Faculty of Science

Laboratory Manual

Basic Immunology

Bachelor of Biotechnology (Hons.)

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Basic Immunology

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LINCOLN UNIVERSITY COLLEGE
FACULTY OF SCIENCE (DEPARTMENT OF BIOTECHNOLOGY)
LABORATORY SAFETY RULES

The following rules must be obeyed by all students in the science laboratory of the faculty. Wilful or repeated in advertent non-compliance may result in dismissal or suspension from the laboratories

- No entry without permission:
  - Outsiders are not allowed to enter the laboratory without permission.
  - No student is allowed to enter the laboratory unless permission has been given by a laboratory assistant or a lecturer.

- At work in the laboratory:
  - No experiment may be attempted without the knowledge and permission of a lecturer.
  - Students must wear shoes in the laboratory. Students wearing slippers or sandals are not allowed to work in the laboratory.
  - Lab coat must be worn at all times during practical work in the laboratory.
  - Do not mouth pipette chemicals.
  - Do not eat or smoke in the laboratory.
  - Do not taste any chemicals, including dilute solutions. If any acid or alkali accidentally enters your eyes or mouth, wash immediately with plenty of tap water. Inform your lecturer, and seek medical attention if necessary.
  - Paper should be used to light up the Bunsen burners.
  - Used match sticks, filter papers, and other solid waste must never be thrown into the sinks. They must be thrown into the dustbins provided. Lighted match sticks and smoldering materials must be extinguished with tap water before thrown in to the dustbins.
  - Any equipment broken or damaged must be reported to the laboratory assistant.

- Before leaving the laboratory:
  - All the equipment and benches must be cleaned at the end of each practical session.
  - Wash hands and arms with soap and water before leaving the laboratory.
  - No student is allowed to take away any chemicals, equipment or other property of the laboratory.

INTRODUCTION

1. The Scientific Method

- Making observations
- Generating hypotheses
- Making predictions
- Designing and carrying out experiments
- Constructing scientific models

2. Practical Exercises

To get the most out of the practical exercises, you need to follow carefully the instructions given. These instructions have been designed to provide you with the experience in the following skills:

- Following instructors
- Handling apparatus
- Having due regard for safely
- Making accurate observations
- Recording results in an appropriate form
- Presenting quantitative results
- Drawing conclusions

3. Following Instructions

Instructions are provided in the order in which you need to carry them out. We would advise that before carrying out the instructions, you read through the entire exercise. This will help you to remember what you have learned.

Each practical exercise in the book begins with a few lines describing its purpose in most cases the following headings are also used:

- Procedure-numbered steps that need to be carried out.
- For consideration -some questions to help you think carefully about the results you have obtained.
- Materials-a list of the apparatus, chemicals and biological materials you need.

4. Handling apparatus

Biologists need to able to use many different types of apparatus, for example, photometers (to measure water uptake by plants), respirometers (to measure oxygen uptake or carbon dioxide production), Petri dishes (for plating out bacteria and other microorganisms) and the light microscope (to magnify specimens). Many of the practical exercises are designed to help you derive the maximum benefit from a piece of apparatus.

5. Having Due Regard for Safety

Surveys have been shown that science laboratories are among the safest places to be. Nevertheless, this is no cause for complacency.

- Always move slowly and carefully in a laboratory.
- Never put your fingers in your mouth or eyes after using chemicals or touching biological specimens until you have washed your hands thoroughly with soap and warm water, and dried them.
- Make sure glass objects (e.g, thermometers, beakers) cannot roll off tables or be knocked onto the floor.
- Wear safely goggles whenever there is a risk of damage to the eyes.

<u>Situations of risk include:</u>

- Heating anything with a Bunsen burner (even heating water has its dangers')
- Handling many liquids, particularly those identified as corrosive, irritant, toxic or harmful

- Handling corrosive or irritant solids
- Some dissection work
- Allow Bunsen burners, tripods, gauzez and beakers to cool down before handling them.
- Never allow your own body fluids (especially blood and saliva) to come into contact with someone else, or theirs into contact with you.
- Keep long hair tied back and do not wear dangly earrings.
- Do not allow electrical equipment to come into contact with water.
- If you are unsure how to carry out a scientific procedure, ask.
- Make sure you understand why you are going to do something before you do it.
- Wear a lab coat when using chemicals or handling any biological specimens.
- Follow exactly agreed procedures with regard to cuts, burns, electric shocks and other accidents (e.g. with chemicals).
- Follow exactly all specific safely instructions given in this book or provided by your teacher for particular practical exercises (e.g. use of gloves, disinfection)

With practice, these procedures should become second nature to you. They will enable you to carry out practical work in safety.

