Laboratory Guidance

IMMUNOLOGY
MIC 451

Prepared by:
Dr. Nagwa M. Aref
Dr. Faheema Khan
Dian Rachma W. MSc.

King Saud University
Department Botany and Microbiology
Kingdom of Saudi Arabia 2011

1. PHAGOCYTOSIS

Background Information

Inflammation is one of the ways our body reacts to an infectious agents or injury. During the inflammatory response, white blood cells with multilobed nucleus, PMNs (Polymorphonuclear neutrophils) migrate out of capillaries and into the infected tissue. PMN attracted to the site of infection through a process known as chemotaxis. In Chemotaxis, phagocyctic cells move migrate towards the infected cite where phagocytosis begin. The phagocytic cells are PMNs, monocytes, and macrophages. Monocytes are white blood cells with one large nucleus. Monocytes circulate in our pheripheral blood for only about 1 day. They may eventually move into tissue where they mature into a cell called a macrophages. Macrophage may life for several month. PMNs and macrophage are the major phagocytic cells of the immune system.

Phagocytosis is the process when phagocytes ingest, kill, and digest infectious organisms and unwanted cellular debris such as old cells or particulate matter. The particulate susceptible to the phagocytosis can be intracellular, such as *Mycobacterium tuberculosis*, or extracellular such as *Streptococcus pneumonia*. The intracellular pathogen are ingested by macrophage when they are released from a dying cell. The process begin after the phagocytes are attracted to the sie of injury and begin to attach to the microorganism(s). Attachment is accomplished because phagocytes have certain receptors (non-speciffic) on their cell surface to which microorganism can be attached.

In this exercise you will see PMNs engulfe yeast cells. Try to observe the entire sequence of events which includes chemotaxis, adherence, engulfment and phagosome formation. You will also stain your cells after phagocytosis has occurred using a common blood cell stain (Wright's stain) and observed ingested yeast cells within the PMNs.

Objectives

- To observe phagocytosis by microscopically examining the PMNs and monocytes as they ingest the yeast cells
- To understand the mechanims of phagocytosis.

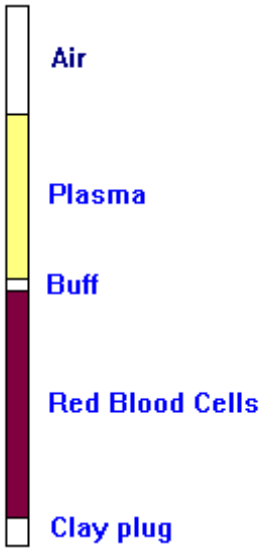
Materials

Lancet
Disposable gloves
Heparanized capillary tubes
Microcentrifuge
Coverslips
RPMI 1640 cell culture medium with 25mM Hepes buffer
Tooth picks
Wright-Giemma stain
Staining racks
Proper discard containers for the lancet, capillary tubes, gauze, slides, etc.
Alcohol swabs
Gauze
Suspension of yeast cells (10^8/ml)

Clay for sealing capillary tubes
Microscopes slides
Humid Incubation Chamber
Pasteur pipets with bulb
Phosphate buffer (pH 6.4)
Safety glasses

Procedures

1. After wiping with an alcohol swab, stick on your finger with the lancet (See Tips on collecting Blood). Your instructor will demonstrate the procedure. **The hand not being stuck should be gloved**.
2. Place the capillary tube in the blood and let the tube fill until at least one-half full.
3. Insert the capillary tube into the sealing clay and remove; This plugs one end of the tube. After bleeding has stoped, this hand can be gloved.
4. Place the tube in the microcapillary centrifuge and balance it with another capillary tube filled with the amount of fluid.
5. Spin the tube for 2 minutes. **Note** : If a microcapillary centrifuge is unavailable, Leave the capillary tube in the clay in a vertical position and let it stand for one hour. Eventually gravity will separate the cells from the plasma. The capillary tube hould look like that shown in the figure. The area on the top of the red blood cell (RBC) layer is called the buffy coat. The buffy coat contains the highest concentration of White Blood Cells (WBCs)and in this layer that you want to extract and observe.

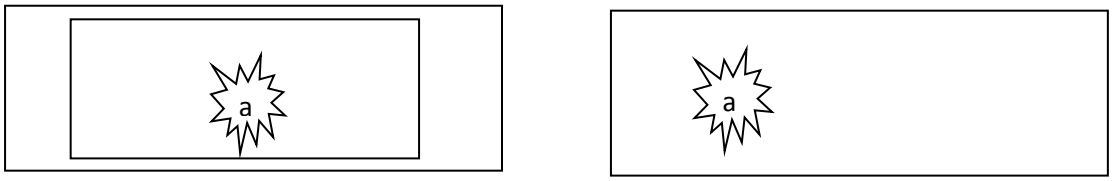


Figure

6. Your next task is to break the capillary tube at the buffy coat. **(Caution: wear eye protection when snapping the tube. Be careful not to cut yourself. The tube may break unevenly and produce sharp edge).** To break the tube, carefully use a glass rod etching device or file to etch

the capillary tube between two hands. Position your forefingers and thumbs at the etched point and snap the tube at the interface.

7. Carefully tap the portion with the fluid (plasma) and cells onto a coverslip. The coverslip is placed on a glass slide for easier handling. Also place a drop at the end of another glass side (figure 1).



(1) (2)

Figure 1.(1). Coverslip on a slide with the fluid (2). Slide with the fluid. (a:Buffy coat and plasma)

8. Dispense 1 drop of RPMI 1640 cell culture medium with Hepes buffer next to the fluid. **The medium ensures that the PMNs do not die while they are phagocytizing the yeast.**
9. Dispense 1 drop of suspended yeast cell (*Saccharomyces cerevisiae*) next to the fluid.
10. Mix the three suspensions together with a toothpick.
11. Place both the slide with coverslip and the second slide in a humid incubation chamber and incubate for 5 to 10 minutes at 37°C. It is important the mixture on the slide does not dry during incubation. Keep the lid closed on the chamber and make sure the paper is thoroughly moistened during incubation.
12. After 5-10 minutes, remove the slide with the coverslip and carefully invert the coverslip onto the slide. Let the fluid on the coverslip settle for one minute before observing. (You have made a wet mount of phagocyte and yeast cells).
13. Continue to incubate the other slide for an additional 45minutes in the humid chamber.
14. Observe the slide with the coversliip under 40xpower. The suspension is best observed using phase microscopy.
15. Watch the PMNs send out pseudopodia and begin to engulf the yeast cells. Record your observations in the RESULTS sheet.
16. After the slide has incubated about 1 hour, remove it from the chamber and spread the fluid over entire slide. This can be done by using another slide or a toothpick. Allow the slide to air dry.
17. Stain the slide according to the following Wright's stain procedure:
    a. Place the slide on a staining rack and cover it with filtered Wright's stain.
    b. Allow the stain to remain on the smear for at least 5 minutes.
    c. Slowly add buffer to the stain until the buffer begins overflow the stain. A metallic luster appearance will show up. (Some stain already contain the buffer, so this step is unnecessary).
    d. After mixing the buffer and stain by gently blowing on the smear, let the slide sit for another 5 minutes.
    e. Gently wash the stain and buffer off the slide with distilled water.
    f. Air-dry the smear or carefully blot the slide between two sheets of bilbulous paper.
18. Examine the smear microscopically. Use 40X or oil immersion (100X) to see the ingested yeast. In a properly stained slide, the red blood cells will appear as small pink- to red-colored cells, whereas the white blood cells will appear blue to dark purple with a dark purple nucleus. If the slide is poor stain, the white blood cells will be faintly stained.

