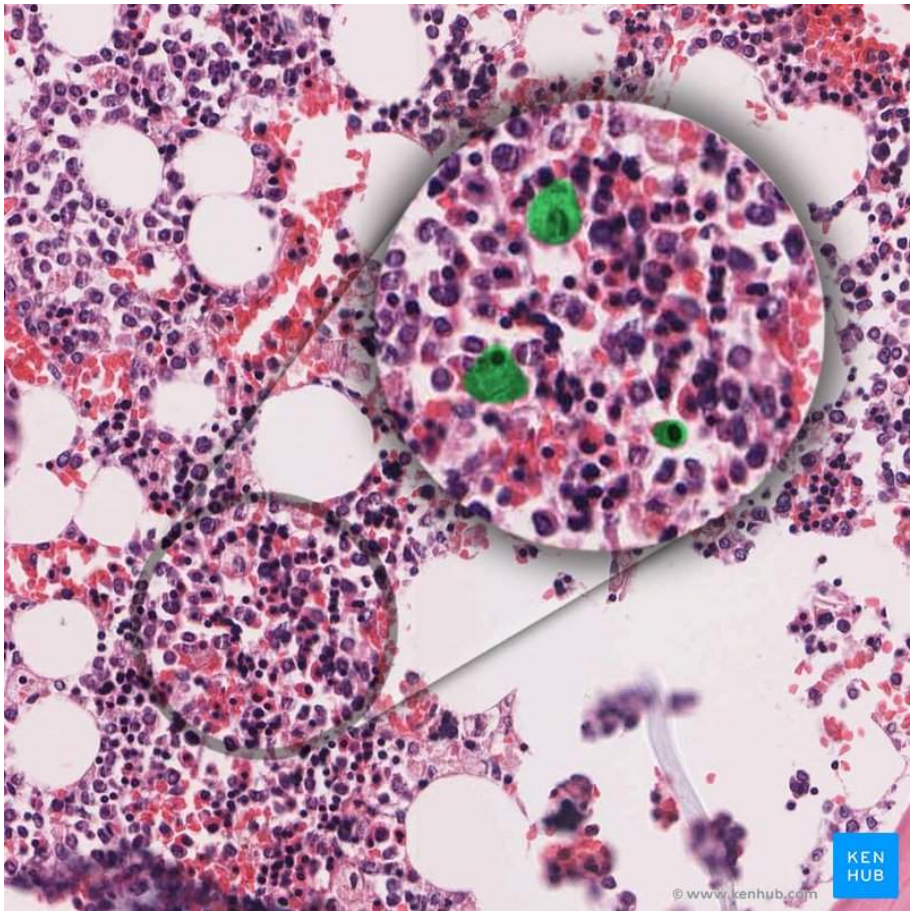
- **Granulocytes** – are a special line of white blood cells that possess secretory granules in their cytoplasm. There are three granulocytes; these are **eosinophils**, **basophils** and **neutrophils**.

- Monocytes – are leukocytes that differentiate into macrophages. Recall that there are different subtypes of macrophages depending on the region of the body that they are found in (i.e. Kupffer cells of the liver).

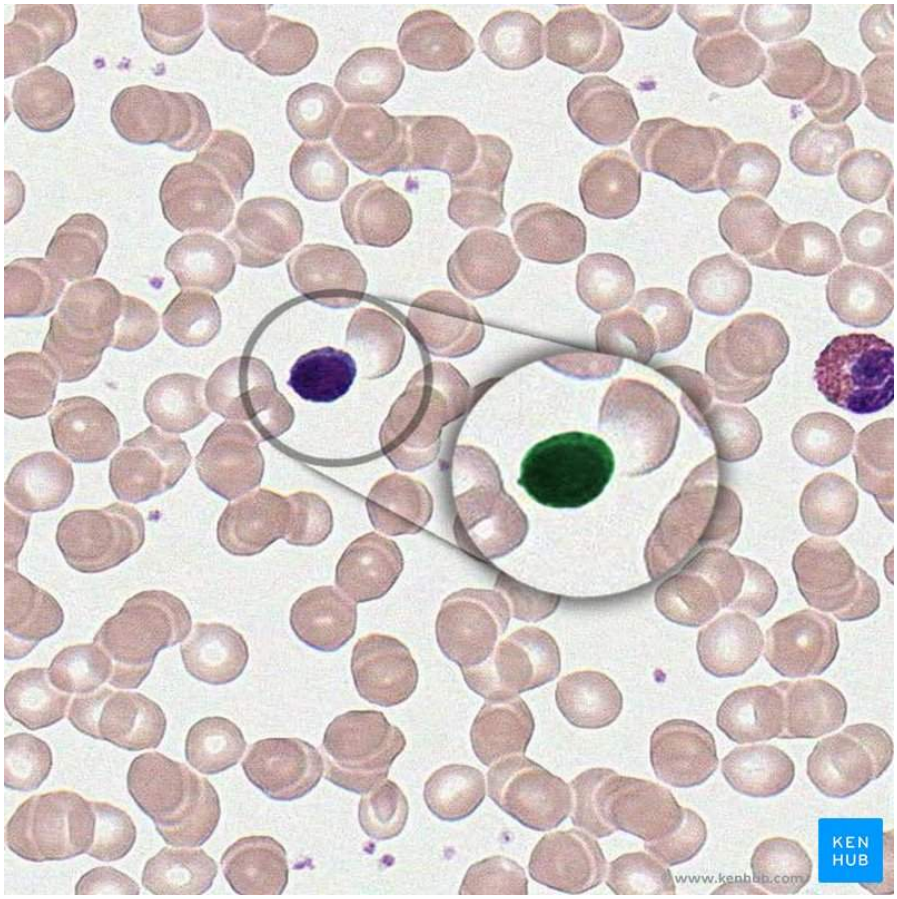
- Erythrocytes – are the anucleate, biconcave, oxygen-carrying species.



- Megakaryocytes – is another large species that is responsible for thrombocytogenesis (i.e. platelet production).



Lymphocytes – are all produced in the bone marrow. However, education and maturation of one subset of lymphocytes occurs in the thymus (i.e. T-lymphocytes)



The stroma also contains a myriad of stem cells of mesenchymal origin. These include **multipotent cell lines** that are capable of differentiating into cartilaginous cell lines (chondrocytes), bone cells (osteoblasts and osteoclasts) in addition to **adipocytes**, **myocytes** (muscle) and **endothelial cells**.

10. **RADIAL IMMUNODIFFUSION**

This protocol is based on the EDVOTEK® protocol "Radial Immunodiffusion".

10 groups of students

5. EXPERIMENT OBJECTIVE

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

6. EXPERIMENT COMPONENTS for 10 groups of students

COMPONENTS	Store
A. Antibody solution	4-8ºC
B. Standard antigen solution	4-8ºC
C. UltraSpec-Agarose™	4-8ºC
D. Buffer powder	4-8ºC
E. Unknown concentration of antigen	4-8ºC
1 Sleeve Petri dishes	
2 10 ml pipettes	
10 Well cutters	
80 Transfer pipettes	
70 Microtest tubes	
1 Graph paper templates	
1 Practice loading solution	

NOTE: Store all components at the indicated temperatures upon receipt.

NOTE: No human material is used in this practice.

NOTE: All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

6.1 Requirements

- Automatic Micropipets and Tips (5-50 µl)
- Pipet Pumps (for 10 ml pipets)
- Ruler
- Plastic Box or Dish
- Plastic Wrap
- Foil
- Paper Towels
- Distilled Water
- Heat plate, Bunsen burner, or microwave
- 400 to 600 ml beaker or Erlenmeyer flask

- 150 ml beaker or flask
- Water bath
- 250 ml Graduated Cylinder
- 37°C Incubation Oven

NOTE: Make sure glassware is clean, dry, and free of soap residue. For convenience, you can buy additional disposable transfer pipettes to the steps of extraction and washing liquids.

