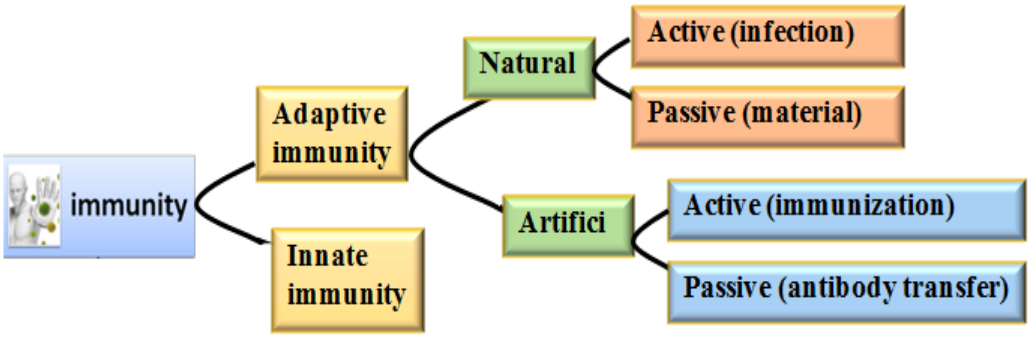
Immunity

Immunity is the ability of the body to defend against infections agent such as pathogens organisms (viruses, bacteria, fungi and parasites) and foreign substances (antigens). The collection of cells, tissues, organs, and molecules that mediate protection against infections agent is called the **immune system** and the coordinated reaction of these cells and molecules to eliminate the infectious microbes called an **immune response** (Humoral & Cellular). **Immunology** is the study of the immune system, including its responses to microbial pathogens and damaged tissues and its role in disease. There are two types of immunity, natural (**innate**) and acquired (**adaptive**) immunity.



- **Innate immunity :**(non-specific) everyone is born with innate immunity, It includes the external barriers of the body, like the skin , mucous membranes (like the nose, throat, and gastrointestinal tract) normal flora & tears … etc, which are the first line of defense in preventing diseases from entering the body & provides immediate protection against microbial invasion. The macrophages and neutrophils also provide the innate immune .
- **The acquired (adaptive) immunity:** (Specific) the second types of protection, which develops throughout our lives in slowly and provides more specificity defense against infections with memory. Adaptive immunity involves the lymphocytes and develops as people are exposed to diseases or immunized

against diseases through vaccination. It may be produced in two ways, namely, **active or passive** immunization:

Active Immunization

Active immunity results when stimulation the immune system in the body to produce antibodies for the foreign antigens that entered the body . Include:

- **Naturally acquired active immunity** : can occur through infection with the actual disease**.**
- **Artificially acquired active immunity:** can occur through introduction of a killed or weakened form of the disease organism through vaccination**.**

Passive Immunity

Passive immunity is provided when a person is given antibodies to a disease rather than producing them through his or her own immune system. Include:

- **Naturally acquired passive immunity:** can occur when a newborn baby acquires passive immunity from its mother through the placenta.
- **Artificially acquired passive immunity**: A person can also get passive immunity through antibody-containing blood products such as immunoglobulin.

The components of the immune system:

1. **Cellular:** blood cells (white blood cells :Neutrophil , Basophil, Eosinophil , Monocyte and Lymphocyte) & dendritic cell.
2. **Humoral :** antibody ,complement, cytokines
3. **Organs:** bone marrow, thymus, lymph nodes, spleen, gut-associated lymphoid tissue, tonsils.

Role of the immune system in:

1. Defense against microbes.
2. Defense against the growth of tumor cells.
3. Destruction of abnormal or dead cells (e.g. dead red or white blood cells, antigen-antibody complex)

Cells of the immune system

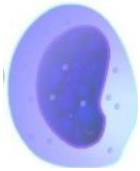
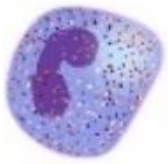
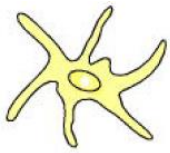
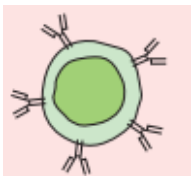
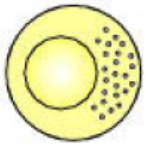
Blood is an important component of the immune system. It contains the cells, proteins, and liquid that circulate throughout the body. Most cells of the immune system arise from the bone marrow. The cells are classified into myeloid progenitor (monocytes, macrophages, neutrophils, basophils, mast cells, eosinophils and erythrocytes), lymphoid progenitor (B-Lymphocyte, T-Lymphocyte and Natural killer cells). A successful immune response to a pathogen depends on interaction among diverse cell types (see table 1). White blood cells (WBC) or leukocytes are cells of the immune system defending the body against both infection disease and foreign material. Both innate immunity and adaptive immune responses depend upon the activities of white blood cells, or leukocytes.

Table (1) normal counts of WBCs.

Cells		**Normal counts**
Total WBCs count		4000- 110000/cu.mm
Differential count	Neutrophils	40-70%
	Eosinophils	1-4%
	Basophils	0-1%
	Monocytes	4-8%
	Lymphocytes	20-40%

Table (1) types of blood cells & immunity cells.

Cell types	**Figure**	**Function**
Erythrocytes (RBC)		O_2 Transport
Platelets		-Blood clotting -Blood vessel repair

Monocytes		-Phagocytosis -Antigen presentation -Inflammation
Neutrophils		-Phagocytosis -Inflammation
Eosinophils		-Phagocytosis , -fight parasites
Basophils		- May prevent clotting in inflammation - IgE responsive
Mast cells		they are important in allergic responses
dendritic cells		- Antigen uptake in peripheral site -Antigen presentation.
T- Lymphocytes		- Cell-mediated immunity (Specific) - Antigen presentation - Help for activation the macrophage
B- Lymphocytes		-Humoral immunity -Antibody production -Antigen presentation
N**atural killer cells**		Cytotoxic or killing for Viral infected cells & Tumor cells.

Examination of the cells in the blood :

Making the blood smear :

1. Collect a blood sample and place drop of blood onto slide on one end.
2. Place 2nd slide onto edge of drop (start from center of slide and back slide into drop) and once fluid is pulled across 2nd slide push the 2nd slide across the 1st slide. Let cells dry, heat fix, and stain with Wright's Stain or Leishman's Stain.

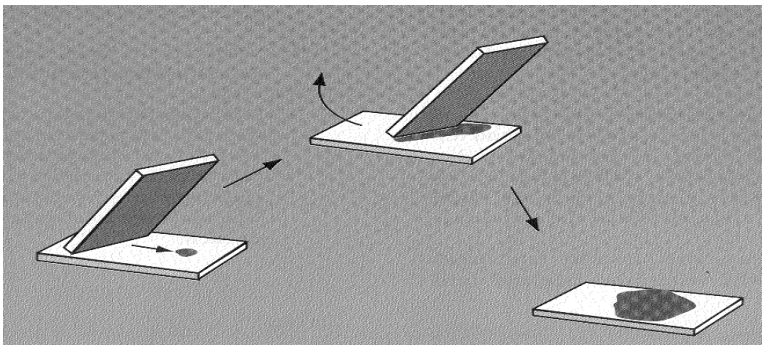


Figure (1): Blood smear preparation.

Leishman's Stain:

1- Place the film on a staining rack, flood with Leishman's stain, and leave for 10 min to fix.
2- Add twice as much buffered distilled water
3- Leave to stain for 10 min and wash off stain with tap water.
4- Examine slide under microscope by oil lens.

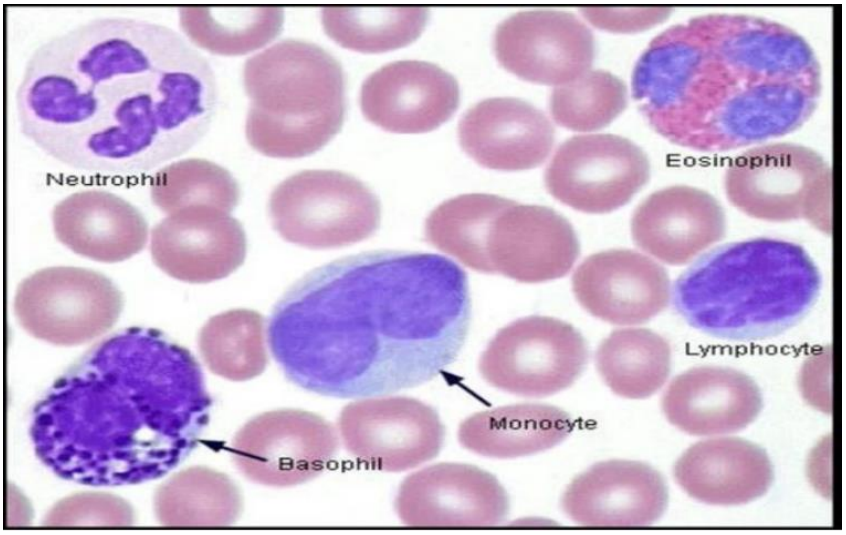


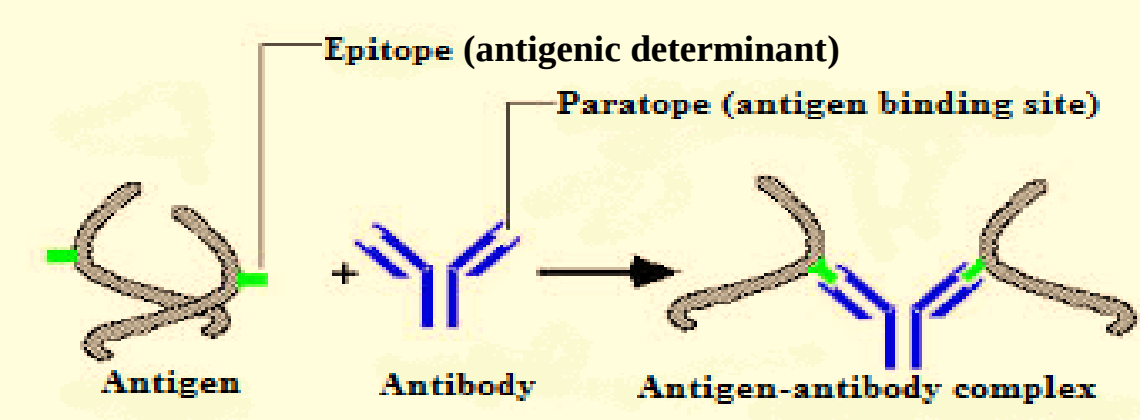
Figure (2): Smear of human blood stained with Leishman's Stain.