6. Making Accurate Observations

In most cases the practical exercise will make it clear what you need to observe, e.g. the time taken for a certain volume of gas to be evolved or the width of a sample cells. Ensure that you know how to use any necessary equipment before starting practical. Think carefully about the precision with which you will make your observations.

7. Recording Results in an Appropriate Form

Results can be recorded in various ways. Often it is helpful to record raw data in a table. Most data will be in the form of numbers, i.e. they will be quantitative data (also known as numerical data). However, some data, e.g. flower colour, will be qualitative data.

One form in which some biological findings can be recorded is a drawing. You don't need to be professional artist to make worthwhile biological drawings. If you follow the following guidelines, a drawing can be of considerable biological value:

- Ensure that your completed drawing will cover at least a third of A4 page.
- Plan your drawing so that the various parts are is proportion and will not be drawn too small. Small marks to indicate the length and breadth of the drawing are a great help in planning and a faint outline can be rapidly drawn to show the relative positions of the parts.
- The final drawing should be made with clean, firm lines using a sharp HB pencil and, if needed, a good quality eraser (not a fluid).If important details are too small to be shown in proportion, they can be put in an enlarged drawing at the side of the main drawing.
- Avoid shading and the use of colour unless you are an excellent artist and they really help, for example when drawing soil profiles.
- When drawing structures seen with the naked eye or hand lens, use two lines to delineate such things as blood vessels and petioles. This will help you to indicate the relative widths of such structures.
- When drawing low power plan drawings from the light microscope, do not attempt to draw individual cells-just different tissues.
- When drawing plant cells at high power under the light microscope, use two lines to indicate the width of cell walls, but a single line to indicate a membrane.
- Always put a scale on each drawing.

8. Presenting Quantitative Results

Presentation of data is all about using graphs or other visual means to make it easier to see what your results tell you. The following four ways of presenting data are the most frequently used in biology: line graphs, bar charts, histograms and scatter graphs (Figure 1).