7. BACKGROUND INFORMATION

Radial immunodiffusion

The fundamental reaction of immunology involves the interaction of **antibodies (Ab)** and **antigens (Ag)**. These interactions are useful in the defense of the body against bacterial and viral infections and toxins. The defense capabilities are dependent upon the recognition of antigens by humoral components of the immune system. Specific antibodies are then produced in response to exposure to the antigen.

The formation of **antigen-antibody complexes** is the first step in removing infectious agents from the body. Because each antibody can bind more than one antigen and each antigen can be bound by more than one antibody molecule, very large macromolecular complexes can form. These complexes form precipitates which can be cleared from the body through various means. These precipitates are also useful for laboratory and diagnostic tests.

When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate at the interface of their diffusion fronts. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing the various antibody-antigen reactions in a system.

Double diffusion in two dimensions is a simple procedure invented by the Swedish scientist, Ouchterlony. Antigen and antibody solutions are placed in separate wells cut in an agarose plate. The reactants diffuse from the wells toward each other and precipitate where they meet at equivalent proportions. A single antigen will combine with its homologous antibody to form a single precipitation line.

Radial immunodiffusion (RID) is a technique that can quantitatively deter- mine the concentration of an antigen. Unlike many gel and liquid precipitation techniques which qualitatively detect antigen, RID is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins.

Antibody is incorporated into molten agarose which is poured into a Petri dish and allowed to solidify. Small wells are cut into the agarose and are filled with known concentrations of antigen which corresponds to the antibody in the agarose. Samples of unknown concentrations are placed

in similar wells. The antigens in solution then diffuse outwards from the well in a circular pattern surrounding the well. Antibody is present in excess and diffusion of the antigen will continue until a stable ring of antigen-antibody precipitate forms. There are antigen-antibody complexes throughout the zone surrounding the well within the precipitin line. At the precipitin line is where the greatest number of complexes can be found because the antigen and antibody are present in roughly equal proportions. This is known as the **equivalence zone** or **equivalence point**. Generally, it takes 24 to 48 hours for optimal diffusion to occur and precipitation to become apparent.

For each antigen, an endpoint precipitation ring of a certain diameter will form. From the known standard concentrations, a standard curve can be drawn by plotting antigen concentration versus the diameter squared measurements of the rings. From this linear calibration curve the concentration of the unknown antigen samples may be determined.

8. EXPERIMENTAL PROCEDURES

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

8.1 Laboratory safety

No human material is used in this experiment.

7. Gloves and safety goggles should be worn at all times as good laboratory practice.

8. NOT PIPETTE WITH THE MOUTH, use appropriate devices.

9. Exercise caution when working with equipment using together heat and mix of reagents.

10. Wash hands with soap and water after working in the laboratory or after using biological reagents and materials.

11. Be careful when using any electrical equipment in the laboratory.

12. Dispose of RID plates using appropriate laboratory waste disposal procedures.

If you are unsure of something, ASK YOUR INSTRUCTOR

8.2 Approximate time requirements for pre-lab and experimental procedures

Your individual schedule and time requirements will determine when the RID plates should be prepared. It takes approximately 30 minutes to prepare the plates (generally 10 minutes of this time is required for solidification).

Students can prepare the plates, if time allows. 4.3 PreLab Preparations

Notes preparations teacher practice

The class size, length of classes of practices and equipment availability are factors that must be considered in the planning and implementation of this practice with their students. These guidelines can be adapted to fit your specific circumstances.

Laboratory notebooks:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Registration laboratory activities

Students must register in their book practices the activities listed below.

Before starting the experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

General instructions

PREPARATIONS BEFORE PRACTICE PREPARE AGAROSE IN BUFFER

13. In a 400 to 600 ml beaker or Erlenmeyer flask, add entire contents of buffer powder package (component D) to 200 ml distilled water. Swirl the flask until the powder is in solution. Remove 50 ml for use as dilution buffer to a separate beaker.
14. Add the entire contents of agarose package (component C) to the flask or beaker containing 150 ml of buffer. Swirl to disperse large clumps. With a marking pen, indicate the level of solution volume on the outside of the flask or beaker.
15. The solution must be boiled to dissolve the agarose. This can be accomplished with a hot plate or microwave. Cover the beaker with foil and heat the mixture to boiling over the burner with occasional swirling. Wear safety goggles and use hot gloves. Boil until all the agarose is dissolved. Check to make sure that there are no small, clear particles of agarose. The final solution should be clear.

    Heat the mixture to dissolve the agarose powder. The final solution should be clear (like water) without any undissolved particles.

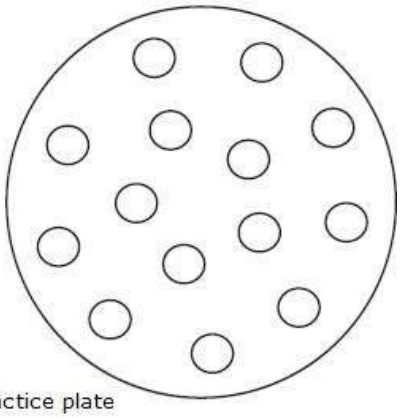
    A. Microwave method:
        - Cover flask with plastic wrap to minimize evaporation.
        - Heat the mixture on High for 1 minute.
        - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

    B. Hot plate method:
        - Cover the flask with foil to prevent excess evaporation.
        - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.

16. If detectable evaporation has occurred, add hot distilled water to adjust the volume of solution up to the original level as marked on the flask or beaker in step 2. Do not use cool water, or the agarose solution may cool too quickly and prematurely solidify.
17. Cool the agarose solution to 55°C in a waterbath. Swirl occasionally while cooling.

PREPARATION OF PRACTICE PLATES

3. If practice plates are to be made, pipette 2.5 ml of molten agarose into each of 10 petri dishes with a 10 ml pipette. Gently spread the agarose with the pipette on the bottom of the plate to cover the entire surface. Return the remaining agarose to waterbath.
4. Allow agarose plates to set up and cool. Refrigerate if plates are not to be used within a few hours.



Practice plate

PREPARATION OF ANTIBODY PLATES

5. Pour 26 ml of molten agarose solution to a large tube or flask.
6. Add entire contents of Antibody Solution (Component A) to the 26 ml warm agarose solution. With a pipet, stir the solution to mix. Keep the solution warm (such as in the 55°C waterbath) so it does not prematurely set up. The antibody concentration will be 1 mg/ml.

NOTE: Ensure that the temperature of the agarose solution is at 55°C by adding the Antibody Solution to prevent degradation of the antibodies.

7. With a 10 ml pipette, dispense 2.5 ml into the bottom of each Petri dish, gently spread the agarose with the pipette to cover the bottom. Allow the agarose to solidify. This will take approximately 10 minutes. If the plates are not to be used the day of the preparation, they can be wrapped in plastic wrap and stored in the refrigerator for no longer than one week.
8. Each group requires 1 antibody plate and 1 practice plate.

PREPARATIONS ON THE PRACTICE DAY PREPARATION OF ANTIGENS

Students will prepare serial dilutions of the Standard Antigen Solution (Component B) to determine the standard curve.

18. Label 10 microtest tubes with “Standard”.
19. Label 10 microtest tubes with “Unknown”.
20. Label 10 microtest tubes with “Buffer”.
21. Aliquot 75 µl of Standard Antigen Solution (Component B) into each tube labeled “Standard”.
22. Aliquot 10 µl of Unknown Antigen Solution (Component E) into each tube labeled “Unknown”.
23. Aliquot 1 ml of Buffer (retained from plate preparation step) into each tube labeled “Buffer”.
24. Each group requires one tube each of Standard, Unknown, and Buffer.

NOTE: Solutions can be aliquoted before the day of practice, in which case they should be stored at 4-8°C until the day of practice.

PREPARATION OF INCUBATION CHAMBER

3. Obtain plastic container or dish with lid. If a lid is not available, the container may be covered with plastic wrap.
4. Line the bottom of the container with several paper towels. Add distilled water to the towels to saturate. There should not be any liquid above the paper towels. All the liquid should be absorbed into the towels. Cover the chamber with the lid or plastic wrap.

4.6 Material that should receive each group

Distribute the following to each student group, or set up a work station for students to share materials.