Antigens

An **antigen (Ag)** is a substance that when introduced in to the body can evoke immune response and induces the formation of a specific antibody which binding the Ag-Ab (humoral response) or cellular immunity response (T cells) or both. Antigen may be bacteria cells and viruses or part of it such as coat, capsule, cell walls, flagellate and toxins ,in adding of other types of the microbe.

Characteristics of Ag

1. **Immunogenicity**: is the ability of inducing immune response.
2. **Antigenicity:** is the ability to combine specifically with the final products of the immune response (antibodies or surface receptor on T-cell).
3. Each antigen molecule has a set of **Epitopes (antigenic determinant)** the region of an antigen that interacts with an antibody. while the part of an antibody, which recognizes and bind to antigen called **Paratope (antigen-binding site)**



Immunogen: is any antigen that is ability of inducing humoral and/or cellular immune response (complete antigen), usually have high molecular weight.

Hapten: is a molecule that can't induce an immune response (incomplete antigen) usually has low molecular weight, unless they are bound to a larger molecule.

Adjuvant: is a substance that enhances the immunogenicity of an antigen.

Super antigens: are a class of antigens which cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release, such as Enterotoxins of *Staphylococcus aureus*

Chemical nature of antigens (Immunogens):

1- **Proteins:** good immunogenic
2- **Poly saccharides:** good immunogenic
3- **Nucleic acid** & **Lipids:** are usually poorly immunogenic. However, they may become immunogenic when it combine with proteins or polysaccharides.

Type of Antigens

1-Exogenous antigens :

These antigens are enters the body and start circulating in the body fluids and trapped by the APCs (Antigen processing cells such as macrophages & dendritic cells) and phagocytosis for these Ags. Examples: bacteria, viruses, fungi ...etc.

2- Endogenous antigens :

These antigens are body's own cells, sub fragments, compounds, or the antigenic products that are produced .Examples: Blood group antigens (RBC Ags) & HLA (Histocompatibility Leucocyte Antigens).

3-Autoantigens:

An autoantigen is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognize by the immune system of patients suffering from a specific autoimmune disease.

These antigens should not be under normal conditions the target of immune system, but due mainly to genetic and environmental factors, the normal immunological for such an antigen has been lost in these patients for Examples: Nucleoproteins, Nucleic acids.

Preparation of antigen from gram-ve bacteria E. coli:

1- Prepare pure culture of E. coli on MacConkey agar and incubate at 37 ºC for 24 h.
2- Add 6 ml of formal saline (0.5 ml of formalin + 99.5 ml normal saline) on the surface of agar then collect suspension in sterile tube .
3- Centrifugation at 4000 rpm for 5 min.
4- Discard supernatant and re-suspend pellet with normal saline, and repeat centrifugation above three times .
5- Place antigenic suspension in water path at 60 Cº for 90 min?
6- Make sterility test by loop full of antigenic suspension and streak it on blood agar ,incubate at 37ºC for 24hr

Antibodies

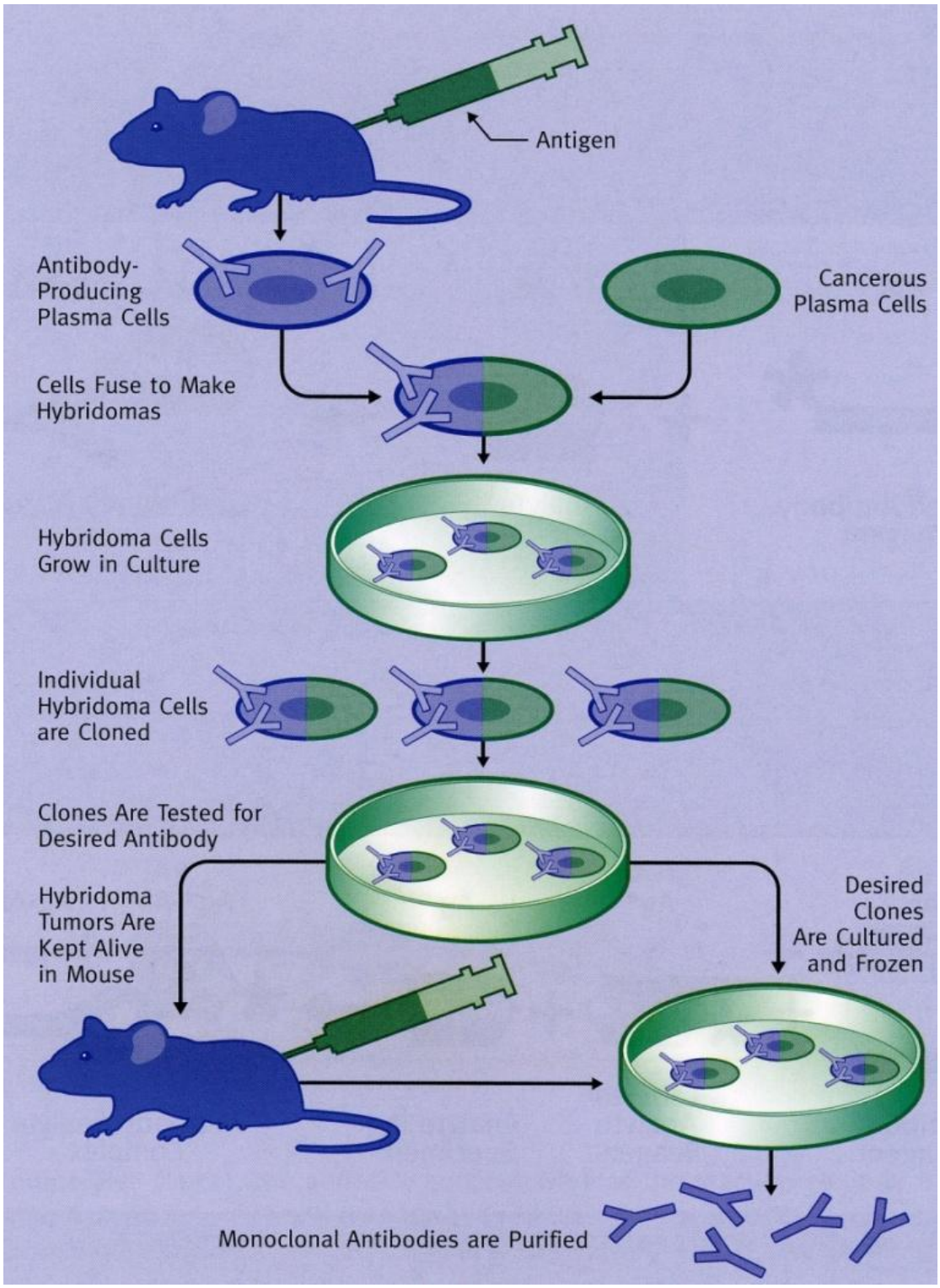
Antibodies : are glycoproteins produced in response to the presence of foreign antigen in the body. They are secreted by plasma cells that it arise from differentiated of B-cells, and circulate throughout the blood and lymph where they bind to foreign antigens & product antibody- antigen complexes **(Ag-Ab)**. Antibodies are immune system-related proteins called **immunoglobulins.**

Preparation of Polyclonal Antibodies:

- **Polyclonal antibodies** is generated in animals (sheep, rabbits or goats) with the introduction of antigens into the animal's bloodstream.
- **The antiserum** (serum from blood containing the desired antibodies) contains a mixture of antibodies, each of which may bind to different antigen binding sites (epitopes).
- Antiserum contains a mixture of antibodies are called polyclonal antibodies.

Preparation of Monoclonal Antibodies :

- **Monoclonal antibodies** − specific antibodies that are identical because they were produced by one type of immune cell, all clones are produced from a single cell line. These Abs are highly specific for a specific epitope on antigen.
- a single cell called **Hybridoma**, it is the cell that is produced through the plasma cells isolated from mouse and laboratory integrated with cancer cells from another mouse to get on the Hybridoma for producing the many copies of the same antibody and grow easily in lab cell culture.
- An advantage of monoclonal antibodies is that the Hybridoma cell line that produces them is potentially - immortal and can produce the same antibodies consistently and indefinitely.


Antigen
Antibody-Producing Plasma Cells
Cancerous Plasma Cells
Cells Fuse to Make Hybridomas
Hybridoma Cells Grow in Culture
Individual Hybridoma Cells are Cloned
Clones Are Tested for Desired Antibody
Hybridoma Tumors Are Kept Alive in Mouse
Desired Clones Are Cultured and Frozen
Monoclonal Antibodies are Purified

Antigens – Antibody binding

The combination of antigen with antibody plays an important role in the laboratory for diagnosing many different diseases.

the laboratory technique that makes use of the binding between an antigen and antibody in order to qualitative and quantitative diagnoses for the specific antigen or antibody in a sample called Immunoassay.

The reaction of an Ag –Ab can be detected in lab by:

1. **Agglutination test.**
2. **Precipitation test.**

Agglutination

Agglutination results when antibody reacts with **particulate antigen**, principally cells such as bacteria & erythrocytes, which it the reaction produces **visible aggregation** of immune complexes because of the larger size of the antigens & formation of a lattice, the lattice is composed of antibodies bridged between antigens. The examination is very fast and often takes a less than 10 min.

This clumping reaction is called **agglutination**, and antibodies that produce such reactions are called **agglutinins,** whereas antigen can interact with agglutinin is called **agglutinogen.**

Application of agglutination test:

1. To determining of blood groups.

2. To assess bacterial infections.

3. To assess inflammation.

4. Diagnosis of autoimmune diseases.

Types of Agglutination Reactions

1) Direct agglutination test:

Direct interaction of specific antibodies with particulate antigens. It is a qualitative tests, such as **Blood grouping** & **Rh antigen**. Also used to detect antibodies in **Widal test** and **Rose Bengal tests**. These tests can be occur on slide of tube agglutination.