19. Draw your observation in the RESULTS section.

Reference

Hudson BK. Sherwood LK. 1997. Exploration in Microbiology a discovery based approach. Prentice hall: USA.

Lab RESULT **1. PHAGOCYTOSIS**

Student name : _____
ID Number : _____
Date : _____

1. Draw and or/describe the events you saw during the microscopic examination of PMNs engulfing yeast cells

2. Draw and or/describe the events you saw during the microscopic examination of PMNs engulfing yeast cells with your stained slide

Reflections

1. The sample placed on the slide contained plasma, not serum (fluid portion of the blood). What is the difference between serum and plasma?

2. Does plasma contain the IgG and C3 necessary for opsonization?

3. How long did it take your PMNs to engulf the yeast cells?

4. What happened during the phagocytosis ?

2. PRECITPITIN: Ring Test

Background Information

The ring test is one of the oldest methods for detecting soluble antigen and antibody. It is simple, quick, and reasonably sensitive for the detection of trace quantities of antigen, as used forensically to detect adulteration of food meats with our meat proteins. It is less sensitive for the detection of antibody. A good antiserum usually gives a ring test visible in minutes at a dilution factor 1:5.

The ring test is based on the propensity of antibodies to form complexes with their corresponding antigens. When antibodies attach to antigen molecules in solution, the molecules become part of an insoluble antibody-antigen complex, and a visible precipitate forms.

Objectives

- To understand how to perform the ring test for detecting antigen and antibody

Materials

Precipitin Tubes (3x50mm)
Saline Solution (0.9%)
Bovine Serum Albumin (BSA) 1 %
Antiserum
Normal rabbit serum

Procedure

1. Obtain 0.5 ml clear antiserum.
2. Prepare a 1:5 dillution of the antiserum by adding 0.1 ml to 0.4 ml saline and mixing, and a 1:10 dilution by adding 0.2 ml of the 1:5 dilution to 0.2 ml of saline.
3. Into 3 precipitin tubes, clearly identified , add 0.2-0.3 antiserum and the dilutions, respectively (Place the serum into the bottom of the tub without creating bubbles)
4. To a fourth tube, add normal rabbit serum.
5. With the precipitin tube held at angel of 45-60 °, carefully allow 2 drops of a 1:20 dilution of the bovine albumin to flow down the side of the tube and over the antiserum. A sharp line will be produced at the interface of the liquids.
6. Observe the precipitin reaction in each tube at minute interval for 5minutes and then at 5-minute interval for 30 minutes. Record it on RESULT sheet.

Reference

Montasser MS. 1999. Experimental Protocols in Virology and Immunology. Academic publication committee. Kuwait University.

Lab RESULT **2. PRECIPITIN: Ring Test**

Student name : _____
ID Number : _____
Date : _____

1. Examine all test tubes for the development of a ring of precipitation at the interface. Indicate the presence or absence of a ring.

2. Determine and indicate the antigen dilution that produce the greatest degree of precipitation that is indicative of the optimal antibody:antigen ratio

Table Ring Test Precipitation on every minutes in the first five minutes

Presence of interfacial ring (+) or (-)	Antigen dilutions				Time
	1:5	1:10	Serum control	Saline control	
					1 min
					2 min
					3 min
					4 min
					5 min

Table Ring Test Precipitation on every minutes in the first five minutes

Presence of interfacial ring (+) or (-)	Antigen dilutions				Time
	1:5	1:10	Serum control	Saline control	
					5 min
					10 min
					15 min
					20 min
					25 min
					30 min

3. MICROPRECIPITIN TEST

Background Information

The viral protein coat has multiple epitopes susceptible to antibodies. Antibodies have two antigen-binding sites. When brought together in optimal proportions, antibodies can link virus particles and for large aggregates. In droplets, such aggregates are visible as whitish precipitate. If either the number of antibodies or virus particles are limiting, no visible precipitate is formed. This is the basis for testing the quality of an antiserum in the microprecipitin test. Drops of a series twofold antiserum dilutions are mixed with drops of a similar series of virus dilutions in a grid titration. The greatest dilution giving a visible precipitate can be determined for both antiserum and virus. For the antiserum, this highest dilution factor signifies the titre of the serum.

By using the test for screening serum from successive bleedings, the titre of the antiserum can be monitored during the immunisation process. If carried out properly, the test requires only properly, the test requires only a small volume of antiserum.

Objectives

- Two learn how to performs : Immunoelectrohphoresis

Materials

Plastic Petri dish, 14 cm diameter
Filter petri dish, 14 cm diameter
Microcentrifuge tube (0.5-1.0 ml)
Rack for microcentrifuge tubes
Micropipettes (200,100 and 20 µl)
Incubator at 37 °C
Stereomicroscope with Iluminator
Glossy black paper sheet, black glass plate, or other black material (11x11 cm)
Tris-NaCl buffer ; Tris-HCL (0.01 M);NaCl (0.85%). Adjust to pH 7.0
Antiserum, in a microfuge tube, undiluted, volume at least 60 µl

virus materials

Plant virus suspension in a microcentrifuge tube, concentration 1 mg/ml, volume at least 240 µl