- 1 tube Buffer
- 1 tube Standard
- 1 tube Unknown
- 4 microtest tubes
- 1 practice plate
- 1 experimental RID plate
- 1 well cutter
- 1 template
- Micropipetting device and tips (or 8 transfer pipets)
- Graph paper
- Ruler
- Marking pen

4.7 Avoiding common pitfalls

6. Follow instructions carefully when preparing gels. Make sure the agarose is completely dissolved.
7. Make neat, clean wells with the well cutters. Take measures to ensure that the wells are properly spaced according to the template on page 5.
8. Add samples to the wells carefully and precisely. Avoid overfilling the wells.
9. Do not tip or invert plates when transferring to the humidity chamber.
10. Placing the humidity chamber in a 37˚C incubation oven will expedite the formation of precipitin arcs.

7. STUDENT EXPERIMENTAL PROCEDURES

A. PREPARATION OF AGAROSE PLATES

1. Place the template under the plate so the pattern is centered.
2. Cut the wells using the well cutter (provided in the kit) in a gentle punching motion. Remove the agarose plugs with a flat-edged toothpick or spatula.

B. PREPARATING THE STANDARDS (SERIAL DILUTION)

1. Label four microtest tubes: 1:2, 1:4, 1:8, and 1:16.
2. Using a micropipet, add 50 microliters of Buffer to each tube.
3. With a fresh pipet tip, add 50 microliters of "Standard" to the tube labeled 1:2. Mix.
4. With a fresh pipet tip, transfer 50 microliters of the 1:2 dilution to the tube labeled 1:4. Mix.
5. With a fresh pipet tip, transfer 50 microliters of the 1:4 dilution to the tube labeled 1:8. Mix.
6. With a fresh pipet tip, transfer 50 microliters of the 1:8 dilution to the tube labeled 1:16. Mix.
7. There are now five antigen samples for the standard curve (see chart).

DILUTION	CONCENTRATION
Undiluted	2 mg/ml
1:2	1 mg/ml
1:4	0,5 mg/ml
1:8	0,25 mg/ml
1:16	0,125 mg/ml

C. PRACTICE WELL LOADING (OPTIONAL)

This experiment contains practice loading solution. This solution is included to allow instructors and students to practice loading the sample wells before performing the actual experiment. Use a micropipetting device or one of the plastic transfer pipettes included in the experiment to practice loading the sample wells with the practice loading solution. Make enough copies of the template for each lab group.

1. One practice plate should be prepared for every two groups. Enough reagents have been provided for this purpose.
2. Using the well cutters provided, cut several wells in the agarose as shown in the template below. Refer to Student Instructions for preparation of sample wells.
3. Practice loading the sample wells with the Practice Loading Solution using a micropipetting device. Load 5 µl per well and make sure the sample covers the entire surface of the well. If a micropipetting device is not available, use the transfer pipets provided, taking care not to overfill the wells. If using transfer pipets, put in just enough sample to cover the bottom of the well.

D. LOADING THE SAMPLES

1. On the bottom of the plate, number the wells on the perimeter of the plate 1 through 5. Leave the center well unlabeled.
2. Load wells 1 through 5 using the same pipette tip or transfer pipette. In well #5,

load 5 µl of the 1:16 antigen dilution. Make sure the sample covers the entire surface of the well by carefully spreading it with the pipette tip.

3. In well #4, load 5 µl of the 1:8 antigen dilution.
4. In well #3, load 5 µl of the 1:4 antigen dilution.
5. In well #2, load 5 µl of the 1:2 antigen dilution.
6. In well #1, load 5 µl of the undiluted antigen.

NOTE: You may use the same pipette tip or transfer pipette to load wells #1 through #5, starting with the most dilute antigen dilution and ending with the most concentrated. Use a fresh tip for the unknown.

7. With a fresh pipette tip or microtipped transfer pipette, load 5 µl of your unknown in the center well.
8. Label the cover of the Petri dish with your lab group number or your initials. Place the cover on the dish, place the dish right side up (do not invert) inside the incubation chamber on the paper toweling. Cover the incubation chamber and place in a 37°C incubation oven or at room temperature for 24 to 48 hours.

11. ELISA technique

There are many instances within the life sciences where detection and quantification of antigens or antibodies within a sample in a timely and cost-effective manner is important. From identifying immune responses in vaccinated or infected individuals, detecting expression of a protein you wish to express on the surface of a cell, to performing quality control testing. Having a tool capable of making such assessments is key.

The enzyme-linked immunosorbent assay (ELISA) is one such test that has proven invaluable as both a research and diagnostic tool.

What is an ELISA test?

An ELISA is an immunological assay commonly used to measure antibodies or antigens, including proteins or glycoproteins, in biological samples. Like other immunoassays, they rely on binding of antibodies to their targets to facilitate detection.

Typically, ELISA assays are performed in 96-well plates, a format that makes them amenable to screening many samples at once. Serum, plasma, cell culture supernatants, cell lysates, saliva, tissue lysates and urine are all common sample types used for these assays, but most liquid sample types could be used in theory. It is, however, important to consider that some sample types may include inhibitory factors, such as buffer components that share similar antigenic epitopes[1] or factors like proteases[2] that may damage the target or detection components, that may interfere with the assay's performance.

There are several different assay formats, but all rely on binding of either the target itself or an antibody/antigen able to capture the target to the surface of the plate. A detection step involving a conjugated antigen, or more often antibody, is then employed to enable successful binding to be detected and quantified, most often by colorimetric detection.

Types of ELISA and ELISA test diagram

The ELISA was originally conceptualized, independently, in 1971 by Eva Engvall and Peter Perlman[3] at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen[4] in the Netherlands. They sought an immunoassay method able to detect the presence of antigens or antibodies to replace the radioimmunoassay, which employed potentially hazardous radioactively labeled antigens or antibodies, and thus devised an enzyme-based alternative.

There are now four main types of ELISA, direct, indirect, sandwich and competitive. The images below (Figure 1) illustrate detection of antigens; however, the same principle applies for antibody detection essentially with the roles of the antigen and primary antibody reversed.

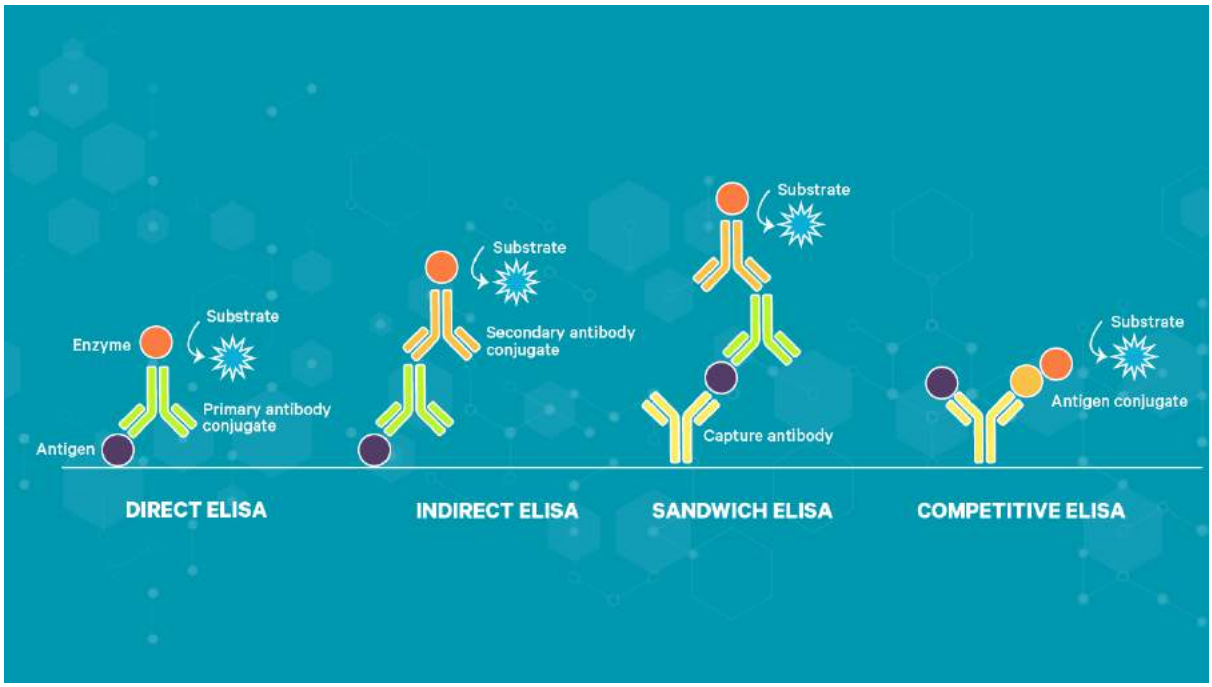


Figure 1: Types of ELISA.