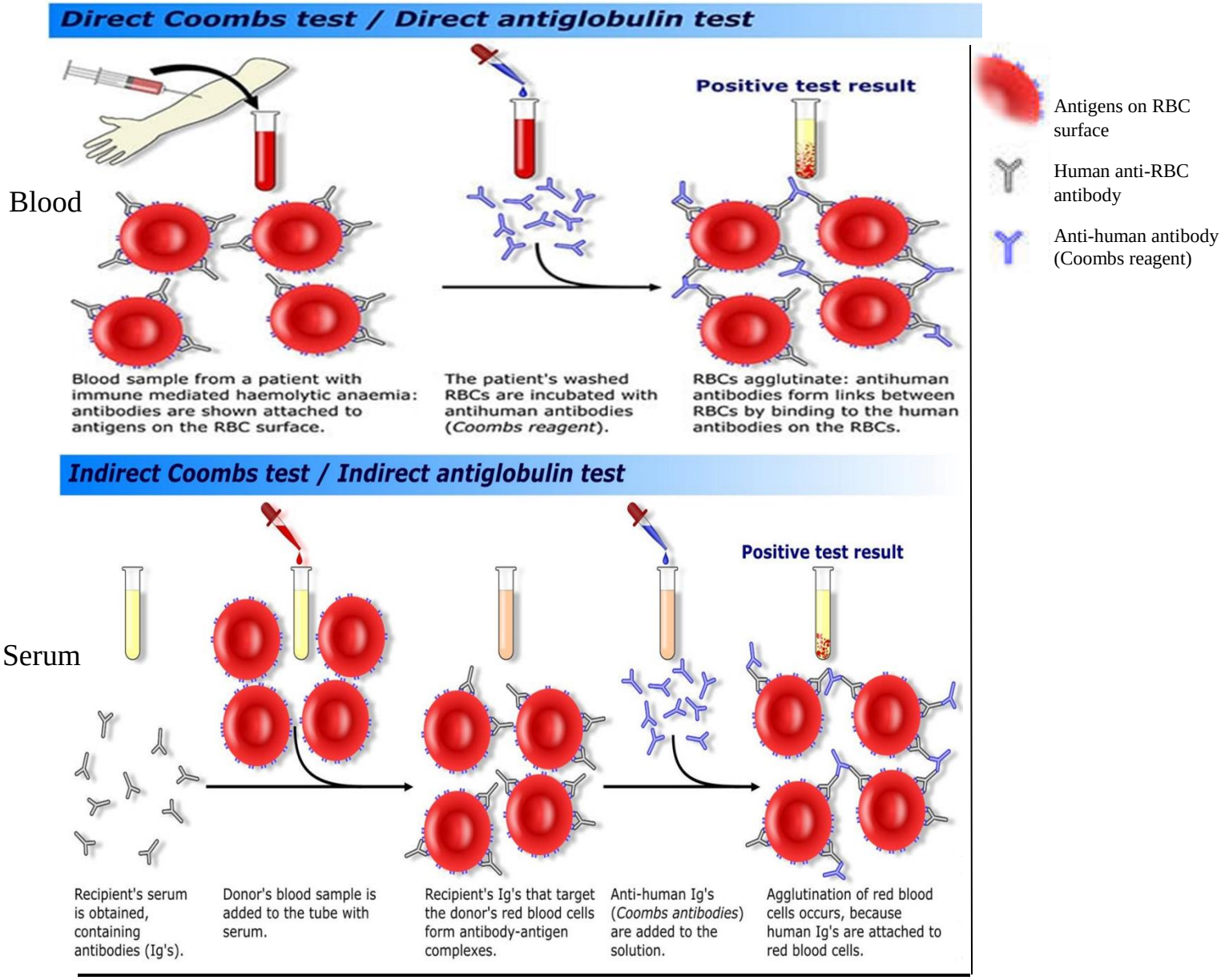
2) Indirect agglutination test :

The most widely used agglutination methods which it the soluble antigens is attached to a carrier particle such as Latex particles, Carbon particles, Bantonite etc, are used as inert carriers. Indirect agglutination test use to detect antibodies in **HCG, RF, CRP**, and **ASOT** tests.

3) Coombs test (Antiglobulin test):

Is the antibody-mediated incomplete agglutination reaction. Antiglobulin antibodies (Coombs reagent) are added to detect subagglutinating amounts of anti-erythrocyte antibodies in tube agglutination**.**

- ✓ **The direct Coombs** :detects antibodies that bind to a patient's erythrocytes. The direct Coombs is designed to identify **anti-Rh antibodies** that are already bound to **infant RBCs** or antibodies bound to RBCs in patients with **autoimmune hemolytic anemia**.
- ✓ **The indirect Coombs** :is a two-stage reaction in which the patient's serum is first incubated red blood cells, after which an antiglobulin antibody is added. The indirect Coombs test is designed to identify **Rh^- mothers** who are producing **anti-Rh antibodies** of the IgG isotype, which may be transferred across the placenta harm in Rh+ fetuses.



Blood grouping

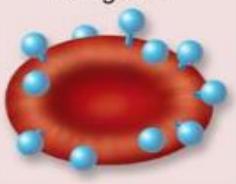
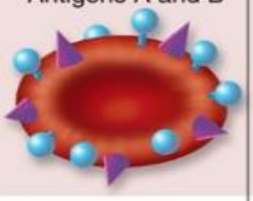
Hemagglutination is a direct agglutination can be used to detect any antigen conjugated to the surface of Red Blood Cells such as ABO antigens.

Hemagglutination is used in determinations the blood grouping, the test using for blood transfusion or when classifying a person's blood for donation.

Blood grouping is important for pregnant women. If the mother is Rh- and the father is Rh+, the mother and child Rh+ will have different blood types in these cases, the mother needs to receive a drug called Rho GAM. This drug will keep

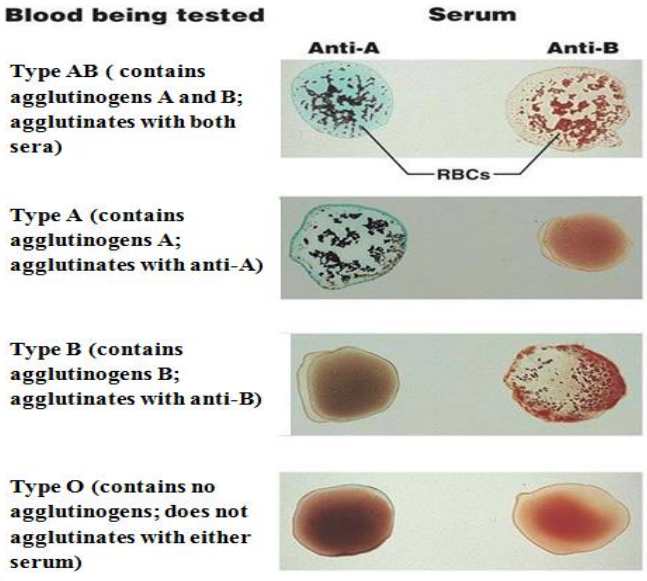
her body from attacking the second baby's blood cells if their blood becomes mixed, which often happens during pregnancy**.**

- ❖ The Ag is cover the surface of erythrocytes and Ab found in the plasma.
- ❖ Rh antigen: another important red blood cell antigen, also important in transfusion reactions, it can be detected with anti-Rh antiserum.
- ❖ **Interpretation of the test**

	Antigen A	Antigen B	Antigens A and B	Neither antigen A nor B
Erythrocytes				
Plasma	Anti-B antibodies 	Anti-A antibodies 	Neither anti-A nor anti-B antibodies	Both anti-A and anti-B antibodies 
Blood type	**Type A** Erythrocytes with type A surface antigens and plasma with anti-B antibodies	**Type B** Erythrocytes with type B surface antigens and plasma with anti-A antibodies	**Type AB** Erythrocytes with both type A and type B surface antigens, and plasma with neither anti-A nor anti-B antibodies	**Type O** Erythrocytes with neither type A nor type B surface antigens, but plasma with both anti-A and anti-B antibodies

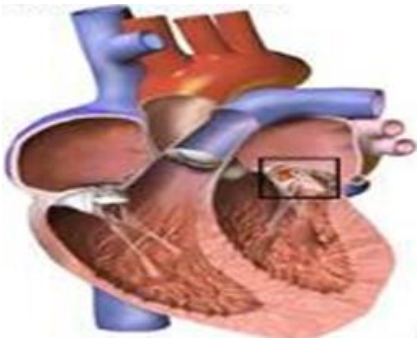
Procedure:

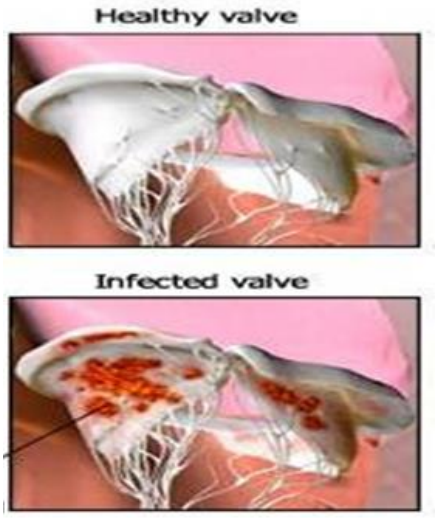
1. Put the three drop of blood to be tested is placed on a slide and mixed with a drop of anti-A ,anti-B & anti –D, respectively.
2. After waiting a few minutes the agglutination reaction is visible complete if homologous antigens is present.
3. If the antigen A is present, red blood cells will be agglutinated by anti-A this type A. Thus, for the rest of the types.
4. A drop contain Rh positive erythrocytes is agglutination with anti-D .