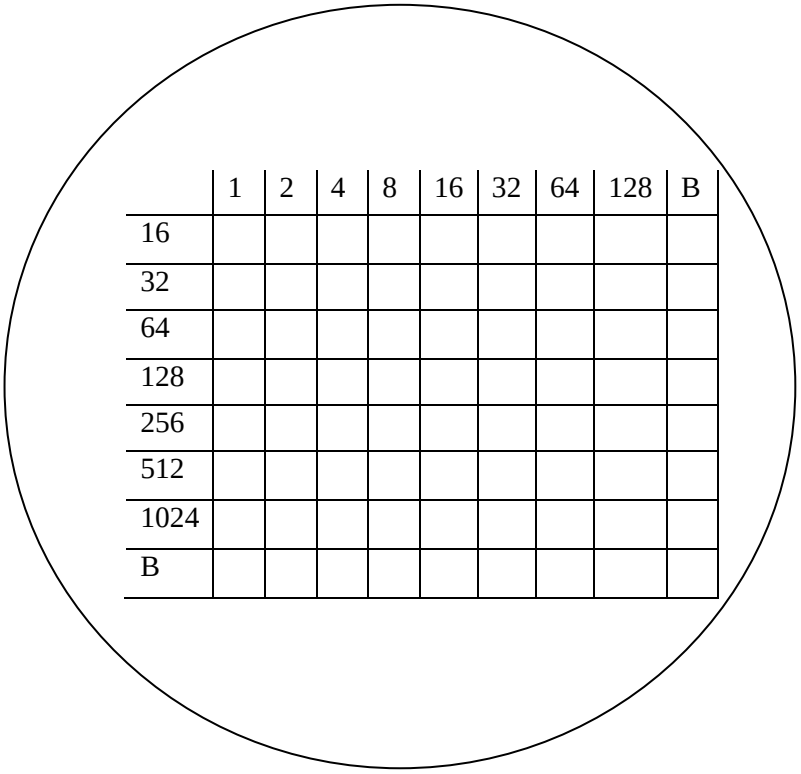
Procedure

Preparing dilution series

1. Place eight microcentrifuge tubes in a rack, with the first one containing undiluted virus suspension. Mark the tubes with the virusdilution factors:1,2,4,8,16,32,64,&128.
2. Pipette 120 µl of Tris-Nacl buffer into each of the tubes 2-128.
3. Transfer 120 µl of virus suspension from tube 1 to tube 2 and mix thoroughly by sucking and expelling the liquid a few times. Avoid the formation of air bubbles or foam as much as possible.
4. With a clean pipette tip transfer 120 µl from tube 2 to tube 4 and mix again.
5. Repeat this until all tubes contain successive virus dilutions.
6. Place seven microfuge tubes in the rack and marks the tubes with the serum dilutions factors 16,32,64,128,256,512, & 1024.
7. Pipette 225 µl of Tris-NaCl buffer into tube 16, and 120 µl in the other six tubes.
8. Transfer 15 µl of undiluted serum into tube 16 and mix.
9. Continue dilution by successively transferring 120 µl to the other tubes.

Grid Titration

1. Draw on the sheet of paper lacctice of 9x8 squares of 1 cm ^{3} Lable the column as shown in the figure .



	1	2	4	8	16	32	64	128	B
16									
32									
64									
128									
256									
512									
1024									
B									

2. Using a 20 µl micropipette, place 12 µl droplets of Tris-NaCl buffer in the center of the squares of the column labelled B. As the droplets are small they may have to be dabbed off.
3. Equally, place 12 µl droplets of virus dilutions in all squares of correspondingly labeled columns
4. Repeat step 2 and 3 with buffer and antiserum dilutions in appropriate rows, placing the droplets on top of the ones with antigen. Start with antiserum dilution 1024 after the application of buffer. If necessary, dab off the droplets on top of the first ones but make the contact of the pipette tip with the two combined droplets as superficial as possible.
5. Place the dish cover after lining it with moist filter paper and incubate at 37 °C for 2 hour
6. Place the dish, without cover, on a glossy black background with oblique to light. Examine the drops with a stereomicroscope for the presence of a whitish precipitate at 16 X or 25 X magnification.
7. Evaluate the amount of precipitate according to the following scale :
   ++++ very heavy precipitate
   +++ heavy precipitate
   ++ slight precipitate
   + slight precipitate
   ± barely visible
   0 no precipitate

8. Keep the dish overnight in a refrigerator for a second evaluation.

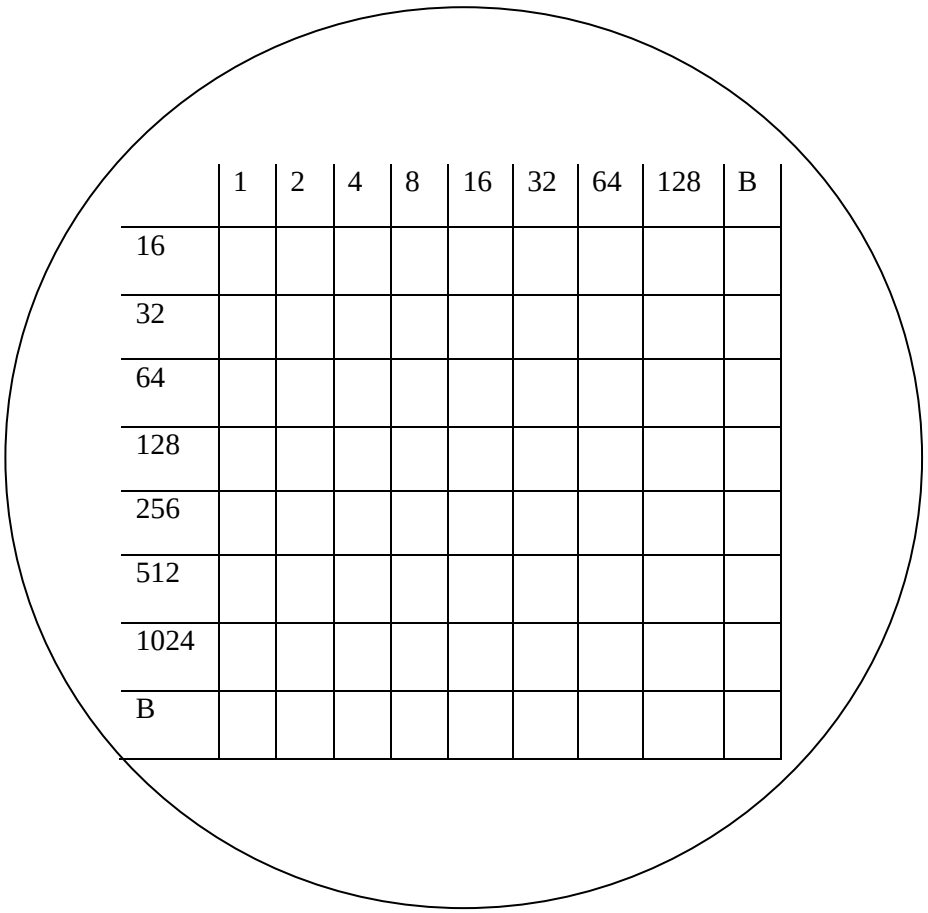
Reference

Djikstra J. Pdee Jager P. 1998. *Practical Plant Virolog,protocols and exercise*. Springer: Heidelberg.