Direct ELISA test

With a direct ELISA, the antigens or antibodies in a sample are adsorbed directly to the test plate in a non-specific manner. A conjugated detection antibody or antigen specific for the target is then applied to the wells. Following this, a detection substrate is used to produce a measurable color change that can be quantified in a plate reader.

As this assay has few steps, it is quicker and offers less opportunity for the introduction of errors than the other ELISA methods. However, as the adsorption step is non-specific, background noise may be high. The absence of a secondary antibody step means there is no signal amplification, reducing assay sensitivity. It also requires conjugated detection antibodies/antigens to be created for each target required.

Indirect ELISA test

Originally developed in 1978[5] for the detection of human serum albumin, the indirect ELISA, or iELISA, works in a very similar way to the direct ELISA except for the addition of a secondary antibody step. This enables amplification of the test signal, overcoming the limitation of the direct ELISA. It also negates the need for target-specific conjugated detection antibodies/antigens as the conjugated secondary antibody need only be species specific for the primary antibody. Where total sample antigens are bound to the plates, like the direct ELISA, background noise remains an issue. However, if the assay is used for the detection of sample antibodies, purified target antigen is coated onto the plates, with the primary antibody coming

from the sample. This greatly reduces background noise and consequently these assays are most popular for determining antibody titers in samples.

Disadvantages of the indirect ELISA include a longer protocol with more opportunities for errors and potential of cross-reactivity with the secondary antibody.

Sandwich ELISA

Developed in 1977[6], as its name suggests, the sandwich ELISA sandwiches the antigen between antibodies. The technique can employ the direct or indirect ELISA format (the sandwich ELISA depicted above is based on the indirect ELISA) except that rather than non-specific binding of antigens to the assay plate, the capture antibody makes this a specific process. This combination further improves assay sensitivity and specificity.

They do, however, require the determination of compatible capture and detection antibody pairs to function effectively with which cross-reactivity can be problematic. This assay typically has the most steps too, offering greater opportunity for error. Due to the selective nature of the antigen binding step, sandwich ELISAs are particularly useful where the antigen is in a complex mix as antigen purification is not required.

Competitive ELISA

A competitive ELISA, also known as an inhibition ELISA or blocking ELISA, is possibly the most complex of the ELISA techniques. Originally developed in 1976[7] for the detection of human choriogonadotropin, the assay works by detecting interference to an expected output signal level, producing an inverse relationship. The more of the target there is in the sample applied, the lower the assay output signal will be. Multiple formats are possible for which the other ELISA types can be adapted into a competitive format. However, there are two general principles; the sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. Alternatively, sample antigen or antibody may compete with a labeled reference for binding to a limited amount of antibody or antigen, respectively.

The competitive ELISA will have some of the same advantages and limitations as the format from which it has been adapted. However, it can be helpful when the antigen is small, limiting the ability of two antibodies to bind concurrently, as required for the sandwich ELISA, or when only one antibody is available.

ELISA steps

While there are variations in the protocols for the different types of ELISA, there are a number of stages to the assay common to most that should be considered.

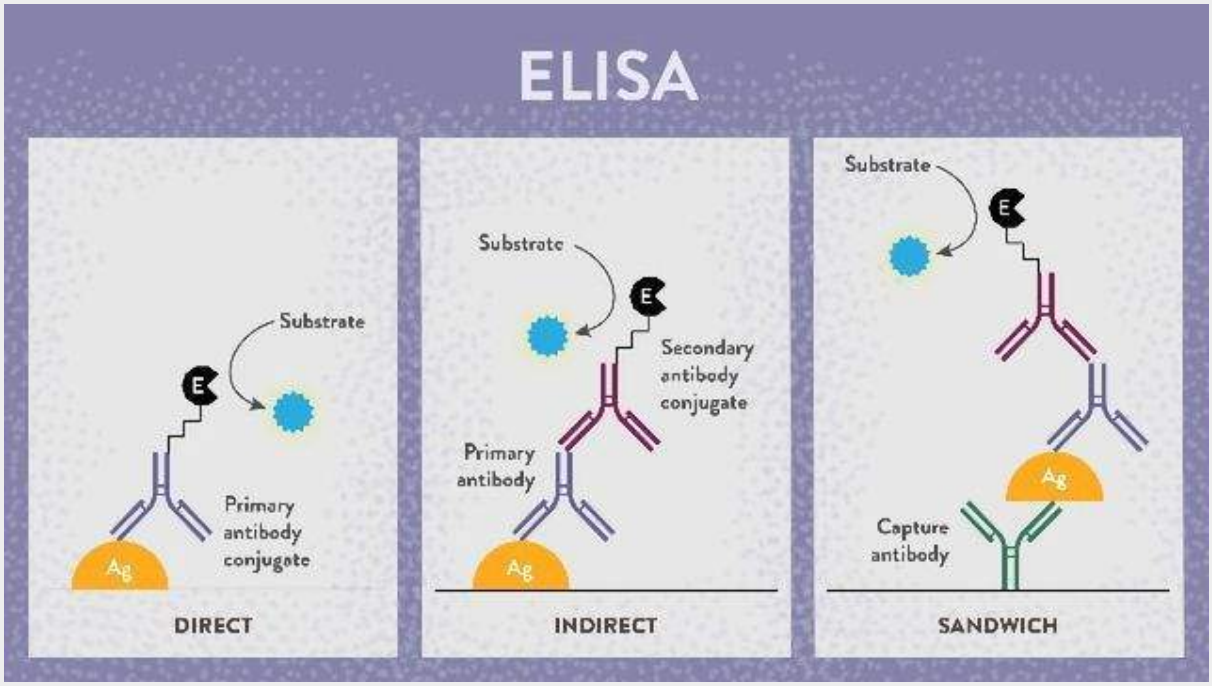


Plate coating

The first step in most ELISAs is the binding of the first component of the assay to the test plate. This is often done through passive adsorption, which is a non-specific process, so the result will depend on what is applied to the plate. If antigen-containing sample is applied, then the result is a plate for which selective steps are still required as a whole variety of antigens will bind, not just those of interest. If, however, purified antigen — as in the case of an indirect ELISA for antibody detection — or capture antibody — as in the case of the sandwich ELISA — is applied, then the result is a plate that already has selective properties.

Multiple plate types, most of which are polystyrene or polystyrene derivatives, with differing binding properties are available depending on the target and nature of the assay. Some targets, including heavily glycosylated proteins, carbohydrates, DNA, lipids, short peptides and proteins in the presence of detergents, do not adsorb well. In these cases, it is advisable to use plates that have been treated to permit covalent linkage to the surface instead.

Incubations

Following the addition of reagents to the ELISA plates, they must be incubated to allow time for binding or reactions to take place. The temperature and duration of each incubation will depend upon the assay step and the action being performed. Incubation for 1 hour at 37 °C is commonly used, for example following sample application. However, steps like blocking may

be performed overnight in a fridge and incubation during detection is often performed at room temperature for much shorter times.

Washing

Plate washing is a vital step between the application of each component of the assay right up until detection. After solutions are emptied from the wells, the wells are washed with a buffer — often phosphate-buffered saline-Tween 20 (PBST) — to remove any residual unbound antigens, antibodies or reagents that remain. This may be done by hand with a multichannel pipette or using an automated plate washer. Failure to wash sufficiently may result in high background signal, whereas too much washing can result in low sample signals. Inconsistent washing is likely to introduce inconsistencies across the plate, resulting in unreliable results.