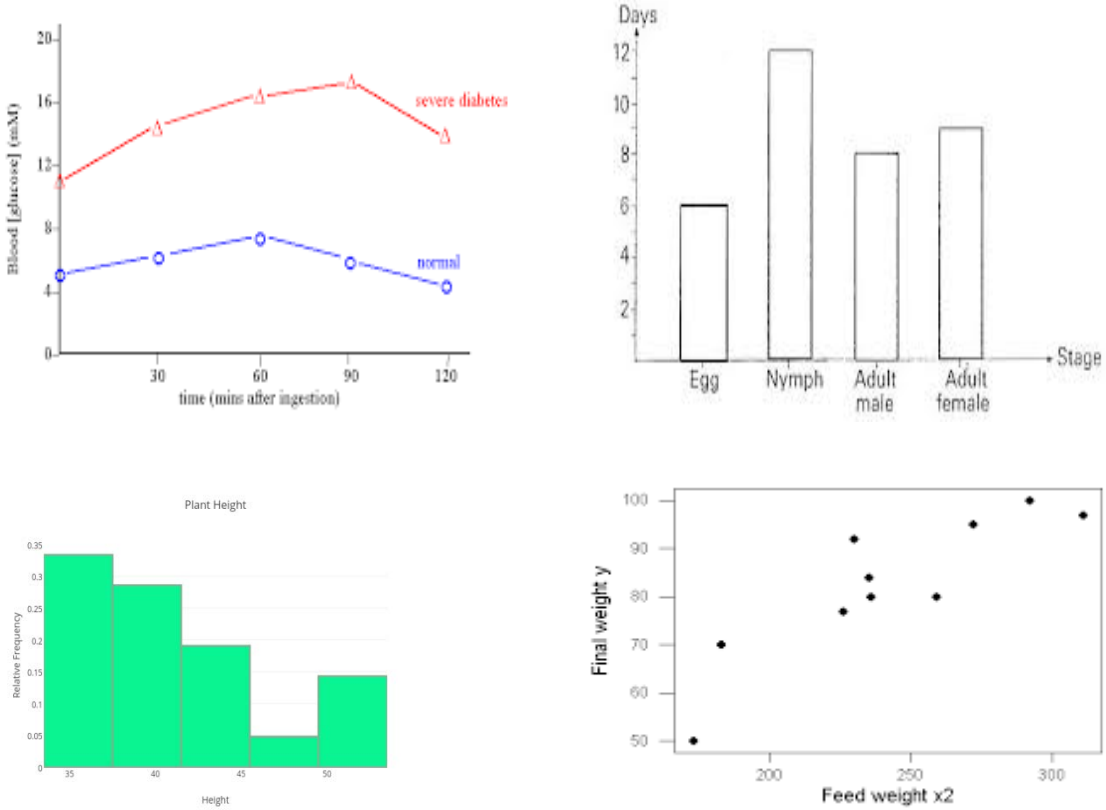


Figure 1: Line graphs, bar charts, histograms and scatter graphs

9. Drawing Conclusions

Finally, you will need to draw conclusions. If your practical exercise has involved the testing of a hypothesis, for example that the enzyme pepsin works better at low pH than in neutral or alkaline conditions, your conclusion should indicate whether the hypothesis has been refuted (i.e. shown not to be the case) or supported. Of course, even if your hypothesis has been supported, it doesn't mean that it has been confirmed with 100% certainty- in other words it isn't proved. Science proceeds more by showing that certain ideas are wrong than by showing that others are right (think about that!). Your conclusion might therefore include further ways of testing the original hypothesis, or might raise new possibilities to be investigated.

Often you will only be able to arrive at your conclusions after statistically analysing your data.

10. Writing a Scientific Lab Report

Title

- Communicate the subject investigated in the paper.

Introduction

- State the hypothesis.
- Give well-defined reasons for making the hypothesis.
- Explain the biological basis of the experiment.
- Cite sources to substantiate background information.

- Explain how the method used will produce information relevant to your hypothesis.
- State a prediction based on your hypothesis. (If the hypotheis is supported, then the results will be.)

Materials and Methods

- Use the appropriate style.
- Give enough detail so the reader could duplicate your experiment
- State the control treatment, replication and standardized variables that were used.

Results

- Summarize the data (do not include raw data).
- Present the data in an appropriate format (table or graph).
- Present tables and figures neatly so they are easily read.
- Label the axes of each graph completely.
- Give units of measurement where appropriate.
- Write a descriptive caption for each table and figure.
- Include a short paragraph pointing out important results but do not interpret the data.

Discussion

- State whether the hypothesis was supported or proven false by the results, or else state that the results were inconclusive.
- Cite specific results that support your conclusions.
- Give the reasoning for your conclusions.
- Demonstrate that you understand the biological meaning of your results.
- Compare the results, with your predictions and explain any unexpected results.
- Compare the results to other research or information available to you.
- Discuss any weaknesses in your experimental design or problems with the execution of the experiment.
- Discuss how you might extend or improve your experiment.

Conclusion

- Restate your conclusion.
- Restate important results.

Literature Cited

- Use the proper citation form in the text.
- Use proper citation form in the Literature Cited section.
- Refer in the text to any source listed in this section.

Acknowledgement

- State any appropriate acknowledgement that you think is necessary.

Practical 1
Title: Agglutination reaction

Objective:
After completing the practical, you will be able:
1. To demonstrate the agglutination reaction through blood grouping test (blood group and Rh factor)

Introduction:

Agglutination is the interactions between insoluble particles (e.g. intact bacteria and cells) or soluble antigens that attached to particles and their corresponding specific antibodies that result in some visible agglutinates if given enough time and the proper concentration of electrolyte. The agglutination of RBCs (hemagglutination) is a result of an immune reaction between the RBCs and antibodies against the corresponding blood type. Hemagglutination caused by antibodies is detected either by human eyes or by imaging techniques in conventional blood typing methods (Ashiba *et al.*, 2015).