Lab RESULT **3. MICROPRECIPITIN TEST**

Student name : _____
ID Number : _____
Date : _____

1. Observe the plate and evaluate the amount of precipitate. Complete the figure below



	1	2	4	8	16	32	64	128	B
16									
32									
64									
128									
256									
512									
1024									
B									

2. Which dilution having a very heavy precipitate ?

3. In the Buffer control, is there any precipitate **?**

4. **IMMUNODIFFUSION : Radial Immunodifussion**

Background Information

In Immunodiffusion tests, antibodies and or soluble antigens are loaded into separate wells of a gel and are allowed to difuse, each reagent moving radially into the gell. An immobile precipitate, visible as a band (precipitin line) in the gel, develops if specific antibody- antigen binding takes place, and if antibody-antigen components are present at optimal proportions.

In radial immunodifussion, the antibodies are equally distribute, loaded into a well, diffuses. When antigens molecules move through the gel, they bind to and carry antibodies, until the ratio of antibodies are optimal for complex formation.

Objectives

- To understand Immunodiffusion technique

Materials

Human IgG, IgA, and IgM "NL" "Bindarit ™
Radial immnodiffusion kit
Human serum
Micropipettte

Procedure

1. Obtain 5 µl of human serum, and pipette it into a designated wells of the RID assay gel.
2. Resserve the three weels for standard concentrations. A high standars, low standard, and a serum control (Provided in the radial immunodiffusion kit)
3. Place the gel into moist box, and incubate for 24 hours at room temperature to allow difussinon and banding (The gel can be stored in refrigerator for several weeks, if the boxis keep moist)
4. ***Read the result of your assay*:** Measure the diameter of the circle of precipitate (in centimeters) for the sample you loaded and for the three standard samples. Record this in the

Reference

Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

Lab RESULT **4. IMMUNODIFFUSION : Radial Immunodifussion**

Student name : _____
ID Number : _____
Date : _____

Record the three standars immunoglobulin (Ig) Concentrations and the diameter of each resulting precipitin ring. Also record the diameter of the precipitin circle for the sample you loaded.

Ig Standard Concntration	Diameter of precipitate circle

Sample precipitin ring diameter :_____

5. IMMUNOELECTROPHORESIS

Background Information

Immunoelectrophoresis involves combination of two techniques of immunodiffusion and electrophoresis. Antigen are first loaded into wells an agarose gel and are separated by charge in an electrophoresis chamber. Then, antibodies are used yo detect the separated antigens after being loaded into a trough that runs the length of the gel, they diffuse towards and complex with the antigens, and form visible lines of precipitate.

Objectives

- To perform one of the immunodiffusion technique : Immunoelectrophoresis

Materials

Hight resolution electrophoresis buffer, pH 8.8
1% Agarose in high resolutionbuffer, pH 8.8
Antigens: bovine serum; bovine albumin 10 mg/ml
Antibodies: anti-bovine albumin; anti-bovine serum

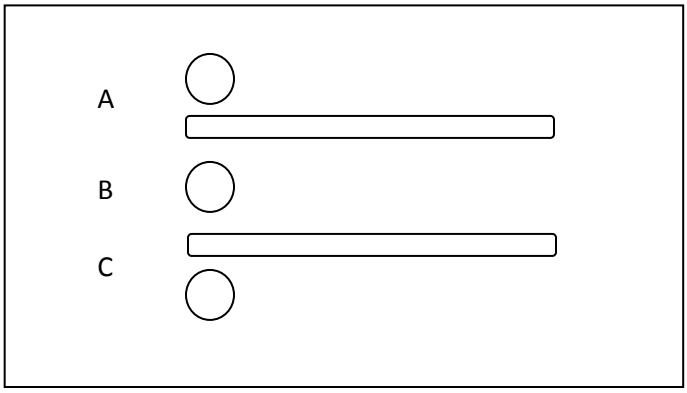
Horizontal gel electrophoresis box
Power supply

160 mm diameter petri dish
Tape
2 glass slides
Glass or plastic dropper
Micropipette/tips (10-100 µl)
Grade no.1 whatman paper or 3MM paper

Procedure

1. Prepare 40 ml of 1% agarose: Add 0.4 g of agarose to 40 ml of high-resolution buffer, PH 8.8 in a 125 ml flask. Microwave the mixture for about 30 seconds, check to make sure it does not boil over. Using a hot glove, gently swirl the flask and return it to the microwave. Heat for 15 seconds, repeating this until no flecks of agarose are visible in the flask. Let the molten agarose cool until the flask is comfortable to handle, but still warm
2. While the agarose cools, prepare a horizontal gel electrophoresis box by putting the dams securely in place. Also prepare a trough-forming apparatus: obtain a 60 mm petri dish with lid, and tape a slide to each side.

3. Once the agarose has cooled so that the flask is comfortable to hold, pour the agarose into the unit until it completely covers the platform. Place the trough-forming apparatus at the center of the platform. Allow the agarose to solidify, about 10 minutes.
4. When the gell is solid, gently remove the dams and trough-forming apparatus. Using the the large end of a plastic or glass dropper (a diameter of about 0.5 cm), cut wells into each gel as shown in the following template



5. Pour high-resolution buffer, pH 8.8, into the electrophoresis box or either side of the gel, being careful not to pour onto the gel itself. 1 or 2 inches deep. Cut two pieces of whatman chromatography paper wicks, and place them into the apparatus. Be sure that the paper is in contact with the gel and the buffer at both ends of the gel.
6. ***Load the antigens:*** Changing micropipette tips between samples, load 20 µlof bovine serum into wells A and C, and 20 µl of bovine albumin into well B. Do not load the troughs.
7. Electrophoresis samples at 70 volts for 1.5 hours
8. Load the antibodies: After electrophoresis is complete, load 50 µ of anti-bovine albumin into trough 1 and 50 µl of anti-bovine serum into trough 2. Again, change tips between samples.
9. Leave the gell in the electrophoresis apparatus, and wrap a moist paper towel and plastic wrap around it to create a moist container. Incubate for 24 to 48 hours at room temperature to allow diffusion and banding.