Blocking

Following the coating of ELISA plates with proteins, blocking is often necessary to prevent any non-specific binding of detection antibodies in the following protocol steps (Figure 2). Mixed proteins unrelated to the assay are added to and incubated in the plate, occupying any available non-specific binding sites. Common protein blocking buffer choices include skimmed dried milk, bovine serum albumin (BSA) and casein. Alternatively, nonionic detergents, such as Tween 20 or Triton X-100, may be used as blocking agents but do not provide permanent blocking like proteins. Ineffective plate blocking can lead to increased background noise and reduce assay sensitivity and specificity.

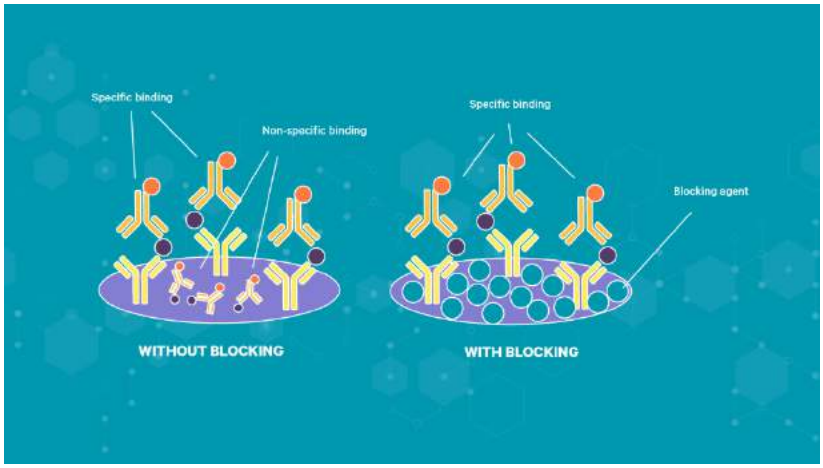


Figure 2: How the blocking process prevents non-specific binding.

Antibodies

Experimental antibodies are the cornerstone of most ELISAs and it is of paramount importance to choose the right ones, especially where multiple antibodies are used. Both monoclonal and polyclonal antibodies can be used, each with their own advantages and limitations. Monoclonal

antibodies offer high specificity but are more costly. Polyclonal antibodies on the other hand can bind a target at multiple binding sites, amplifying the signal and improving sensitivity.

The use of a methods employing a secondary antibody adds extra steps, prolonging the assay time, increasing the opportunity for mistakes and requiring more optimization to find an appropriate compatible antibody pair. However, sensitivity gains from the use of a polyclonal secondary antibody may make this worthwhile or essential to the development of an effective assay.

Detection

Irrespective of the ELISA type used, all end in a detection step, most often utilizing enzyme-mediated visible color change chemistry which can then be measured using UV-Vis spectrophotometry. An enzyme-conjugated antigen or antibody is applied to test wells where it will bind if its target is present. When an appropriate substrate for the enzyme is added to the plate, it causes a color change that is proportional to the amount of target bound inside the well. Horseradish peroxidase (HRP) is a common conjugate used in partnership with the substrate 3,3',5,5'-tetramethylbenzidine (TMB), which turns blue in response to HRP and then yellow on the addition of a sulfuric acid solution that stops the reaction.

The absorbance values for each well can then be determined using a microplate reader (at 450 nm in the case of TMB following the addition of stop solution) — a type of UV-Vis spectrophotometer — and corrections and calculations, such as subtraction of average blank well values, averaging of technical replicates or ratio calculation to standards, made according to the assay design.

An example of an ELISA workflow are depicted in Figure 3.

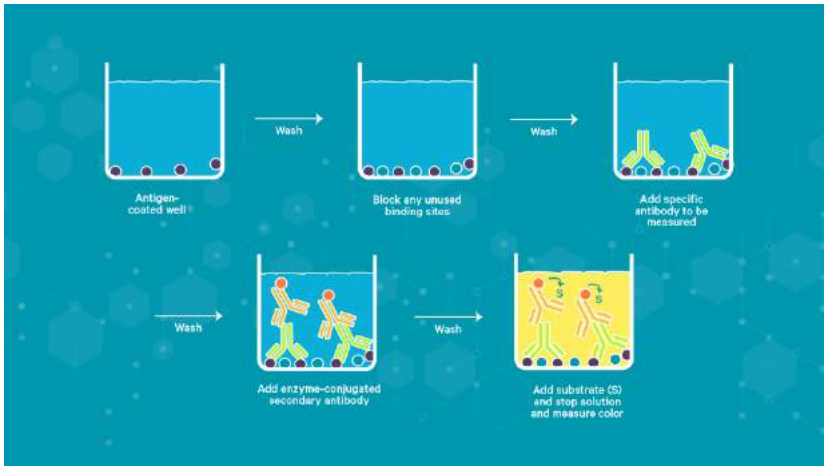


Figure 3: Example of an iELISA workflow.

ELISA test results,

ELISA results may be interpreted **quantitatively, qualitatively** or **semi-quantitatively**. In a quantitative assay, a serial dilution of a known standard is used to enable the generation of a standard curve, normally of optical density (OD) versus concentration. From this, the precise quantities of target in the unknown samples can be calculated (Figure 4).

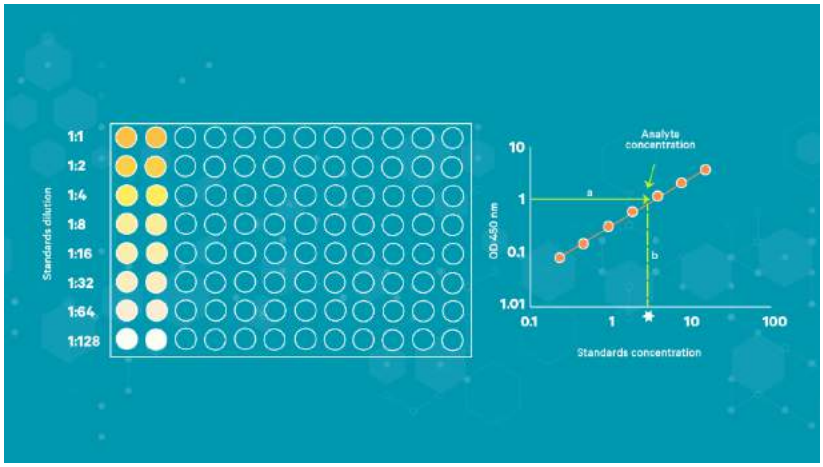


Figure 4: Example of standard curve calculation and target concentration determination of an unknown for an ELISA experiment.

12. Western Blot: Technique

Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest.

The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present. The paper will first describe the protocol for western blot, accompanied by pictures to help the reader and theory to rationalize the protocol. This will be followed by the theoretical explanation of the procedure, and in the later section, troubleshooting tips for common problems.

Technique

Cell lysis to extract protein

Protein can be extracted from different kind of samples, such as tissue or cells. Below is the protocol to extract proteins from adherent cells.

Adherent cells:

1. Wash cells in the tissue culture flask or dish by adding cold phosphate buffered saline (PBS) and rocking gently. Discard PBS. (Tip: Keep tissue culture dish on ice throughout).
2. Add PBS and use a cell scraper to dislodge the cells. Pipette the mixture into microcentrifuge tubes.
3. Centrifuge at 1500 RPM for 5 minutes and discard the supernatant.
4. Add 180 µL of ice cold cell lysis buffer with 20 µL fresh protease inhibitor cocktail. (Tip: If protein concentration is not high enough at the end, it is advised to repeat the procedure with a higher proportion of protease inhibitor cocktail).
5. Incubate for 30 minutes on ice, and then clarify the lysate by spinning for 10 minutes at 12,000 RPM, at 4°C.
6. Transfer supernatant (or protein mix) to a fresh tube and store on ice or frozen at -20°C or -80°C.
7. Measure the concentration of protein using a spectrophotometer.