Blood grouping is the classification of blood based on the presence or absence of two inherited antigenic substances on the surface of red blood cells (RBCs). The ABO and Rh are the major clinically significant and the most important of all the blood group systems. ABO and Rh(D) blood typing is also one of the most important tests that are performed prior to blood transfusion. The ABO blood group system was first discovered by Karl Landsteiner in 1900. The associated Anti A and Anti B antibodies usually belong to IgM class of immunoglobulins. The Rhesus system (Rh) is the second most important blood group system in humans. The most significant and immunogenic Rhesus antigen is the RhD antigen. The individuals carrying the Rh antigen are considered to have positive blood group whereas those individuals that lack this antigen are considered to have negative blood group.

Slide agglutination reaction is the direct agglutination carried out on the slides, by directly mixing the antibody with a particle antigen under the certain concentration of electrolyte. The result is positive when there is visible agglutinate, otherwise negative.

The ABO blood group antigens are O-linked glycoproteins in which the terminal sugar residues exposed at the cell surface of the red blood cells determine whether the antigen is A or B. Blood group A individuals have A antigens on RBCs and anti-B antibodies in serum. Similarly, blood group B individuals have B antigens on RBCs and anti-A antibodies in serum. Blood group AB individuals have both A and B antigens on RBCs and neither anti-A nor anti-B antibodies in serum. Whereas, blood group O individuals have neither A antigens nor B antigens, but possess both anti-A and anti-B antibodies in serum. The Rh antigens are transmembrane proteins in which the loops exposed on the surface of red blood cells interact with the corresponding antibodies.

Antigens	Antibodies	ABO Blood Group	Genotype
A	Anti B	A	A/A or A/O
B	Anti A	B	B/B or B/O
A and B	Neither Anti A nor Anti B	AB	A/B
Neither A nor B	Anti A, Anti B, Anti AB	O	O/O

Materials

- Human peripheral blood
- Standard sera: Anti-A, anti-B sera and Anti RhD
- Saline
- Slides, needles, cotton ball, etc.
- Reagents: 70% Alcohol/ Spirit

Procedure

1. Dangle the hand down to increase the flow of blood in the fingers.
2. Clean the fingertip to be pierced with spirit or 70% alcohol (usually ring or middle finger).
3. With the help of the sterile lancet, pierce the fingertip and place one drop of blood in each of the cavities.
4. Add one drop of antiserum into each cavity as shown below:

Blood drop	Blood drop	Blood drop
+	+	+
Anti A	Anti B	Anti RhD

5. Mix each blood drop and the antiserum using a fresh mixing stick.
6. Observe agglutination in the form of fine red granules within 30 seconds. Anti RhD takes slightly longer time to agglutinate compared to Anti A and Anti B.
7.

Results and Observation:

1. Record your group results in the table below:

Student Name	ID No.	Anti A	Anti B	Anti RhD	Blood Group

1. Paste a photograph of your blood grouping result in the space provided below:

Questions:

1. Is ABO blood compatibility enough for the safety of blood transfusion? Justify your response.
2. Describe the relevance of Rh factor.

Practical 2
Title: Complement fixation test

Objective:
After completing the practical, you will be able:
1. To determine whether the complement has been fixed using sheep RBCs and antibodies against sheep RBCs

Introduction:

The complement fixation test (CFT) is a common serological test which is used to detect the presence of specific antibodies or antigens to diagnose infections, particularly diseases caused by microbes that are not easily detected by standard culture methods (Li *et al*., 2016)

The complement fixation test was extensively used in syphilis serology after being introduced by Wasserman in 1909. Erythrocytes are used as the target cell, because complement-induced leakiness of the membrane can be visualized or measured calorimetrically as an increase in free hemoglobin.

In the presence of specific antibodies to an infectious agent, any complement in the system is bound, leaving no residual complement for reaction with antibodies to the erythrocytes. Thus, the presence of specific antibody is indicated by the absence of hemolysis.

It is the nature of the complement to be activated when there is formation of antigen-antibody complex. The first step is to heat the serum at 56°C to destroy patient's complement. A measured amount of complement and antigen are then added to the serum. If there is presence of antibody in the serum, the complement is fixed due to the formation of Ag-Ab complex. If no antibody is present then the complement remains free.