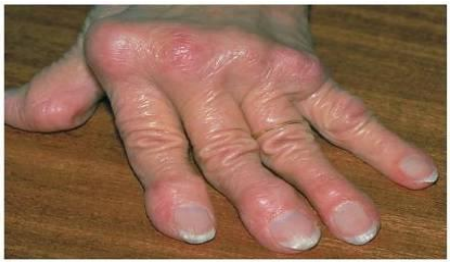
Anti-streptolysin O Test (ASOT)

- **Anti-streptolysin O** : is the antibody produced against an Ag which is secreted by group A *Streptococcus* bacteria (this Ag a toxic enzyme called streptolysin O involved in hemolysis) the bacteria responsible for many of diseases.
- When the body is infected with *Streptococcus pyogenes*, it produces antibodies against the specific antigens that the streptococci produce. ASO is one such antibody so, a raised or rising levels can indicate past or present infection. Increased levels of ASO titer in the serum could cause damage to the heart and joints.
- ASOT is used to detect infection by Group A *Streptococcus*, the bacteria responsible for diseases such as:
  - ★ Bacterial endocarditis
  - ★ Glomerulonephritis
  - ★ Rheumatic fever
  - ★ Scarlet fever





- ASOT perform by:
  1. Latex agglutination.
  2. ELISA to detect the exact titer value.
  3. Tube agglutination using a serial dilution technique, to detect the titer value.



Latex agglutination of ASO Test

Materials:

1) **ASO Antigen:** A stabilized buffered suspension of polystyrene latex particles coated with Streptolysin O and 0.1% sodium azide as preservative. Shake well prior to use.
2) **ASO Positive Control**: Human serum containing more than 200 IU/ml ASO and 0.1% sodium azide as preservative.
3) **ASO Negative Control**: Human serum containing 0.1% sodium azide.
4) micropipettes.
5) Tips.
6) Glass test slide.

Procedure for Anti-streptolysin O Test:

1. Bring all test reagents and samples to room temperature.
2. Put the drop of serum , Positive Control & Negative Control on the slide.
3. Mix the ASO latex reagent by gently shaking. Add one drop of reagent to each control and sample.
4. Using the flattened end of the tips as a stirrer thoroughly mix each sample with reagent within the full area of the circle.
5. Discard the tips.
6. Slowly rock the slide for exactly two (4) minutes and observe for agglutination under a high.
7. Record results appear clumping +.
8. Re-wash glass slide for future use.

Dilutions

- **A dilution** is a process that reduces the concentration of a substance in a solution and is a common laboratory technique used to obtain the desired concentration.
- The serology tests (usually refers to the diagnostic concentration of antibodies in the serum) can be classified into:
  1) Qualitative tests (positive and negative).
  2) Quantitative tests (such as 300 µl).

  To make the quantitative tests we have to make several dilutions. Usually, in immunology lab the specimen is the serum and the diluent is the normal saline.

- **Serum:** is that part of blood which is similar in composition with plasma but exclude clotting factors of blood, which can be obtained from put the blood in the plane tube and left stand at room temperature for 15- 30 minutes to coagulate and a centrifuge for 10 minutes at 3000 rpm.
- It may be found that the concentration of the substance being measured is too high for measurement with a certain instrument . In such cases, a dilution is necessary.
- **Antibody Titer**: the relative amount of antibody present in an antiserum that can bind to a constant amount of antigen. The relative amount is usually determined by making dilutions of an antiserum and testing the dilutions for their ability to react with an antigen then greatest reacting dilution is taken as the Titer.
- The diluent is **Normal Saline** (N.S) prepared by weighing the 85 gm NaCl to 1L distilled water.

Examples: Dilute the patient's sample 1 to 2 ?

- ✓ This means add one volume of the serum to 2 volumes of the normal saline.
- ✓ The final volume will be 3, we say 3 parts.

Questions: Prepare 1to 2 dilution, the final volume is 6, 1.5 ?

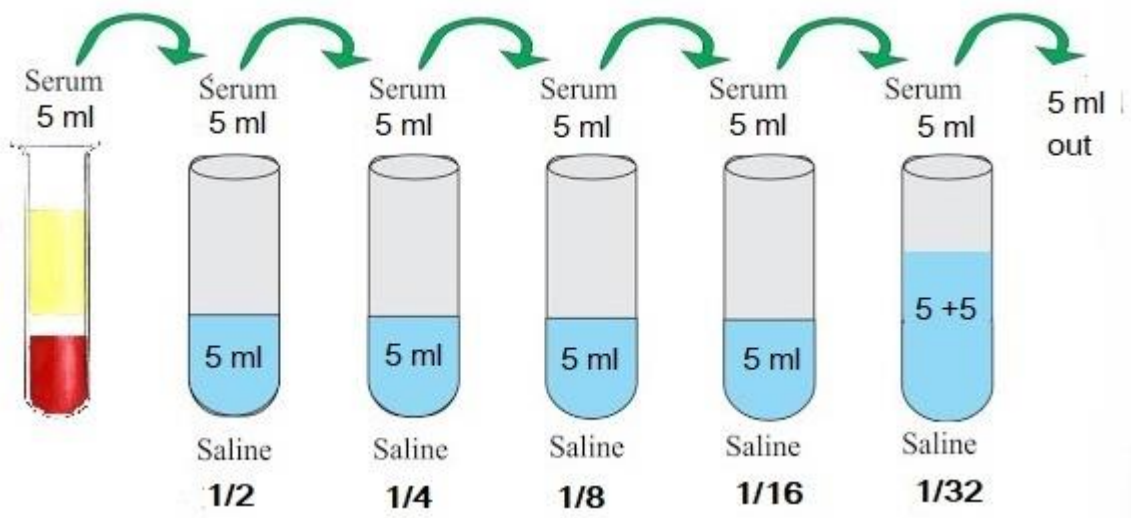
Serum	Diluent	Final volume
2 ml	4 ml	6 ml
0.5 ml	1 ml	1.5 ml

Examples: But if we say 1 in 2, this means add 1 volume of the serum to 1 volume of diluent, because the final is 2 volumes. Dilution 1 in 8 is a dilution made by mixing 1 volume of serum with 7 volumes of diluents.

Questions: dilute the patient's serum 1 in 4 , 3 in 8 **?**

Dilution	Serum	Diluent	Final volume
1 in 4	1	3	4
3 in 8	3	5	8

Double dilution



Dilution calculation:

$$\text{Dilution} = \frac{\text{Serum volume}}{\text{Total volume}} = \frac{\text{S.V}}{\text{T.V}}$$

D.V (diluent volume)

S.V. (Serum volume)

T.V.(Total volume)

Total volume = S.V. + D.V.

S.V = T.V. – D.V.

D.V. = T.V. – S.V.

Q1 /What is the dilution of this tube test?

1- SV = 0.5 ml DV (diluent volume) = 1 ml

$$\text{Dilution} = \frac{\text{Serum volume}}{\text{Total volume}} \Rightarrow \frac{0.5}{1 + 0.5} = \frac{1}{3}$$

2- SV = 2000 µL DV (diluent volume) = 12 ml

$$\frac{2000}{1000} = 2 \text{ ml} \Rightarrow \text{Dilution} = \frac{2}{2 + 12} = \frac{2}{14} = \frac{1}{7}$$

Q2/ How do you prepare 1:4 dilution on a total volume of 8 ml?

$$\text{S.V} = \frac{1}{4} = \frac{?}{8} = 2 \text{ ml} \Rightarrow \text{D.V} = 8 - 2 = 6 \text{ ml}$$

Q3 / Prepare 1: 2 dilution in a total volume 0.4 ml?

$$\text{S.V} = \frac{1}{2} = \frac{?}{0.4} = 0.2 \text{ ml} \Rightarrow \text{D.V.} = 0.4 - 0.2 = 0.2 \text{ ml}$$

Rose Bengal Test

The Rose Bengal test (RBT) is a simple, rapid slide- agglutination assay performed with a stained *Brucella abortus* suspension (Ags) coloured with rose bengal at pH 3.6–3.7 and serum for the detection of anti-Brucella. Rose Bengal test used for diagnosis Brucella bacteria that responsible of human Brucellosis (a zoonotic disease). *Brucella* gram-negative coccobacilli without a capsule, survive as intracellular organisms, transmitted to human by direct or indirect contact with infected animals or their products. Humans are the accidental host.

Materials required:

- Serum (sample)
- Rapid latex kit …… read the leaflet …….. Brucella antigen (Rose Bengal) - Positive control - Negative control -White glass slide - Stirring sticks.

Result of Rose Bengal Test:

Rose Bengal reagent (Ags) Brucella **+** Serum (anti-Brucella) ⟹ Agglutination

Negative : no visible agglutination

Positive : visible agglutination

LORNE LABORATORIES LTD.

GREAT BRITAIN

RAPID LATEX KIT

DIRECTIONS FOR USE

ROSE BENGAL: For Detection Of Anti-Brucella antibodies.

SUMMARY

The Rose Bengal test is a slide agglutination test for the qualitative and semi-quantitative detection of anti-Brucella antibodies in human serum. The reagent, because of its formulation in an acid buffer, is reactive with both IgG and IgM antibodies and very useful for the diagnosis of chronic individuals, which present a high level of IgG antibody that is difficult to detect by the reference tube method (Wright).

PRINCIPLE

When used by recommended techniques, a suspension of Brucella abortus in the reagent will agglutinate (clump) in presence of anti-Brucella antibodies. No agglutination generally indicates absence of anti-Brucella antibodies (see **Limitations**).

KIT DESCRIPTION

Lorne Rose Bengal Kit is for the detection of anti-Brucella antibodies. The reagent is a suspension of Brucella abortus strain S99, that agglutinates in the presence of anti-Brucella antibodies. All reagents are supplied at optimal dilution for use with all recommended techniques without the need for further dilution or addition. For lot reference number and expiry date see **Vial Labels**.

STORAGE

Do not freeze. Reagent vials should be stored at 2 - 8ºC on receipt. Prolonged storage at temperatures outside this range may result in accelerated loss of reagent reactivity.

SPECIMEN COLLECTION

Specimens should be drawn with or without anticoagulant using an aseptic phlebotomy technique. If testing is delayed specimens can be stored at 2-8ºC for 7 days or for up to 3 months at or below –20ºC. Specimens must be free from bacterial contamination, fibrin, gross lipaemia and gross haemolysis.