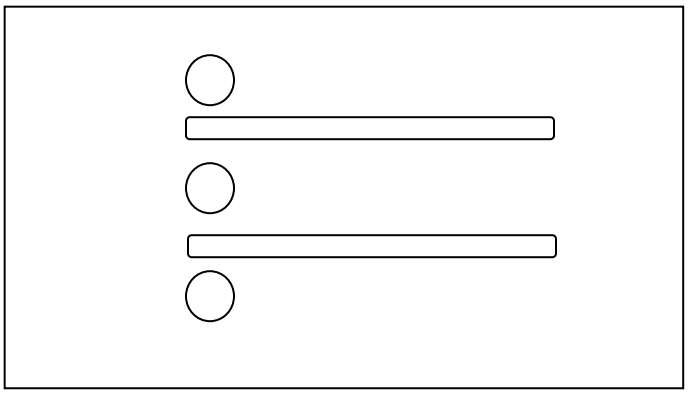
Reference

Prescott, LM. 2005. Study Guide for Use With Microbiology. McGraw-Hill: Montana State University.

Lab RESULT **5. IMMUNOELECTROPHORESIS**

Student name : _____
ID Number : _____
Date : _____

Diagram the result of the IEP Assay



6. THIN LAYER IMMUNOASSAY

Background In formation

Many antigens adsorbs firmly to the hydrophobic surface of polystyrene. The exposed antigenic determinants on the monomolecular film are fully reactive with antibody. Antibody bound to the antigen may be detected by fluorescence in ultraviolet light after reaction with fluorescein labeled anti-immunoglobulin.

Thin-layer immunoassay (TIA) is a technically simple and accurate method for the assay of antigen-antibody reaction. The technique basically use the principle that macromolecules may be absorbed as a thin layer to a polystyrene surface. These macromolecules most often preserve their serological reactivity and hence capable of binding antibodies. The presence of antibodies on the antigen surface is visualized as a distinct hydrophilic condensation pattern when surface is exposed to water vapor. TIA has been used for the determination of antibodies against purified proteins, polysaccharides, and lipopolisaccharides also proved suitable for screening of serum samples.

Objectives

- To know the Thin-layer Immunoassay technique

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Materials

Petri plates, 100 mm polystyrene
Ethanol (70%)
Microtiter plates
Microtiter diluters (25 µl)
Capillary tubes (20 µl)
Bovine Serum Albumin (BSA), 1%
$NaHCO_3$ (0.05 M)
NaCl (0.15M)
Water bath, 60°C

Procedure

1. Rinse a polystyrene Petri plate with 70% ethanol. Blow the plate dry with a jet of air.
2. Using a 0.05M $NaHCO_3$ as diluents, prepare 20 ml of a BSA solution containing 10 µg/ml. Pour the solution into the dry petri plate. Incubate the plate for 30-60 minutes at 37°C. (Plates with antigen may be stored at 4°C until ready for use).
3. Pour off the antigen solution; wash plates thoroughly with distilled water. Blow dry.
4. With marker pen, draw a grid on the underside of the plate to provide 20-40 squares.
5. Prepare 10^{-2} serial dilutions of antiserum in NaCl (0.15M) using the microdillution equiptment. Starting with the highest dilution and using a capillary tube, spot 5µl of

antiserum onto a square. Proceed with the remaining dilutions of antiserum. Repeat with a control normal serum.

6. Place the petri plate with dilutions in a humid chamber 37°C for one hour.
7. Invert the plate with the serum drops still in place over a beaker filled with water at 60°C for one minute/ Rinse the plate thoroughly with distilled water blow dry, and again invert the plate over the beaker filled with water at 60°C.
8. Observe the condensation patterns in the squares. Record as positive those dilution of serum that give water droplets obviously larger than droplets appearing over the background.

Reference

Montasser MS. 1999. Experimental Protocols in Virology and Immunology. Academic publication committee. Kuwait University.

Jeansson S. Elwing H. Nilsson L. 1978. *Thin-Layer Immunoassay for Determination of Antibodies to Herpes Simplex Virus*. *Journal of Clin Microbiol*.

Lab RESULT **6. THIN LAYER IMMUNOASSAY**

Student name : _____
ID Number : _____
Date : _____

Diagram your Thin-Layer Immunoassay result, and give explanation on the picture

7. POLYCLONAL ANTIBODIES

Background Information

The Immunization of laboratory animals to induce a humoral and or/cellular immune reponse has been a routine procedure performed worldwide. Several animal species are used for the production of antibodies, rabbits and mice are the most frequently used species for the production polyclonal antibodies (pAbs).

The mammals immune system are believed to be comprised of approximately 1000 clolnal populations of limphocytes as characterized by their antigen-receptor specificity. The diversity allows immune response to a broad range of iimunogens, for example: foreign proteins, carbohydrates, peptides, bacterial, and viral components.Apolyclonal humoral response, making use of the entire range of antibodies result in high avidity (defined as the product of the affinity constants of all binding antibodies) and gives the ability to the organims to defend themselves against pathogen.

Objectives

- To understand the protocols of polyclonal antibody production in laboratory animal

Materials

Young rabbits (2.5-3.0 Kg; 10-16 weeks of age), 2 animals per antigen
Complete Freund's adjuvant
Bovine serum albumin (BSA) 2%
Alumunium Chloride, 2%
Vaccine vials
Pipettes
Sterile small test tube
Syringe, 3ml; 10 ml
Cotton
Alcohol
Test tube
Animal clippers

Procedure

Preparation of antigen with adjuvant

1. To 4 ml Freund's adjuvant, add 1 ml 2% BSA. Vortex in a sterilized small (5 ml) test tube for about15 min. The emulsion should not separate on standing.
2. To 3 ml 2% BSA , add 1 ml $AlCl_3$ (2%). Add sufficient 0.5 N NaOH to produce maximum flocculation. Label and store in refrigerator.

Immunization

1. Procure a normal rabbit and collect 2-3 ml blood and preserve the serum.
2. Inject 0,2 ml antigen-Complete Freund's Adjuvant (CFA), subcutaneous (SQ) and Intramuscular (IM).
3. One week after injection test bleed the rabbit (see no.1).
4. After two weeks give 0,2 ml antigen-Freund's Incomplete Adjuvant (IFA)
5. One week after injection test bleed the rabbit (see no.1).
6. Collect the blood from the rabbit (follow the instructor).
7. Write your Lab RESULT in the form of a **' Laboratory report'.**

Immunization Schedule

Day 0 Pre-immune bleed, First immunization
Day 7 Test Bleed
Day 14 Second immunization
Day 21 Test Bleed
Day 25 Blood collection

Reference

Montasser MS. 1999. Experimental Protocols in Virology and Immunology. Academic publication committee. Kuwait University.