In the positive test: The available complement is fixed by Ag-Ab complex and no hemolysis of sheep RBCs occurs. So the test is positive for presence of antibodies. (Figure 1)

In the negative test: No Ag-Ab reaction occurs and the complement is free. This free complement binds to the complex of sheep RBC and it's antibody to cause hemolysis, causing the development of pink color. (Figure 1)

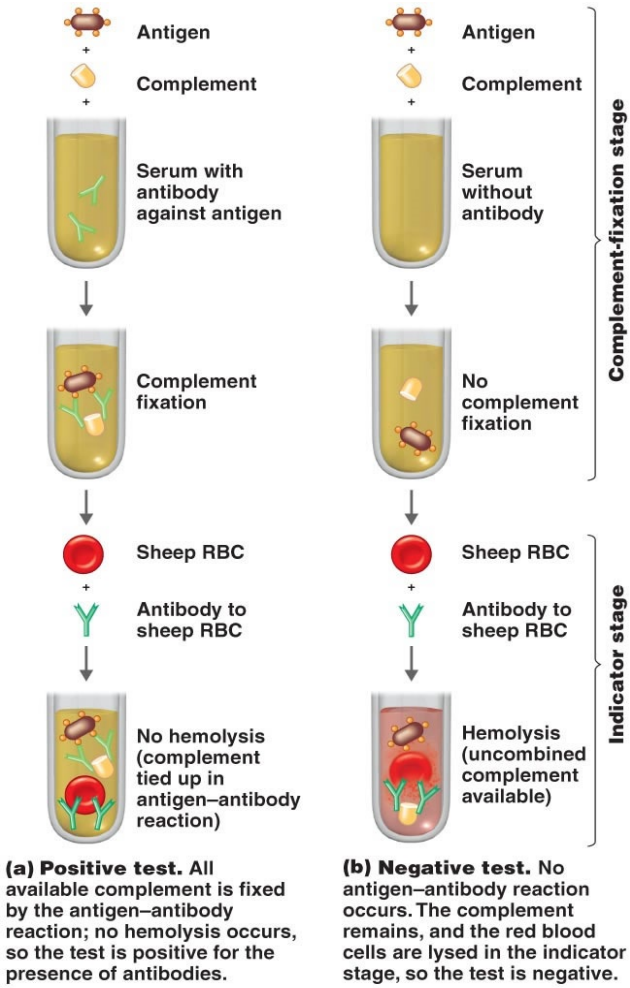


Figure 1: Positive and negative test

Materials:

- Sheep erythrocytes suspension (5% suspension of washed sheep RBCs)
- Hemolysin (rabbit anti-sheep red-cell antibody)
- Guinea pig complement, free of antibodies to the agent of interest (Note: Guinea pig is the commonest source of fresh complement)
- Barbital-buffered diluents
- Plastic microtitre plate
- Centrifuge adapter for microtitre plates
- Water bath for incubation of plates
- Color standards for judging hemolysis (prepared by lysing various concentrations of red cells)

Procedure:

Complement Fixation Test (CFT) consists of two stage:

Complement fixation stage:

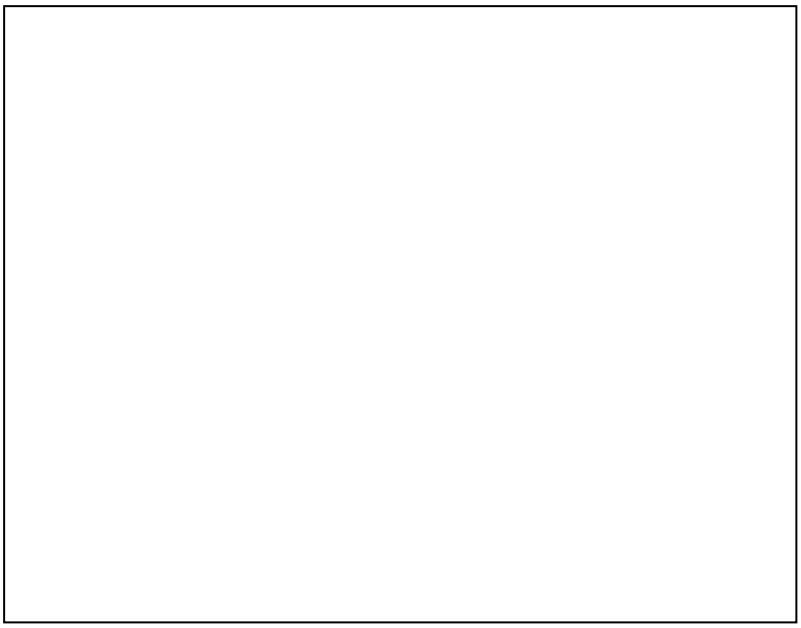
1. Heat the serum at 56°C for 30 minutes to destroy patient's complement.
2. Incubate a known antigen and inactivated patient's serum with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement

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.

Indicator Stage:

3. The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system including sheep erythrocytes suspension (5% suspension of washed sheep RBCs) and haemolysin
4. 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. There won't be any lysis of RBCs.
   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.

Results: Place the photographs of relevant result from your work:



Question:

1. Discuss the advantages and disadvantages of complement fixation test.

Practical 3

Title: Radial immuno-diffusion techniques

Objective:

After completing the practical, you will be able:

1. To determine the concentration of immunoglobulin (IgG) in a dry lab experiment

Introduction

Gel precipitation is an immunologic assay in which soluble antigen and antibody are allowed to diffuse through a gel medium. As the antigen and / or antibody diffuse from the point of application (usually a well cut in the agar) through the gel, their concentration decreases until they eventually arrive at their own zone of equivalence (optimal concentration of antigen and antibody) and forming visual precipitation within the gel.

Explanation of terms often applied to gel diffusion tests:

Diffusion - refers to the number reactants are moving through the gel. For instance, single-diffusion tests have only one reactant (usually the antigen) moving, while the other reactant is fixed in the gel. Double-diffusion testing has both of the reactants moving though the medium.

Dimension - refers to the direction of movement. Reactions in tubes essentially have only one effective dimension, up and down and are called single dimension. When a reactant is able to move both up and down as well as radially through a gel the term double dimension can be applied. Therefore using this criterion, there are four combinations or classification of reactions in gels:

1. single diffusion - single dimension
2. single diffusion - double dimension
3. double diffusion - single dimension
4. double diffusion - double dimension

Principle:

When the antigens and corresponding antibodies are allowed to react in gels or other mediums, they will diffuse toward one another, and at the point in which they meet in optimal proportions, they will form a visible precipitate (Burtis, Ashwood, & Bruns, 2012).

Ouchterlony gel diffusion (double diffusion, double dimension)

When an antigen solution is placed in a well / hole cut into an agar plate and the corresponding antibody is placed in an adjoining well, they will diffuse radially from their respective wells toward one another. When an optimal ratio of the two materials is reached, a visible line of precipitation is formed in the gel. The test provided *qualitative* identification of individual proteins.

Radial immunodiffusion (RID) [single diffusion, double dimension]

RID testing is used to obtain a *quantitative* level of an antigen when agar containing an appropriate antiserum (antibody is "fixed" in the agar) is poured in plates and carefully cut circular wells are removed. A series of three (3) standards containing known concentration of antigen are placed in three of the wells, while control and "unknown" samples are placed in other wells. As the antigen diffuses radially, a ring of precipitate will form in the area of optimal antigen - antibody concentration. A standard curve is prepared using the ring diameters of the standards versus their concentrations. This curve is then used to determine the concentration of the control and unknown samples.