Sample preparation

1. $\text{Using, concentration} = \frac{\text{mass}}{\text{volume}}$

   determine the volume of protein extract to ensure 50 µg in each well.
2. Add 5 µL sample buffer to the sample, and make the volume in each lane equalized using double distilled H_2O (dd H_2O). Mix well. (Tip: Total volume of 15 µL per lane is suggested).
3. Heat the samples with dry plate for 5 minutes at 100°C.

Gel preparation

10% Stacking gel	dd H_2O	3 mL
	1 M Tris-HCl	2.1 mL (pH 8.9)
	30% Acr Bis	2.8 mL
	10% SDS	80 µL
	10% APS*	56 µL
	TEMED*	6 µL
6% Separating gel	dd H_2O	2 mL
	1M Tris-HCl	400 µL (pH 6.7)
	30% Acr Bis	600 µL
	10% SDS*	36 µL
	10% APS*	24 µL
	TEMED	4 µL

*, APS: Ammonium Persulfate ; TEMED: Tetramethylethylenediamine; SDS: Sodium dodecyl sulfate

1. After preparing the 10% stacking gel solution, assemble the rack for gel solidification [Figure 1]. (Tip: 10% AP and TEMED solidify the solution; therefore, both gels can be prepared at the same time, if the abovementioned reagents are not added until the end).

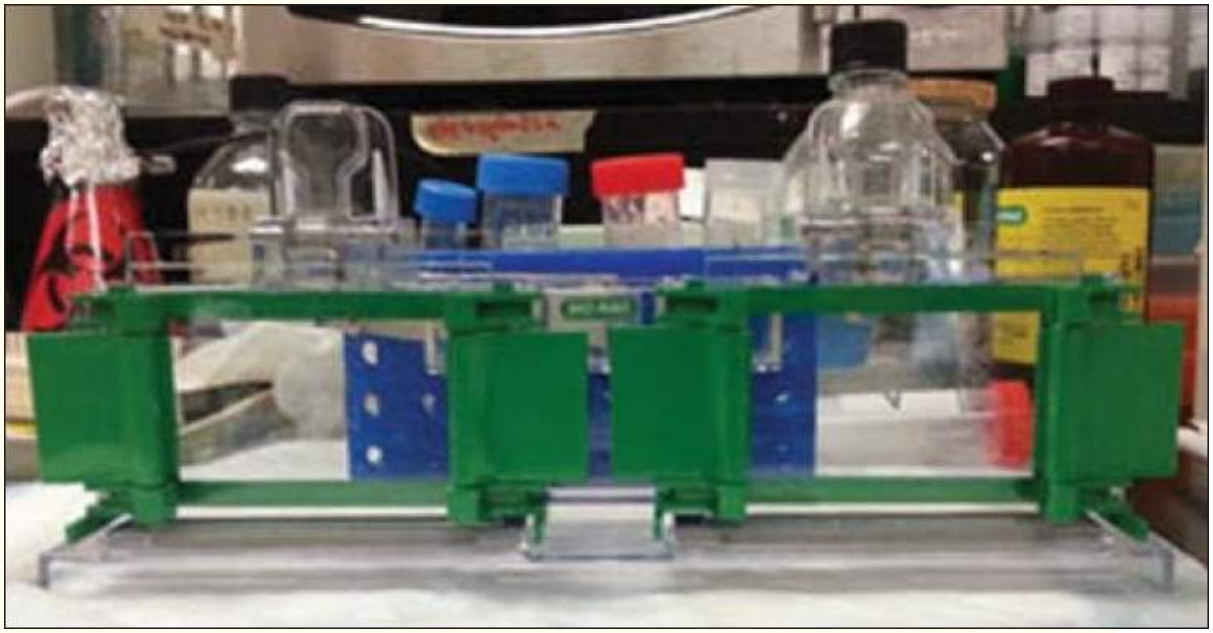


Figure 1

Assembled rack for gel solidification

2. Add stacking gel solution carefully until the level is equal to the green bar holding the glass plates [Figure 2]. Add H_2O to the top. Wait for 15–30 minutes until the gel turning

solidified. (Tip: Using a suction pipette can make the process of adding the gel to the glass plate easier).

Open in a separate window

Figure 2

Add gel solution using a transfer pipette

3. Overlay the stacking gel with the separating gel, after removing the water. (Tip: It is better to tilt the apparatus and use a paper towel to remove the water).
4. Insert the comb, ensuring that there are no air bubbles.
5. Wait until the gel is solidified. (Tip: Solidification can be easily checked by leaving some gel solution in a tube).

Electrophoresis

1. Pour the running buffer into the electrophorator [Figure 3].

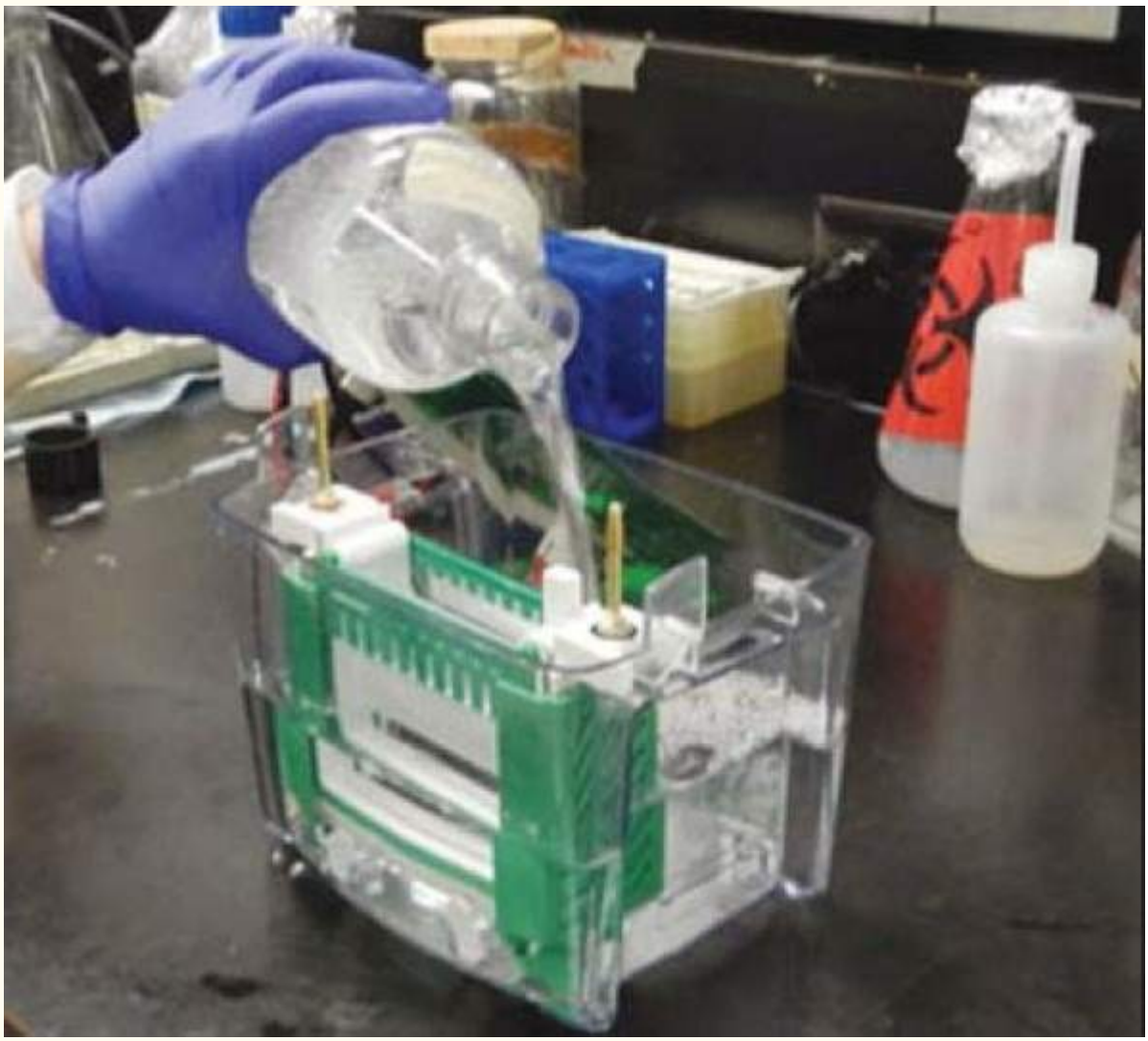


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Figure 3

Add running buffer to the electrophorator

2. Place gel inside the electrophorator and connect to a power supply. (Tip: When connecting to the power source always connect red to red, and black to black).
3. Make sure buffer covers the gel completely, and remove the comb carefully.
4. Load marker (6 µL) followed by samples (15 µL) in to each well [Figure 4].