Florida State University. 2007. Polyclonal Antibody Production Protocol-Rabbit. Florida State University.

8. Two Step DAS-ELISA

ELISA (enzyme-linked immunosorbent assay) or ELA (enzyme immunosorbent assay) is the most widely used immunoassay labs today. Immunoassays are based on detectable interactions between antigens and antibodies such as precipitation, agglutination, or complement fixation.To increase sensitivity in detecting antigens, antibodies can be labeled with substance such as radioactive chemicals (E.g., Iodine-125), fluorescent compounds, magnetic beds, or enzymes.

The ELISA take advantage of the strong and specific attachment that occurs between an antibody and antigen (thus the term is imumunosorbent) An enzyme covalently attached to the tail portion of the antibody. The enzyme linked to the antibody is one that catalyzes the conversion of a colorless substrate into a colored product.

In this exercise we will perform the direct-double antibody sandwich (DAS)-ELISA. In this procedure, test sample and conjugate are incubated simultaneously in the wells, so that one washing step is omitted. This technique has advantage than the standard DAS-ELISA. It is less time consuming and, in general, more sensitive, with less background reaction. However it is less sensitive when the virus concentration are higher than 50 ng. Hence, this technique is recommended for the detection of small amounts of virus.

Objectives

- To practice modified ELISA technique
- To understand how ELISA test can be use clinically to detect antibodies or antigen

Materials

General

Polystyrene mictotitre plates (96 wells)
Glass vials
Medium-speed centrifuge with tubes
Microtiter and pestle or small bags of thick plastic
Cheesecloth
Micropipettes (200 µl; 300 µl) with tips
Dialysis tubing prepared by boiling for 10 minnutes in 0.01 EDTA
Incubator at 30-37°C

Virus and plant materials

Virus-free and virus containing materials, for instance, leaves of noninfected and infected plants, respectively.
Purified virus suspension

Chemicals, solutions and suspensions

Purified immunoglobulins to the virus
PBS (see appendix)
Tween-20
PBS with 0.05% (v/v) Tween-20 (PBS-Tween)

Sodium carbonate coating buffer: 0.05M, pH 9.6 (see appendix)
Diethanolamne
Diethanolamine susbtrate buffer: 10%(v/v), pH 9.8(see appendix)
p-Nitrophenyl phosphate (Substrate)
Sodium azide (NaN_3)
Glutaraldehyde (Electronmicroscope grade)
Alkaline phosphate
Bovine Serum Albumin
Glycerol: 50% (v/v)
PVP (*M*r 44,0000)
Ovalbumin crystallized
NaOH: 3M
Na-DIECA : 0.012 M
Na_2-EDTA : 0.012 M

Preparations virus and control samples

Grind noninfected, and infected, leaf material separately in PBS-Tween (0.2-1.0 g fresh weight per 10 ml buffer) in a mortar with pestle. Some plants, especially woody ones, contain substances, such as tannins, which may interfere with reactions in ELISA. In that case, it is advised to add 20g PVP and 0.2 ovalbumin to 11 of the PBS-Tween. PVP is a synthetic polymer which binds tannins and other phenolic substances.

When the concentration of virus in the leaf samples is expected to be low and the leaves do not contain much fibrous materials, they may be crushed with some PBS-Tween in a small plastic bag, by placing the latter on the bench and rubbing over it with a test tube or a similar cylinder. Usually dilution series of leaf extracts obtained are made in PBS-Tween.

Immunoglobulins

For purification of immunoglobulins (see appendix). Purified immunoglobulins to be used for conjugation should not contain sodium azide. Glycerol in stored immunoglobulins does not interfere with adsorption to the polystyrene plates provided its concentration does exceed 1%. Hence, glycerol-perserved immunoglobulins can be used for coating after appropriate dilution

1. Dissolve 5 mg alkaline phosphatase in 2 ml purified immunoglobulin suspension of 1 mg/ml.
2. Dialyse 3 h at 4 °C against 1Lof PBS (three changes)
3. Add 50 µl of freshly prepared 2.5% (w/v) glutaraldehyde.
4. Incubate the mixture at 22 °C for 4 h.
5. Remove the glutaraldehyde by dialysing at least three times against 1L of PBS.
6. Remove any precipitate formed by low-speed centrifugation
7. Store the conjugate thus obtained wih approx. 1% BSA (w/v) (final concentration) at 4°C; for storage up to 6 months, add NaN_3 to a final concentration of 0.02% (w/v).
8. Dilute the conjugate with PBS-Tween before use.

Substrate

Dissolve P-nitrophenyl phosphate powder in freshly prepared diethanolamine substrate buffer to a concentration of 0.67 mg/ml. (Note: Glassware used for substrate solutions should be very clean!)

Optimum concentration of coating and enzyme-labelled antibodies

Before starting the actual experiment, the optimum dilutions of coating immunoglobulin and conjugate are tested with sap from virus-free (control) and infected (test) leaf material. Filling of wells and washing of plates are done as described below for DAS-ELISA. The combination which gives maximum distinction between the control samples and the least infected test sample is chosen for the actual; experiment

Procedure

1. Coating: fill all wells of the microtiter plate (except those the top and bottom rows and the row on the extreme right, which may give nonspecific reaction) with 200 µl aliquots of immunoglobulin diluted in coating buffer. Cover the plate and incubate at 30-37°C for 2-4 hour or at 4-6°C over night.

2. Washing: Remove the immunoglobulin suspension by vigorously shaking out the plate over the wash basin. Fill the wells with PBS-Tween, empty the plate and fill it again with PBS-Tween. Remove the PBS-Tween after 3 min. Repeat this washing and soaking in PBS-tween twice and finally beat the plate dry on paper towels spread on the bench.

3. Test sample fill all coated wells (except those of the extreme left row) with 100 µl aliquots of test samples, diluted in PBS-Tween (addition of chelating agents such as Na-DIECA or Na_2-EDTA) to a concentration of 0.012 M may further improve the sensitivity of the assay.

4. Conjugate: fill each well with 200 µl aliquots of enzyme labeled immunoglobulin, diluted in PBS-Tween. Cover the plate at 4-6°C overnight.

5. Washing: see step 2

6. Substrate : Fill each well with 200 µl aliquots of substrate. Add 200 µl PBS-Tween to the wells of rows on the top. Bottom and extreme right, for uniformity.

7. Incubate at room temperature unit a yellow colour is clearly visible in the positive controls (usually between 30-90 min). If desired, the reaction can be stopped by adding 50 µl of 3M NaOH to each well (mix the component by agitating the plate carefuly).