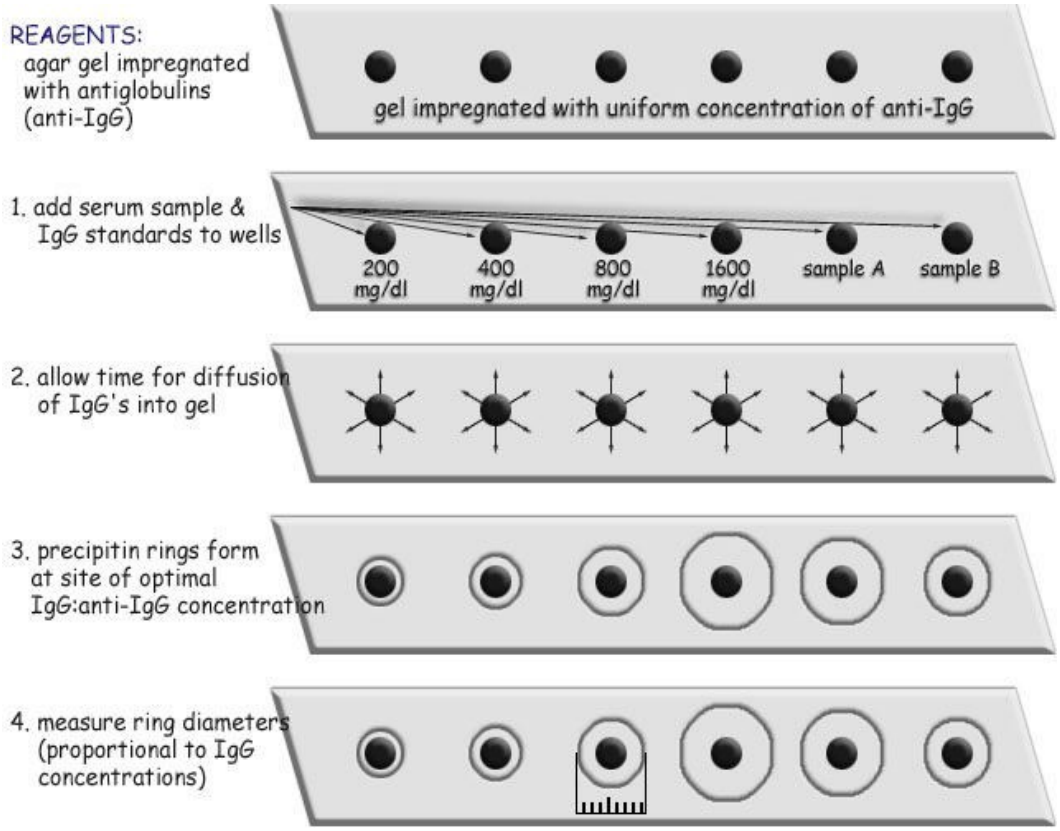
The relationship of immunoprecipitation ring size and antigen concentration was described in the mid- 1960s by Mancini et al. Mancini observed that the precipitin ring diameter stopped increasing at the point where diffusible antigen had been reduced and antigen-antibody complexing had attained equivalence. At equivalence, or endpoint, a linear relationship exists between the antigen concentrations and their corresponding ring diameters squared. A reference curve on linear graph paper is constructed by plotting the square of the precipitin ring diameters of reference sera against their corresponding concentrations.

Materials:

- Lab paper representing the RID rings of standards, controls, and patients
- Ruler capable of providing accurate measurement in millimeters
- Calculator
- Linear graph paper

Procedure:

1. For each diagram of a precipitin ring below, measure the diameter in millimeters and record the results in the space provided.



2. Square the diameter of the precipitin ring and record in the space provided.
3. Use the graphing paper provided, create a standard curve of the results of the standards provided.
4. Read the results of the controls and patients from the standard curve.
5. Record the results in the spaces provided using correct units.
6. Using product insert or other reference information, determine acceptability of the controls. Indicate your evaluation of each control by circling YES or NO.

7. Using textbook or other references, evaluate each patient result as to being low, normal, or high.

Results:

Record your results in the format provided below:

Sample & standards (mg/dl)	Diameter (mm)	Diameter^2 (mm^2)
200		
400		
800		
1600		
Sample A		
Sample B		

Place the photographs of the standard curve:

Questions:

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?

Practical 4

Title: Dot enzyme linked immunosorbent assay (Dot ELISA)

Objective:

After completing the practical, you will be able:

1. To learn the technique of Dot ELISA for the detection of an antigen

Introduction

Enzyme linked immunosorbent assay (ELISA) is a sensitive immunological technique commonly used to detect the presence of a specific antigen (Ag) or antibody (Ab) in a biological sample. ELISA is extensively used for diagnostic purposes. It requires an immobilized antigen/antibody bound to a solid support (e.g. microtitre plate or membrane).

There are different types of ELISAs for the detection of a protein of interest in a given sample. One of the most common ELISA is dot ELISA which can visually detect the presence of an antigen very quickly. The nitrocellulose dot technique was first developed for screening large number of hybridoma antibodies in 1983.