PRECAUTIONS

1. The kit is for *in vitro* diagnostic use only.
2. Do not use kit past expiration date (see **Vial and Box Labels**).
3. Protective clothing should be worn when handling the reagents, such as disposable gloves and a laboratory coat.
4. The Rose Bengal reagent contains Phenol: Toxic (T) R24/25: Toxic in contact with skin and if swallowed. R34: Causes burns. S28.2: After contact with the skin, wash immediately with plenty of water. S45: In case of accident, seek medical advice immediately.
5. All the reagents must be allowed to reach 18-25ºC before use.
6. Materials used to produce the kit were tested at source and found to be negative for HIV 1+2 and HBsAg using approved microbiological tests. However, no known tests can guarantee that products derived from human or animal sources are free from infectious agents. Care must be taken in the use and disposal of each vial and its contents.

DISPOSAL OF KIT REAGENT AND DEALING WITH SPILLAGES

For information on disposal of kit reagent and decontamination of a spillage site see **Material Safety Data Sheets**, available on request.

CONTROLS AND ADVICE

1. It is recommended the Brucella Positive and Negative Controls be tested in parallel with each batch of tests. Tests must be considered invalid if controls do not show expected results.
2. All the reagents must be allowed to reach 18-25ºC before use.
3. Shake the reagents well before use to ensure homogeneity.
4. Do not interchange components between different kits.
5. Use of kit and interpretation of results must be carried out by properly trained and qualified personnel in accordance with the requirements of country where kit is in use. The user must determine the suitability of the kit for use in other techniques.

KIT COMPONENTS SUPPLIED

1) Rose Bengal Reagent (2.5 mL): Brucella abortus suspension, strain S99, in lactate buffer 1 mol/L, phenol 5 g/L, Rose Bengal, pH 3.6.
2) Positive Control (Red cap, 1 mL): Animal serum with an anti-Brucella antibody concentration ≥ 50 IU/mL and a preservative.
3) Negative Control (Blue cap, 1 mL): Animal serum and a preservative.
4) Pipette-Stirrers.
5) Reusable Agglutination Slide.

MATERIALS AND EQUIPMENT REQUIRED

- Mechanical rotator with adjustable speed of 80-100 rpm.
- Pasteur and Graduated Pipettes.
- Vortex mixer.

RECOMMENDED QUALITATIVE TECHNIQUE

1. Allow the reagents and samples to reach room temperature. The sensitivity of the test may be reduced at low temperatures.
2. Place 50 µL of the sample and one drop each of the Positive and Negative controls into separate circles on the slide test.
3. Mix the Rose Bengal reagent vigorously or on a vortex mixer before using and add one drop next to the sample to be tested.
4. Mix the drops with a stirrer, spreading them over the entire surface of the circle. Use different stirrers for each sample.
5. Place the slide on a mechanical rotator at 80-100 r.p.m. for 4 minutes. False positive results could appear if the test is read after 4 minutes.

INTERPRETATION OF QUALITATIVE RESULTS

1. **Positive:** Visible agglutination constitutes a positive result and within the accepted limitations of the test procedure, indicates a level of anti-Brucella antibodies in the specimen ≥ 25 IU/ml.
2. **Negative:** No visible agglutination in a milky liquid constitutes a negative result and within accepted limitations of test procedure, indicates a level of anti-Brucella antibodies in the specimen < 25 IU/ml.

RECOMMENDED SEMI-QUANTITATIVE TECHNIQUE

1. The semi-quantitative test can be performed in the same way as the qualitative test using dilutions of the serum.
2. Make doubling dilutions of serum specimen in 9 g/L saline as follows:

Dilution	Serum	Saline
1/2	100 µl undiluted serum	100 µl
1/4	100 µl 1/2 diluted serum	100 µl
1/8	100 µl 1/4 diluted serum	100 µl
1/16	100 µl 1/8 diluted serum	100 µl

3. Test the specimen dilutions in the same way as for the quantitative technique above.
4. Agglutination of the sera indicates:

Dilution	Brucella antibodies (I.U/ml)
1/2	50 (25 x 2)
1/4	100 (25 x 4)
1/8	200 (25 x 8)
1/16	400 (25 x 16)

5. Normal levels of anti-Brucella antibodies in adults is <25 IU/ml.

RESULTS

The titre is expressed as the reciprocal of the highest dilution showing macroscopic agglutination: e.g. if this occurs in dilution 1/8, the titre is (8 * 25 IU/mL) = 200 IU/mL.

STABILITY OF THE REACTIONS

Slide tests should be interpreted immediately after the 4-minute rotation period to avoid the possibility that a negative result may be incorrectly interpreted as positive due to drying of the reagent.

LIMITATIONS

1. Diagnosis should not be solely based on the results of the Rose Bengal method but also should be complemented with a clinical examination.
2. Hemoglobin (≤ 10 g/L), Rheumatoid factors (≤ 300 IU/mL) and lipemia (≤ 10 g/L), do not interfere. Bilirubin (≥ 2.5 mg/dL) interferes. Other substances may interfere[4].
3. False positive or false negative results may also occur due to:
   a) Contamination of test materials
   b) Improper storage of test materials or omission of reagents
   c) Deviation from the recommended techniques

SPECIFIC PERFORMANCE CHARACTERISTICS

1. The kit has been characterised by all the procedures mentioned in the **Recommended Techniques**.
2. The Rose Bengal sensitivity is calibrated against the 2º International WHO Preparation of anti-Brucella abortus from NIBSC (UK).
3. Analytical sensitivity: 25 (±5) IU/mL, under the described assay conditions.
4. Prozone effect: No prozone effect was detected up to 1000 IU/mL.
5. Diagnostic sensitivity: 100 %.
6. Diagnostic specificity: 98 %.

DISCLAIMER

1. The user is responsible for the performance of the kit by any method other than those mentioned in the **Recommended Techniques**.
2. Any deviations should be validated prior to using established laboratory procedures.

BIBLIOGRAPHY

1. Young E J. Clinical Infectious Diseases 1995; 21: 283-290.
2. Alton GC. Techniques for Brucellosis Laboratory INRA Paris, 1988.
3. Ariza J. Current Opinion in Infectious Diseases 1996; 9: 126-131.
4. Young DS. Effects of drugs on clinical laboratory test, 4th ed. AACC Press, 1995.

AVAILABLE KIT SIZES

Kit Size	Catalogue Number
50 Tests Per Kit	155050A

For the availability of other sizes, please contact:

Lorne Laboratories Limited
Unit 1 Cutbush Park Industrial Estate
Danehill
Lower Earley
Berkshire RG6 4UT
England
Tel: +44 (0) 118 921 2264
Fax: +44 (0) 118 986 4518
E-mail: info@lornelabs.com

TABLE OF SYMBOLS

LOT	Batch Number	IVD	*In-vitro* Diagnostic
REF	Catalogue Reference		Store At
	Expiry Date		Manufacturer
	Read Pack Insert		

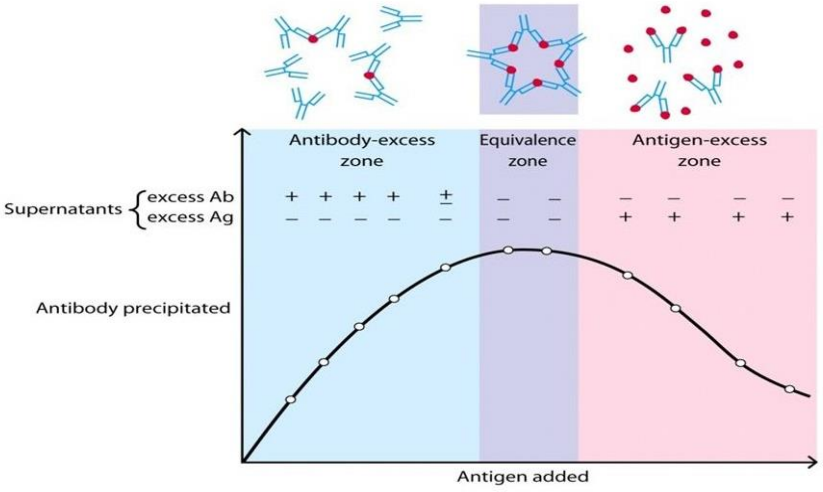
Precipitation Reactions

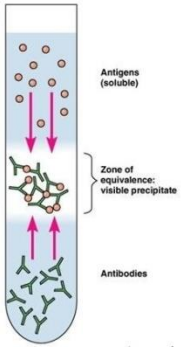
The reaction which occurs when specific antibody combines with soluble antigen in medium (solution or gel) to form a lattice that develops into a visible precipitate. **Antibodies** that aggregate soluble antigens are called **precipitins** while **antigen** known as **precipitinogen**. Formation of the **visible precipitate** (Ag- Ab) complex insoluble occurs more slowly and often takes a day or two to reach completion**.** Detection may be qualitative or quantitative. Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

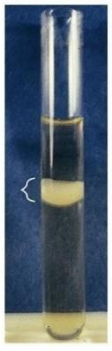
- The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.
- The antigen must be either bivalent or multivalent.

A precipitation curve for a system of one antigen and its antibodies. The amount of antibody precipitated versus increasing antigen concentrations reveals **three zones:**

1) **Zone of antibody excess (prozone):** in which precipitation is inhibited and antibody not bound to antigen can be detected in the supernatant.
2) **Equivalence zone**: antibody and antigen form large insoluble complexes and precipitation
3) **zone of antigen excess (postzone):** in which precipitation is inhibited and antigen not bound to antibody can be detected in the supernatant.