8. Asses result by either visual observation or by measurement of absorbance of the hydrolysed substrate (*p*-nitrophenol) at 405 nm wavelength in a microtitre plate reader. Record your result.

9. Write your Lab RESULT in the form of a **‘ Laboratory report’.**

Reference

Djikstra J. Pdee Jager P. 1998. *Practical Plant Virolog,protocols and exercise*. Springer: Heidelberg.

9. Amplified ELISA

Background Information

This is a very sensitive ELISA, developed by Stanley in 1985. It may amplify signals up to 500-fold, thus enabling detection of very small amounts of virus, for instance in individual vector insects or seeds.

In this procedure, the alkaline-phosphatase-labelled antibody dephosphorylates nicotinamide adenine dinucleotide phosphatase monosodium salt (NADP) to nicotinamide adenine dinucleotide (NAD) which catalyses a redox cycle: alcohol dehydrogenase converting ethanol into acetaldehyde leading to reduction of NAD by ethanol. In the presence of diaphorase, the reduced NAD (NADH) thus formed in turn reduces a violet coloured tetrazolium salt (*P*-iodonitro-tetrazolium violet) to a red product (formazan). Limitation of the technique is the highcost of the chemicals in the amplification mixture.

Objectives

- To practice modified ELISA technique
- To understand how ELISA test can be use clinically to detect antibodies or antigen

Materials

The same ingredients as the two step DAS-ELISA escept for the substrate.

- β-Nicotinamide adenine dinucelotide phosphate monosodium salt (NADP): 0.2mM
- Diethanolamine buffer: 0.05 M, pH9.5 The Buffer is prepared by adding 5,28 ml diethanolamine to 1L of deionized water.
- Phosphate buffer: 0.025 M, pH 7.0
- Amplification mixture (see appendix)
- Nitrophenyl o-phosphate disodium-salt: 0.5M

Procedure

1. Coating: fill all wells of the microtiter plate (except those the top and bottom rows and the row on the extreme right, which may give nonspecific reaction) with 200 µl aliquots of immunoglobulin diluted in coating buffer. Cover the plate and incubate at 30-37°C for 2-4 hour or at 4-6°C over night.

2. Washing: Remove the immunoglobulin suspension by vigorously shaking out the plate over the wash basin. Fill the wells with PBS-Tween, empty the plate and fill it again with PBS-Tween. Remove the PBS-Tween after 3 min. Repeat this washing and soaking in PBS-tween twice and finally beat the plate dry on paper towels spread on the bench.

3. Test sample fill all coated wells (except those of the extreme left row) with 200 µl aliquots of test samples (each samples in duplicate or triplicate). Fill each of the wells on the extreme left with 200 µl PBS-Tween (They serve as a blanks in the ELISA reader).

4. Washing: see step 2

5. Conjugate: fill each well with 200 µl aliquots of enzyme labeled immunoglobulin, diluted in PBS-Tween. Cover the plate and incubate for 2-4 hour at 30-37°, or at 4-6°C overnight.

6. Washing: see step 2.

7. Fill the well with 100 µl aliquots of 0.2 M NaDP in 0.05 M diethanolamine buffer pH 9.5.

8. Incubate at 20 °C for 30 minutes

9. Terminate the enzyme-substrate reaction by adding 15 µl of 0.5 Nitrophenyl *o*-phosphate disodium salt.

10. Add 150 µl of the amplification mixture to the coated wells; add 250 µl PBS-Tween to the wells of rows on the top, bottom and on the extreme right.

11. Measure clour development at 492 nm wavelength. Record the result.

12. Write your Lab RESULT in the form of a **‘ Laboratory report’.**

Reference

Djikstra J. Pdee Jager P. 1998. *Practical Plant Virolog,protocols and exercise*. Springer: Heidelberg.

10. LATEX AGGLUTINATION SLIDE TEST FOR *Staphylococcus aureus*

Background Information

Agglutination reactions involve visible aggregation of particles because of the specific antigen-antibody combination. Antibodies that produce such reaction are known as agglutinins. Agglutination is a two-step process, involving sensitization or initial binding followed by lattice formation, or formation of large aggregates. Latex agglutination test are very popular in clinical laboratories. The tests have been applied to the detection of over 100 infectious diseases, and many other applications are currently available. The test was first performed by Singer and Plotz in 1956. Afterwards, the tests have been developed to diagnose microbial and viral infection, autoimmune diseases, hormones, drugs, and serum proteins.

In latex agglutinations test, an antibody (or antigen) coates the surface of latex particles (sensitized latex). When a sample contains the specific antigen (or antibody) is mixed with the milky-appearing sensitized latex. Many of the latex agglutination tests developed are performed manually and the agglutination is detected by visual observation. In spite of, its usefulness and less costly this manual assays suffer from lack of consistency in endpoint readouts.

Objectives

- To know how to perform the latex agglutination
- Understand principle of the technique

Materials

Latex agglutination kit
Pipettes of 10,20,35, and 50 µl
Gloves
Loop inoculation
Bunsen burner
Timer
Staphylococcus aureus
Staphylococcus epidermidis

Procedure

1. Allow reagents, controls, and specimens to reach room temperature.
2. Mix the latex reagent by shaking; expel any latex from the dropper for complete mixing.
3. Dispense 1 drop of Test Latex onto one of the circles on the reaction card and add 1 drop to another circle.

4. Using a microbiological loop pick up and smear 5 suspect colonies onto the Test Latex-containing circle and mix this into the Test Latex reagent. Spread to cover the circle.
5. Repeat step 3 for the Control Latex.
6. Pick up and hand rock the card for up to 20 sec and observe for agglutination under normal lighting conditions. Read macroscopically; do not use a magnifying glass.
7. Dispose of the reaction card in an appropriate biohazard container.
8. Re-cap the bottles and return to the refrigerator.

Interpretation of the test result

Positive result

A positive result is obtained if agglutination of the blue latex particles occurs within 20 sec in the test circle, with no agglutination in the control circle. The result is positive when there is noticeable clearingof the blue background in the test latex. This indicates the present of *S. aureus*

Negative result

A negative result is obtained if no agglutination occurs and a smooth suspension remains at 20 sec in the test circle. The result is negative when there is no noticeable clearing of the blue background in the test latex.

Uninterpretable Result

The test is uninterpretable if the control reagent shows agglutination or autoagglutination.

Reference

Laboratory Procedure BBL™ Staphyloslide™ Latex Test for *Staphylococcus aureus*. http://www.bd.com/ds/technicalCenter/clsi/clsi-staphyloslide.pdf

Stevens CD.2010. *Clinical Immunology and Serology A Laboratory Prespective: Third Edition*. F.A.Davis Company: Philadelphia.