Principle

Dot ELISA, a qualitative ELISA test, can be performed very quickly with the end detection done visually (Li, Huang, Zhang, Ye, & Li, 2017). Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA. In Dot-ELISA, small volumes of antibodies are immobilized on a protein binding membrane (Nitrocellulose) and the other antibody is linked to an enzyme Horse radish peroxidase (HRP). The test antigen at first reacts with the immobilized antibody and later with the enzyme-linked antibody. The amount of enzyme linked antibody bound is determined by incubating the strip with an appropriate substrate (Hydrogen peroxide, H_2O_2) and a chromogen [Tetramethylbenzidine (TMB)]. HRP acts on H_2O_2 to release nascent oxygen, which oxidizes TMB to TMB oxide, which gives, a blue colored product. The latter precipitates onto the strip in the area of enzyme activity and appears as a colored dot, hence the name Dot-ELISA. The results can be visualized in naked eye. The enzyme activity is indicated by intensity of the dot, which is directly proportional to the antigen concentration. Figure 2 describes the principle of Dot ELISA.

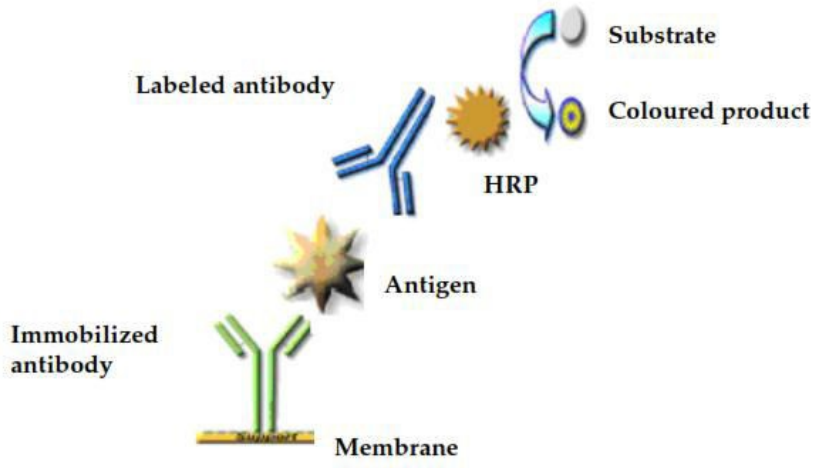


Figure 2: Principle of Dot ELISA

Materials

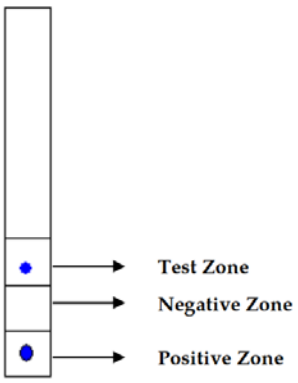
- HiPer® Dot ELISA Teaching Kit
- Glassware: Test tubes
- Distilled water,
- Micropipette and tips

Procedure:

1. Pour agarose solution containing the antiserum on to a grease free glass plate.
2. Take 2 ml of 1× Assay Buffer in a test tube and add 2 µL of the test serum sample. Mix thoroughly by pipetting. Insert a Dot-ELISA strip into the tube.
3. Incubate the tube at room temperature for 20 minutes. Discard the solution.
4. Wash the strip two times by dipping it in 2 mL of 1× Assay Buffer for about 5 minutes each. Replace the buffer each time.
5. Take 2 ml of 1×Assay Buffer in a fresh test tube, add 2 µL of HRP conjugated antibody to it. Mix thoroughly by pipetting. Dip the ELISA strip into it and allow the reaction to take place for 20 minutes. Wash the strip as in step # 3 for two times.
6. In a collection tube (provided in the kit) take 1.3 ml of TMB/H_2O_2 and dip the ELISA strip into this substrate solution.
7. Observe the strip after 5 - 10 minutes for the appearance of a blue spot.
8. Rinse the strip with distilled water.

Results and Observation:

Look for the appearance of blue dot as shown below:



Zone	Spot
Positive	
Negative	
Test	

Question:

1. Illustrate the various types of ELISA.

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Notes

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