Type of Precipitation Reactions

1- Precipitation reactions in solution:

A quantitative precipitation reaction can be performed by placing a **constant amount of antibody** in a series of tubes and adding **increasing amounts of antigen** to each tubes. The complexes Ag-Ab, when formed, will precipitate in a solution resulting in a turbid or cloudy appearance that can be detected by measures turbidity or cloudiness of a solution by measuring the amount of light passing through the solution **(Turbidimetry**) or by measuring the amount of scattered light from sample **(Nephelometry).** Potting the amount of precipitate Ab against increasing antigen concentrations yields a precipitin curve. Example:

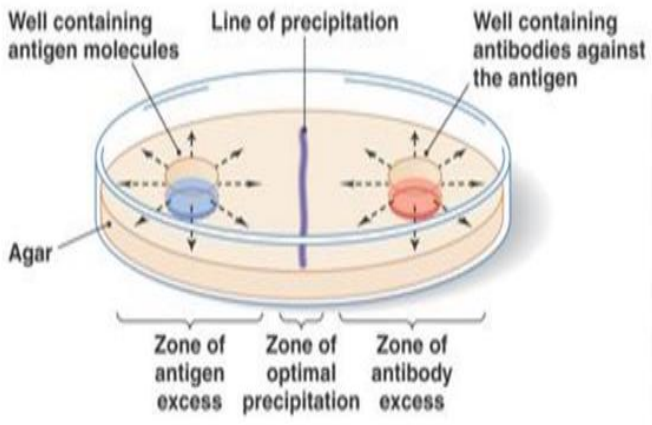
1) Test for anthrax diagnosis.

2) Test for syphilis.

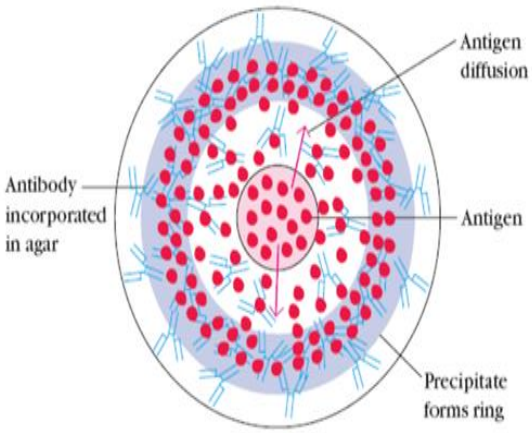
2- Precipitation reactions in gels (Immunodiffusion):

Immune precipitates can form not only in solution but also in an agar matrix (semisolid medium), the immunodiffusion reactions can **be used to** determine relative concentrations of antibodies or antigens ,to compare antigens or to determine the relative purity of an antigen preparation. They are two types of immunodiffusion :

A) Double immunodiffusion

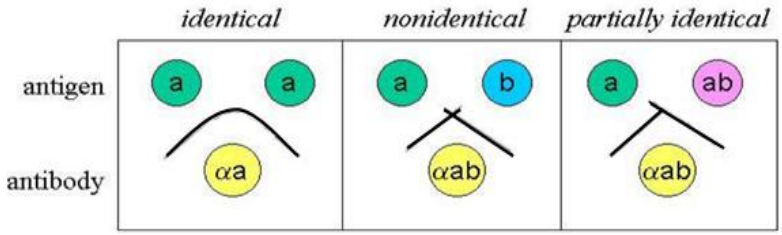


B) Single immunodiffusion



A) Double immunodiffusion (Ouchterlony method):

Antigen and antibody are placed in different wells in agar and allowed to diffuse toward each other in agar and form visible precipitation line at the points of optimal concentrations, whereas no visible precipitate forms in regions of antibody or antigen excess. This method is used to determine whether antigens are related: identical , non –identical & partially identical.



B) Single immunodiffusion (Radial immunodiffusion):

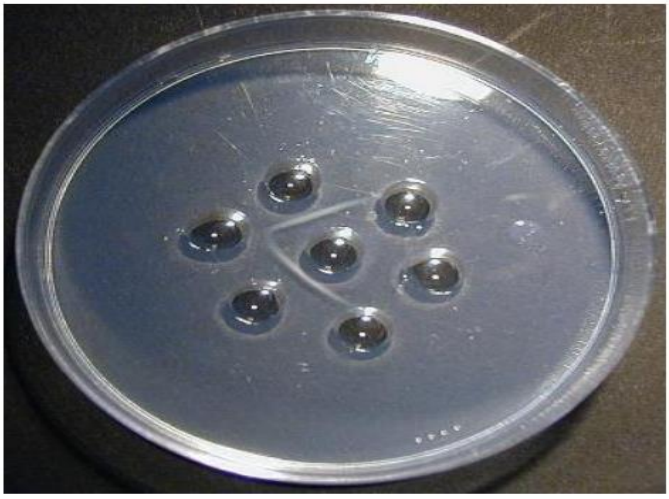
when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible ring precipitation will form & depending on the concentration of the antigen . visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. This method using for measurement of immunoglobulins IgA, IgG, IgM and complement C3& C4.

Procedure (1): Ouchterlony method

1. Dissolve agarose gel powder (20 g/l) in boiling phosphate buffered saline PBS, pH 7.4, after cool 50^{o}C then pour into petri dishes, thickness of less than 1mm.
2. Make holes by use the wide end of a Pasteur pipette to create several wells in the agarose. one central and other surrounded it.
3. Antibody is added to the central well, antigens are added to the outer wells.
4. Cover each plate (do not invert), and seal it around the edges with parafilm. Incubate 48 hours at room temperature
5. Antibody and antigen will diffuse through the agarose. When antibody meets with its specific antigen at their equivalent zone, the precipitation reaction occurs. Antibody-antigen precipitates in agarose appear as a **light white band** between the antibody and the antigen wells.

6. The position of the bands formed between the antigens allows for comparison of the antigens to each other.

The result of Ouchterlony method



Procedure (2): Radial immunodiffusion

We use the supply of kit C3 or C4 as radial immunodiffusion to appear ring precipitation :

1) Leave the dish after removing the lid and for 5 minutes at room temperature
2) add5 µl of each sample(serum) to the well in the dish and return the lid of the dish after placing the sample and put a damp filter paper and leave at room temperature for 48 hours.
3) After 48 hours, the precipitation ring appeared and can measured by a special lens called optical reader.
4) Calculation of the concentration (C3 and C4) by reference to the standard table attached to the kit by adopting the precipitation ring diameter.

The result of Radial immunodiffusion

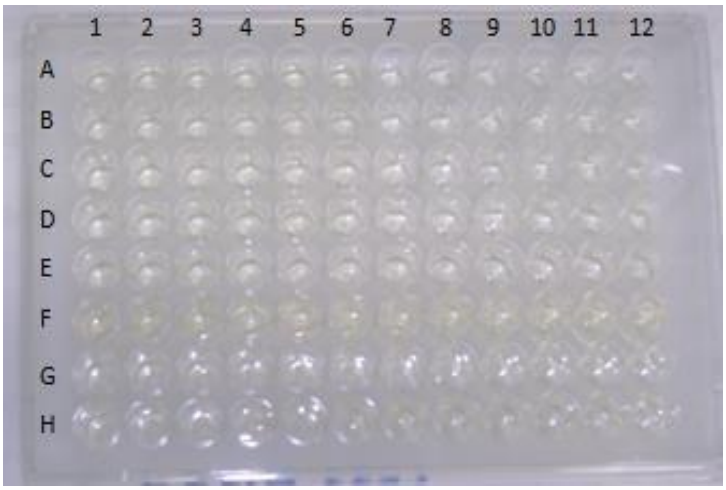
ELISA

- ELISA technique: (referred to as **E**nzyme **L**inked **I**mmunosorbent **A**ssay) is an immunological assay commonly used to measure **antibodies, antigens, proteins** and **glycoproteins** concentration in biological samples. Some examples include: diagnosis of bacteria ,viruses, hormones, and measurement of cytokines or soluble receptors in cell supernatant or serum.
- **Principle :-** ELISA reaction is combine the specific antibody/antigen complexes to produce a signal in present a labeled enzymes for detect the reaction which has occurred that can be measured and related to the concentration of a Ab or Ag in solution.
- **Why using the ELISA technique? Advantages of ELISA?**

1. Some antigen & antibody reactions not detected by precipitation or agglutination due to their small size or low concentrations.
2. Easy to perform, quick procedures & Used many sample at same time.
3. High sensitivity and specificity 99%.
4. No radiation hazards occur during labeling or disposal of waste.
5. It can be used on most type of biological samples like plasma, serum, urine & cell extracts.

Equipment:

❖ **Solid Phase:** Usually a micro-well plate is flat bottom polystyrene plate, contains 8 x 12 wells holding 350 µL each. It found in ELISA Kits. The 96 well plate is labeled (1,2,3…12 & A,B,C,…H) and the first wells are used to draw the standard curve.



❖ **Multipipette:**

An 8-channel or 12-channel pipette is a good help for ELISA procedure & washing

❖ **Washing Device:**

manually operated washing devices. may be of use particularly when there is a risk that the samples tested in ELISA contain infectious material, so must be collected for subsequent disinfection.

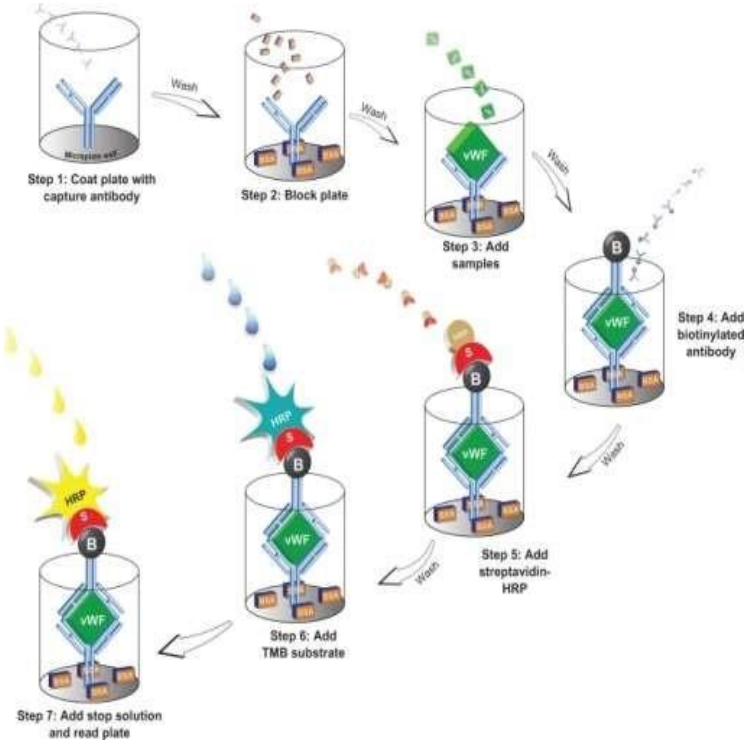
❖ **ELISA- plate Reader**

The optical density of each wells is estimated immediately after the previous step at specific wavelength by a micro-plate reader The intensity of the color gives an indication of the amount of antigen or antibody

❖ **Printer:** for prints the results**.**

The process in ELISA technique:

1) **Antibody capture OR Antigen:** the coating antibody on solid phase, It is prepared by the manufacture company for kit.
2) **Adsorption:** The process of adding sample contains an antigen or antibody, may be diluted in buffer, so it attaches to the solid phase and incubated it.
3) **Washing:** The simple flooding & emptying of wells with a buffered solution to separate bound from un-bound reagents in ELISA.
4) **Blocking :** The other sites are not bound in each well , then bound by add the casein or bovine serum albumin, to block or prevent other proteins in the test sample from adhering.
5) **Enzyme conjugate:** An enzyme that is attached irreversibly to an antibody or antigen. Enzymes occur naturally and catalyze biochemical reactions. e.g: Horseradish peroxidase, Alkaline phosphatase &beta galactosidase. An enzyme conjugated with an antibody reacts with colorless substrate to generate a colored product.
6) **Chromogen substrate:** A chemical material alters color as a result of an enzyme interaction with substrate e.g. Trimethyl benzidine (TMB).
7) **Stopping:** The process of stopping the action of an enzyme on a substrate.
8) **Reading:** The intensity of color is measured by a micro-plate reader.