Lab RESULT 10.LATEX AGGLUTINATION SLIDE TEST FOR *Staphylococcus aureus*

Student name : _____
ID Number : _____
Date : _____

1. What is the result of the test ? (positive and negativve)

2. What s the conclusion of the result ?

11. ROCKETIMMUNOELECTROPHORESIS

Background Information

Rocket immunoelectrophoresis also known as single crosses immunoelectrophoresis, spike immnoelectrophoresis, (the Laurell technique) or electroimmnuoassay. This is a quantitative method for the estimation of antibody titre and concentration of antigens. It is a relatively quick method and can be adopted to determine antibody titer since the area enclosed by the precipitation line is lineary related to the amount of antigen and inversely to antibody content in the gel.

Objectives

- Practice Rocket Immunoelectrophoresis Technique

Materials

Rocket Immunoelectrophoresis kit
Electrophoresis unit
Agarose
Barbitone buffer 0.1 M
Calcium lactate 0.2 mM
Antiserum
Coomassie brilliant blue
Methanol acetic acid buffer

Procedure

1. Prepare 1% (w/v) agarose gels containing 2% polyethylene glycol 6000 to stabilize and enhance the immune precipitate, in 0.1 M barbitone buffer 2mM Calcium lactate, pH 8.6 and containing 0.7-3.0 % antiserum.
2. Pour the above agarose gels on a glass plates (10x10cm) to give a gel 1.5 mm thick.
3. Punch 3 mm diameter hole at about 8-10 mm centres in a line 2 cm from side edges.
4. By using different antigen or diluting the Original antigen , place 10 µl of each sample in a hole.
5. Carry the electrophoresis at a high voltage, 10 V/cm for 2-4 hours, using the same buffer in the gels.
6. Press the gels onto the glass plates under 0.5 cm of filter paper and soak in 0.1mM NaCl twice for 20 minutes each and then in running water once for 15 minutes.
7. At the end of this time, press again the gels onto a glass plate and dry with a portable hair dryer.

8. Stain the plates with 0.25 % Coomassie brilliant blue for 15 minutes and destain in methanol-acetic-acid-water (25:7.5: 67.5, vol/vol/vol).
9. Measure peak heights on a dry surface of the glass plate. The antigen concentration are proportional to the peak heights.

Reference

Montasser MS. 1999. Experimental Protocols in Virology and Immunology. Academic publication committee. Kuwait University.

12. ELECTROBLOT IMMUNOASSAY

Background Information

Immunoblotting assays generally make use of the strong protein-binding capacity of nitrocellulose or nylon membrane. The protein under investigation may be either electroblotted onto the membrane from a gel through which they have been separated by electrophoresis (electroblot immunoassay, western blotting) or applied directly to the membrane (dot-blot and tissue blot immunoassays).

Electroblot immune assay (EBIA;western blotting) is a technique based on electrophoresis and serology and thus combine the protein-separating capacity of the former with the specificity of the latter. Therefore, very small quantities of protein can be detected. In EBIA, protein samples are electrophoresed on an SDS polyacrylamide gel; then the separated protein are covalently bound to a nitrocellulose or nylon membrane by electrotransfer to make them more accessible for reaction with subsequently added antibodies.

Materials

Preparation of crude protein extract

The following cultures and reagent are available in the identification of Bacterial Protein Profiles Kit.

Bacterial strain grown as lawns on LB agar plates
E.coli B, *Serratia marcescens*, *Micrococcus luteus*, *Bacillus subtillis*,

Reagents

Tris-EDTA-glucose (TEG) solution (25mM Tris-Cl, pH 8.0, 50 mM glucose, 10 mM EDTA)
TEG containing lysozyme 5 mg/ml, prepared the day of lab and stored cold.
Sample loading buffer

APPENDIX

1. Phosphate Buffer Saline

NaCl	137mM
KH_2PO_4	1.5 mM
Na_2HPO_4	8.0 mM
KCL	2.7 mM
NaN_3	3.0 mM

Check the pH of the final solution. It may be convenient to preparte a stock solution at 10X the desired concentration. In that case, no preservative such as NaN_3, has to be added. The pH of concentrate will be lower than that of the working solution. Note: NaN_3 is highly toxic and it bind to metals, forming explosive compounds when kept dry. Wear gloves to dispense.

2. Ampification mixture

Prepare 15 ml of 0.025 M phosphate buffer, pH 7.0, containing: 700 U alcohol dehydrogenase; 100 U diaphorase; 3% (v/v) ethanol; 1 mM *p*-iodonitrotetrazolium violet.

3. Purification of Immunoglobulin

In some cases it may be necessary to purify the immunoglobulin (the predominantly IgG) from the serum, e.g. for raising the titre of the antiserum, for eliminating other interfering proteins or for working with known quantities or concentrations of antibodies. Several methods exist for purification of immunoglobulins. The simplest methods involving precipitation by ammonium sulphate, is given below.

Materials

General

Medium speed centrifuge with fixed-angel rotor and tubes
Spectophotometer
Deep-freeze at -20°C
Dialysis tubing

Chemicals, solution and suspensions

Antiserum
Half-Strenght PBS (1/2xPBS); see appendix for PBS
Saturated ammonium sulphate: 75 g in 100 ml deionised water
Sodium azide (NaN_3)
Na_2-EDTA

Preparations

Before use, the dialysis tubing should be boiled for 5 minutes in deionised water to remove glycerin. Add to the water Na_2-EDTA to 0.01 M to neutralize polyvalent cations. After boiling, rinse the tubing thoroughly with deionised water.

Procedure

1, To 1 ml of antiserum add 9 ml of half-strength (1/2X) PBS.
2. Add 10 mol of saturated ammonium sulphate, dropwise with constant stirring.
3. Leave mixture for 1 hour at room temperature.
4. Collect the precipitate by centrifugation at 8000 g for 15 min
5. Resuspend the pellet in 2 ml of 1/2XPBS
6. Dialyse three times against 500 ml of ½ PBS
7. Determine the immunoglobulin concentration spectrophotometrically, using a specific absorbance of 1.4 for 1 mg/ml at 280 nm wavelength.
8. Add NaN_3 to a concentration of 0.01% (w/v)
9. Divide the immunoglobulin preparation in small portions (to avoid repeated freezing and thawing) and stored at -20°C. Immunoglobulins can be stored for longer periods with 50% (v/v) glycerol.

Caution : do not add the Immunoglobulins will be used for conjugation.