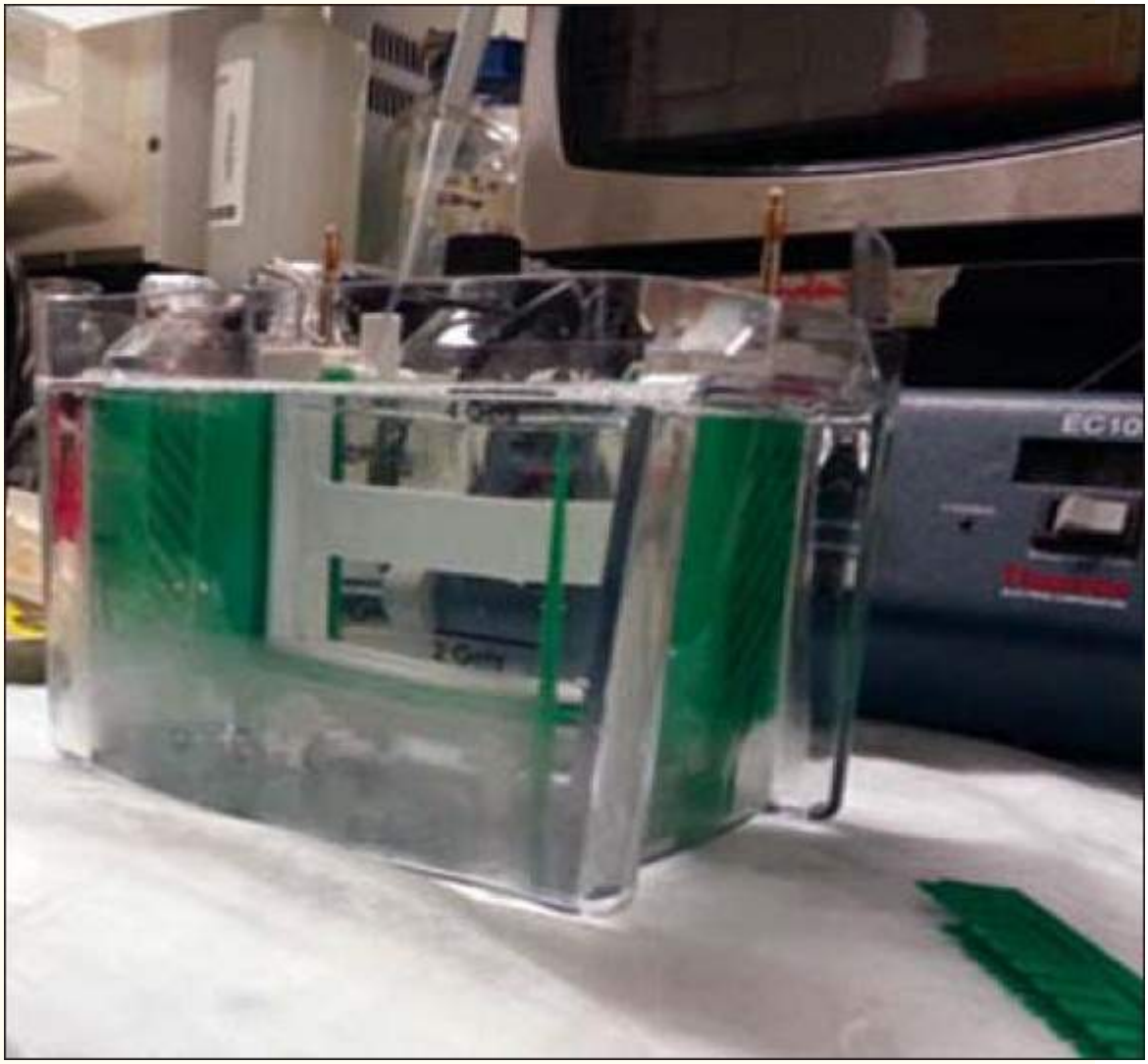

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Figure 4

Add samples and molecular marker to the gel, after removing the combs

5. Run the gel with low voltage (60 V) for separating gel; use higher voltage (140 V) for stacking gel [Figure [Figure5a5a and andbb].

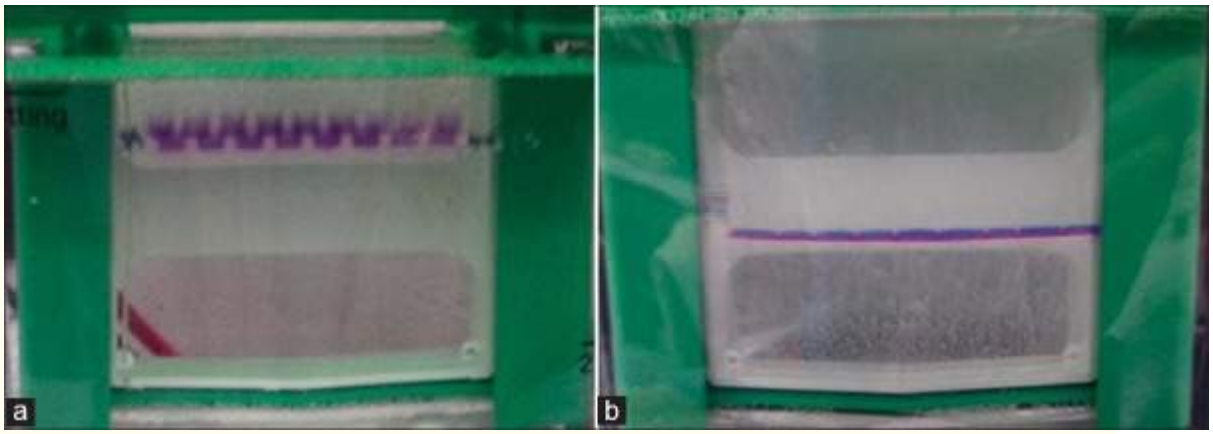

Figure 5

(a) Samples running through the stacking gel (lower voltage). (b): Samples running through the separating gel (higher voltage)

6. Run the gel for approximately an hour, or until the dye front runs off the bottom of the gel [Figure 6].

Figure 6

Run the gel to the bottom of the electrophorator

Electrotransfer

1. Cut 6 filter sheets to fit the measurement of the gel, and one polyvinylidene fluoride (PDVF) membrane with the same dimensions.
2. Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol.
3. Separate glass plates and retrieve the gel.
4. Create a transfer sandwich as follows:

   Sponge

   3 Filter Papers

   Gel PVDF

   3 Filter Papers

   (Tip: Ensure there are no air bubbles between the gel and PVDF membrane, and squeeze out extra liquid).
5. Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4°C. Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the PVDF membrane is between the gel and a positive electrode [Figure 7].

Figure 7

Transfer should be done on ice

6. Transfer for 90 minutes [Figure 8]. (Tip: The running time should be proportional to the thickness of the gel, so this may be reduced to 45 minutes for 0.75 mm gels).

Open in a separate window

Figure 8

Membrane after transfer

Blocking and antibody incubation

1. Block the membrane with 5% skim milk in TBST* for 1 hour.
2. Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4°C on a shaker [Figure 9].

Figure 9

Use a shaker to incubate the membrane with antibody

3. Wash the membrane with TBST for 5 minutes. Do this 3 times. (Tip: All washing and antibody incubation steps should be done on a shaker at room temperature to ensure even agitation).
4. Add secondary antibody in 5% skim milk in TBST, and incubate for 1 hour.
5. Wash the membrane with TBST for 5 minutes. Do this 3 times
6. Prepare ECL mix (following the proportion of solution A and B provided by the manufacturer). Incubate the membrane for 1–2 minutes [Figure 10]. (Tip: Use a 1000 µL pipette to ensure that ECL covers the top and bottom of the membrane).