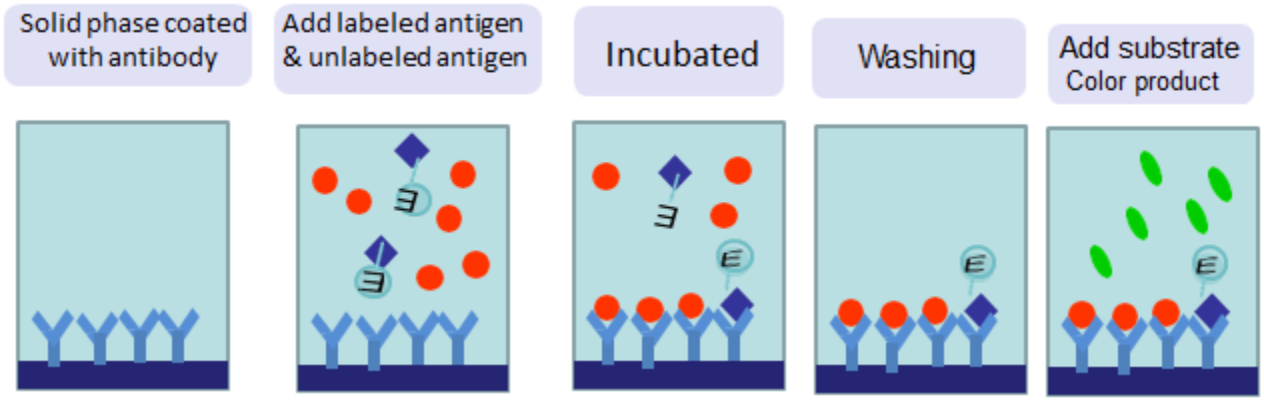


The ELISA technique is divided into:

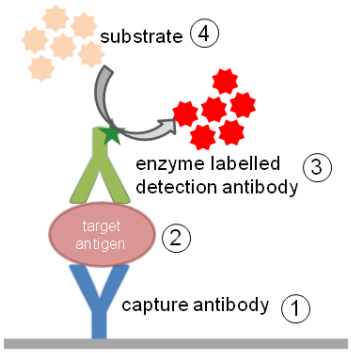
1. Competitive ELISA
2. Sandwich ELISA
3. Indirect ELISA.

1) Competitive ELISA

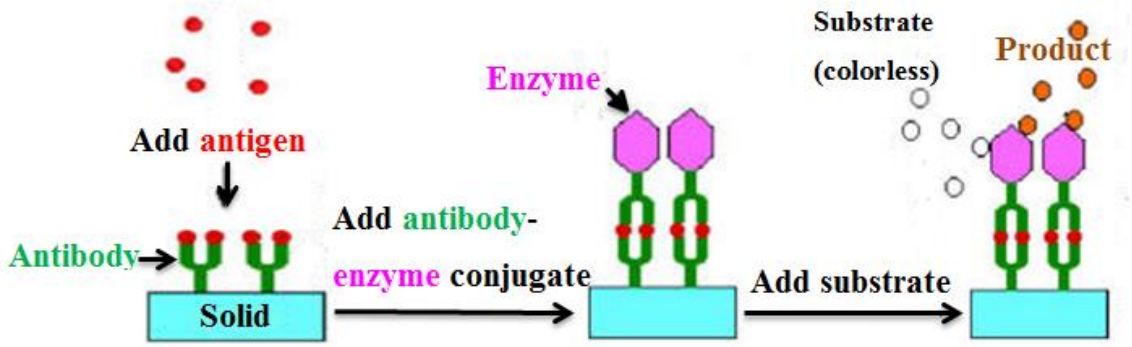
- In a competitive ELISA for antigen, Enzyme labeled antigen competes with unlabeled patient antigen for antibody sites on solid phase.
- After incubation and washing to remove unbound reactants.
- A substrate for the enzyme used to label the antigen is added to the system, which causes color change.
- The intensity of which can be measured by spectrophotometry
- Results: Color intensity is directly proportional to the amount of bound labeled antigen, which in turn is inversely proportional to the concentration of unlabeled antigen added to the mixture.
- More patient antigen bound, less color while if little or no patient antigen bound, dark color.
- Used to measure small antigens such as Insulin and Estrogen.



2- Sandwich ELISA



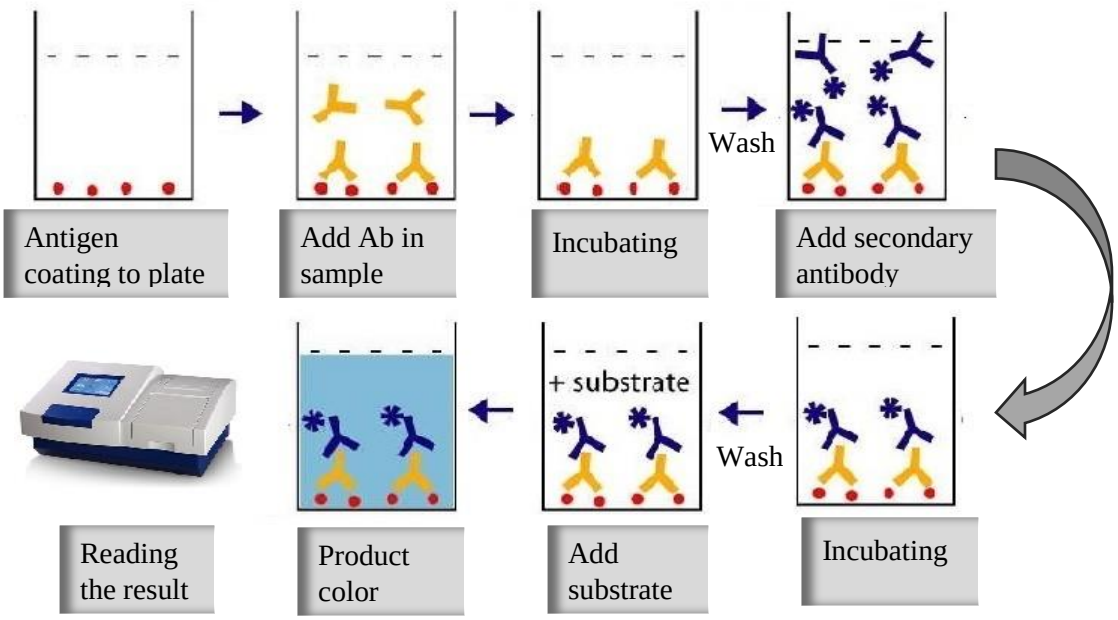
- Sandwich" Technique ELISA (also called **direct ELISA**)
- Antibody bound to solid phase (Micro-well plates).
- Add patient sample with target antigen.
- Antigen will bind to antibody bound to solid phase.
- Add enzyme labeled antibody directed against the antigen.
- Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
- Add substrate, which is converted by the enzyme into a coloured product.
- Measure the absorbency of the plate wells to determine the presence and quantity of antigen.

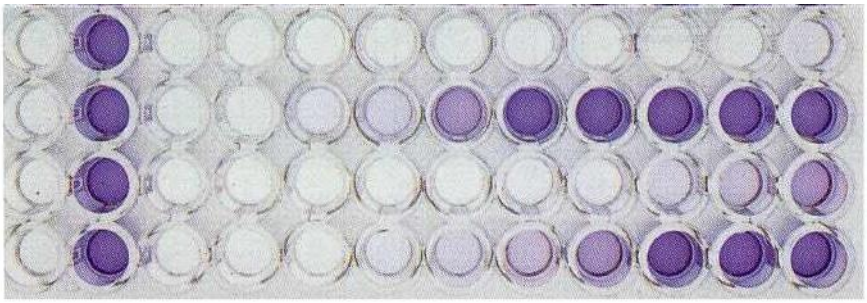


3-Indirect ELISA

- ❖ The antigen coating to each well of ELISA plate.
- ❖ Then the serum is added, which contains a mixture of the serum antibodies, of unknown concentration, some of which may bind specifically to the test antigen that is coating the well.

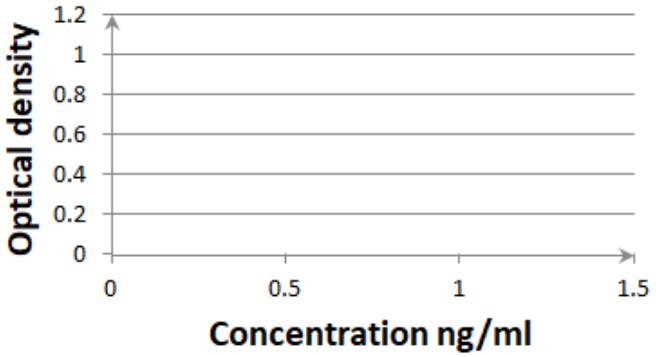
- a secondary antibody is added, which will bind to the antibody bound to the test antigen in the well. This secondary antibody often has an enzyme attached to it.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The color change shows that secondary antibody has bound to primary antibody.
- The higher the concentration of the primary antibody that was present in the serum, the stronger the color change. Often a spectrometer is used to give quantitative values for color strength.



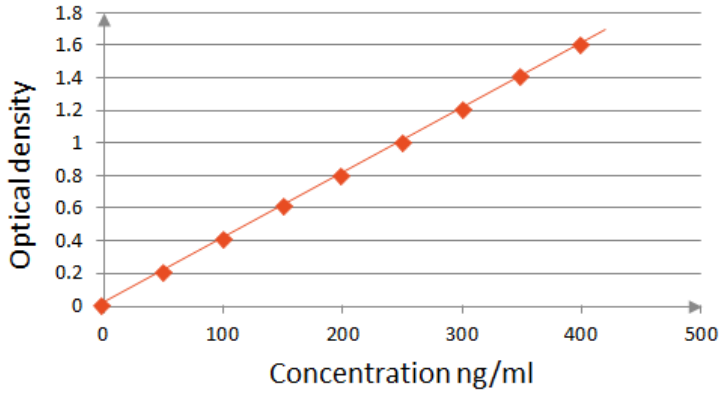


ELISA result:

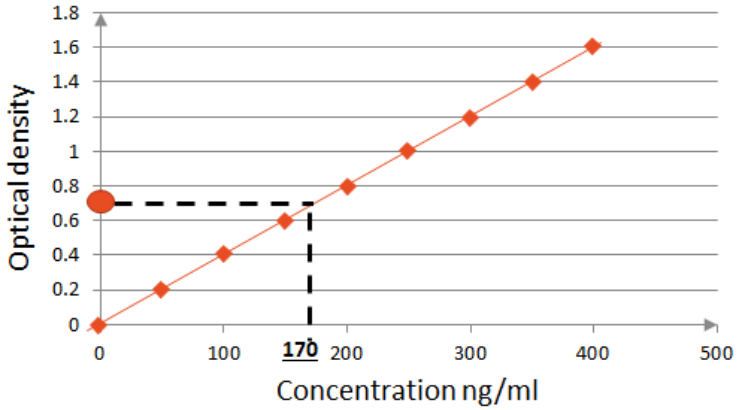
After reading the results the standard curve is drawn were the **Concentration** is blotted on the X-axis and the **Optical density** on the Y-axis:



The standards concentrations is specified on the x-axis and the reading optical density of each standard is specified on the y-axis and the **standard curve** is drawn:



This standard curve is used to determine the **unknown concentration** of each sample by finding the opposite concentration to the optical density of samples:



The quality control sample concentration is determined from the standard curve and if the result is in the range given by the kit manufacturer the results could be accepted.

Disadvantages of ELISA:

1) Measurement of enzyme activity can be more complex than the measurement of activity of some type of radioisotopes.
2) Enzyme activity may be affected by plasma constituents.
3) Kits are not cheap.
4) Very specific to particular antigen but won't recognize other antigens.
5) False positive/ negative possible, especially with mutated/ altered antigen.

Application of ELISA

1. Presence of antigen or antibody in a sample can be evaluated.
2. Determination of serum antibody concentrations in a virus test .
3. Measurement of hormone levels e.g. :HCG (as a test for pregnancy) , TSH, T3 and T4 (for thyroid function).
4. Used in food industry when detecting potential food allergens.
5. Detecting illicit drugs.
6. 6.Detection of some infection e.g. HIV, bird flu, common colds, Hepatitis C Hepatitis B, Cholera, Toxoplasma, syphilis and chlamydia. etc.

IL-27

animal labratory Report

Date: 05/13/2015 Time: 13:27:34

Well	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S2	001	009	017	025	033	041	049	057	065	073	081
Abs	0.162	0.149	0.074	0.040	0.102	0.257	0.041	0.051	0.060	0.084	0.088	0.091
QTA	62.500	48.035	-36.541	-75.206	-4.837*	95.989	-73.788	-62.490	-52.110	-25.991	-21.545	-17.329*
QLA	Standar	Pos+	Neg—	Neg—	Neg—	Pos+	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—

Well	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S1	002	010	018	026	034	042	050	058	066	074	082
Abs	0.135	0.165	0.031	0.059	0.128	0.060	0.039	0.029	0.062	0.028	0.052	0.035
QTA	31.250	63.603	-85.335	-54.054	24.300	-52.275	-75.858	-86.979	-50.353	-88.682	-61.569	-80.280*
QLA	Standar	Pos+	Neg—	Neg—	Pos+	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—

Well	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S3	003	011	019	027	035	043	051	059	067	075	083
Abs	0.339	0.098	0.059	0.126	0.321	0.034	0.446	0.019	0.282	0.039	0.075	0.028
QTA	125.000	-9.730*	-53.924	22.219	118.628	-81.761	177.540	-98.365	105.015	-76.463	-36.123	-88.771*
QLA	Standar	Neg—	Neg—	Pos+	Pos+	Neg—	Pos+	Neg—	Pos+	Neg—	Neg—	Neg—

Well	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S4	004	012	020	028	036	044	052	060	068	076	084
Abs	0.594	0.056	0.258	0.040	0.045	0.022	0.168	0.044	0.040	0.028	0.079	0.018
QTA	250.000	-57.133	96.259	-74.726	-69.594	-95.377	64.490	-70.631	-74.676	-88.543	-31.159	-99.494*
QLA	Standar	Neg—	Pos+	Neg—	Neg—	Neg—	Pos+	Neg—	Neg—	Neg—	Neg—	Neg—

Well	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S5	005	013	021	029	037	045	053	061	069	077	085
Abs	0.917	0.195	0.052	0.035	0.091	0.120	0.182	0.052	0.051	0.027	0.051	0.071
QTA	500.000	74.076	-61.947	-80.471	-17.215	15.196	69.623	-60.958	-62.714	-89.952	-62.674	-40.141*
QLA	Standar	Pos+	Neg—	Neg—	Neg—	Pos+	Pos+	Neg—	Neg—	Neg—	Neg—	Neg—

Well	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S6	006	014	022	030	038	046	054	062	070	078	086
Abs	1.998	0.138	0.078	0.017	0.201	0.083	0.070	0.366	0.109	0.098	0.027	0.039
QTA	1000.000	34.724	-31.745	-101.007	6.082	-26.630	-41.048	138.399	2.478	-9.692*	-89.113	-76.337*
QLA	Standar	Pos+	Neg—	Neg—	Pos+	Neg—	Neg—	Pos+	Pos+	Neg—	Neg—	Neg—

Well	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
Program	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample	S7	007	015	023	031	039	047	055	063	071	079	087
Abs	2.390	0.239	0.496	0.028	0.026	0.019	0.082	0.046	0.043	0.063	0.025	0.030
QTA	2000.000	89.666	202.187	-88.217	-91.079	-98.953	-28.250	-68.698	-71.069	-48.828	-91.860	-86.050*
QLA	Standar	Pos+	Pos+	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—	Neg—

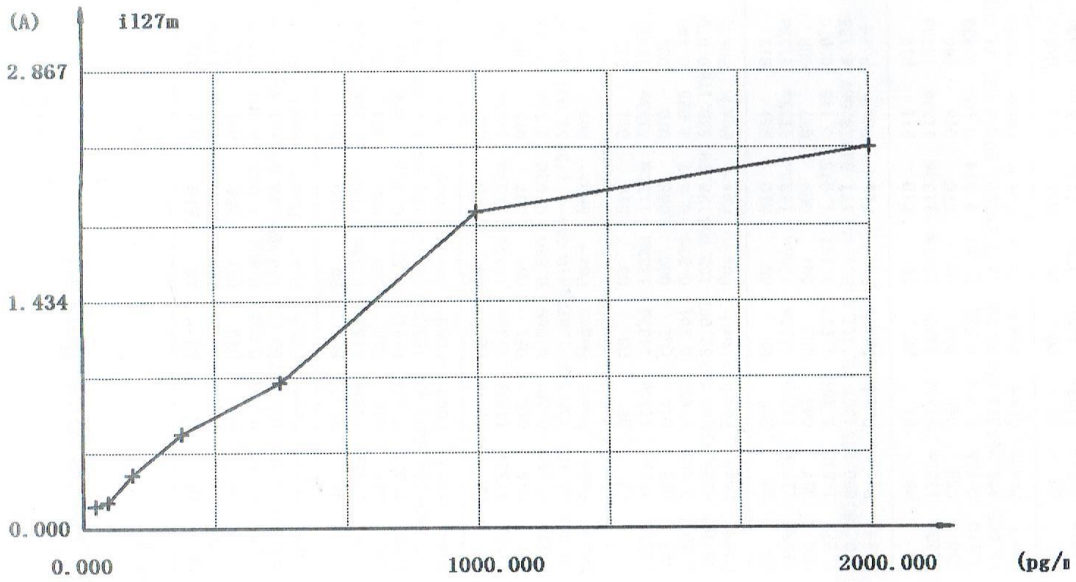
Well	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Program		il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m	il27m
Sample		008	016	024	032	040	048	056	064	072	080	088
Abs		0.167	0.060	0.032	0.529	0.500	0.199	0.265	0.271	0.123	0.130	0.162
QTA		63.970	-51.979	-83.806	218.185	204.075	75.495	98.722	101.000	18.510	26.093	61.575
QLA		Pos+	Neg—	Neg—	Pos+	Pos+	Pos+	Pos+	Pos+	Pos+	Pos+	Pos+

Operator: Checker:
Print date: 05/13/2015

IL-27

IL-27

Standard curve



Program: il27m Caculation: Curve Wavelength(nm): 450/630

Standard sample	#1	#2	#3	#4	#5	#6	#7
Concentration(pg	31.250	62.500	125.000	250.000	500.000	1000.000	2000.000
Abs(A)	0.135	0.162	0.339	0.594	0.917	1.998	2.390

Operator: Checker:
Print date: 05/13/2015