Figure 10

Incubate the membrane with ECL mix using a 1000 µL pipette to help the process

7. Visualize the result in the dark room [Figure 11]. (Tip: If the background is too strong, reduce exposure time).

Figure 11

Use the cassette to expose the membrane in the dark room

Recipe

1. Dissolve the following in 800 ml of distilled H_2O
    - 8.8 g of NaCl
    - 0.2g of KCl
    - 3g of Tris base
2. Add 500ul of Tween-20
3. Adjust the pH to 7.4
4. Add distilled H_2O to 1L
5. Sterilize by filtration or autoclaving

Theory

Sample preparation

Cell lysates are the most common form of sample used for western blot. Protein extraction attempts to collect all the proteins in the cell cytosol. This should be done in a cold temperature with protease inhibitors to prevent denaturing of the proteins. Since tissue sample display a higher degree of structure, mechanical invention, such as homogenization, or sonication is needed to extract the proteins.

After extracting the protein, it is very important to have a good idea of the extract's concentration. This eventually allows the researcher to ensure that the samples are being compared on an equivalent basis. Protein concentration is often measured using a

spectrophotometer. Using this concentration allows to measure the mass of the protein that is being loaded into each well by the relationship between concentration, mass, and volume.

After determining the appropriate volume of the sample, it is diluted into a loading buffer, which contains glycerol so that the samples sink easily into the wells of the gel. A tracking dye (bromophenol blue) is also present in the buffer allowing the researcher to see how far the separation has progressed. The sample is heated after being diluted into a loading buffer, in order to denature the higher order structure, while retaining sulfide bridges. Denaturing the high structure ensures that the negative charge of amino acids is not neutralized, enabling the protein to move in an electric field (applied during electrotransfer).

It is also very important to have positive and negative controls for the sample. For a positive control a known source of target protein, such as purified protein or a control lysate is used. This helps to confirm the identity of the protein, and the activity of the antibody. A negative control is a null cell line, such as β-actin, is used as well to confirm that the staining is not nonspecific.

Gel electrophoresis

Western blot uses two different types of agarose gel: stacking and separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Protein is thus separated by their size more so in this gel, as the smaller proteins to travel more easily, and hence rapidly, than larger proteins.

The proteins when loaded on the gel have a negative charge, as they have been denatured by heating, and will travel toward the positive electrode when a voltage is applied. Gels are usually made by pouring them between two glass or plastic plates, using the solution described in the protocol section. The samples and a marker are loaded into the wells, and the empty wells are loaded with sample buffer. The gel is then connected to the power supply and allowed to run. The voltage is very important, as a high voltage can overheat and distort the bands.

Blotting

After separating the protein mixture, it is transferred to a membrane. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. The sandwich includes a fiber pad (sponge) at each end, and filter papers to protect the gel and blotting membrane [Figure 12]. Here two things are very important: (1) the close contact of gel and membrane to ensure a clear image and (2) the placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins.

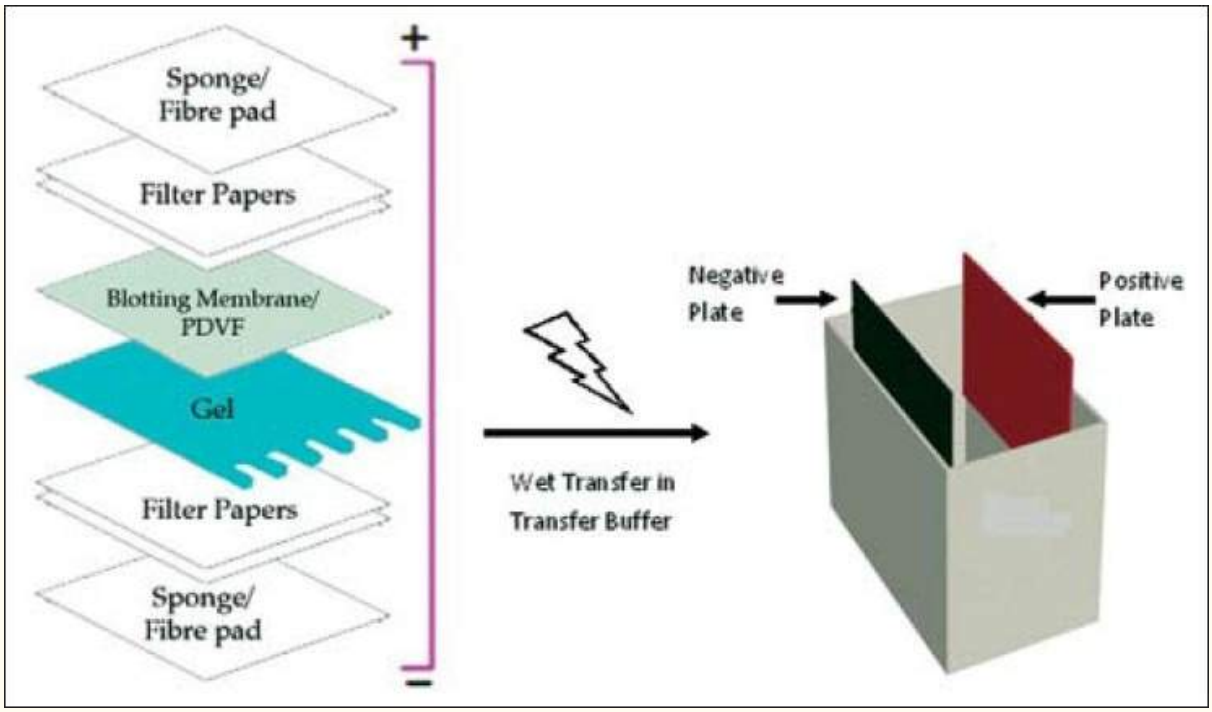

Figure 12

Assembly of a sandwich in western Blot

The membrane, the solid support, is an essential part of this process. There are two types of membrane: nitrocellulose and PVDF. Nitrocellulose is used for its high affinity for protein and its retention abilities. However, it is brittle, and does not allow the membrane to be used for reprobing. In this regard, PVDF membranes provide better mechanical support and allow the blot to be reprobed and stored. However, the background is higher in the PVDF membranes and therefore, washing carefully is very important.

Washing, blocking and antibody incubation

Blocking is a very important step of western blotting, as it prevents antibodies from binding to the membrane nonspecifically. Blocking is often made with 5% BSA or nonfat dried milk diluted in TBST to reduce the background.

Nonfat dried milk is often preferred as it is inexpensive and widely available. However, milk proteins are not compatible with all detection labels, so care must be taken to choose the appropriate blocking solution. For example, BSA blocking solutions are preferred with biotin and AP antibody labels, and antiphosphoprotein antibodies, since milk contains casein, which is itself a phosphoprotein and biotin, thus interfering with the assay results. It is often a good strategy to incubate the primary antibody with BSA since it is usually needed in higher amounts than the secondary antibody. Putting it in BSA solution allows the antibody to be reused, if the blot does not give good result.

The concentration of the antibody depends on the instruction by the manufacturer. The antibody can be diluted in a wash buffer, such as PBS or TBST. Washing is very important as it minimized background and removes unbound antibody. However, the membrane should not be left to wash for a really long time, as it can also reduce the signal.

The membrane is then detected using the label antibody, usually with an enzyme such as horseradish peroxidase (HRP), which is detected by the signal it produces corresponding to the position of the target protein. This signal is captured on a film which is usually developed in a dark room.

Quantification

It is very important to be aware that the data produced with a western blot is typically considered to be semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of quantity. There are two reasons for this; first, there are variations in loading and transfer rates between the samples in separate lanes which are different on separate blots. These differences will need to be standardized before a more precise comparison can be made. Second, the signal generated by detection is not linear across the concentration range of samples. Thus, since the signal produced is not linear, it should not be used to model the concentration.